Technology Transition Workshop

Overview of the Ibis mtDNA Tiling Assay

Tom Hall, Ph.D.

Ibis Biosciences, Inc.

Outline

- Ibis mtDNA assay format
- Base composition analysis of mtDNA samples
  - Data processing and analysis
  - Blinded samples
- Information content relative to sequencing
- Heteroplasmy detection
- Sensitivity
- Reproducibility

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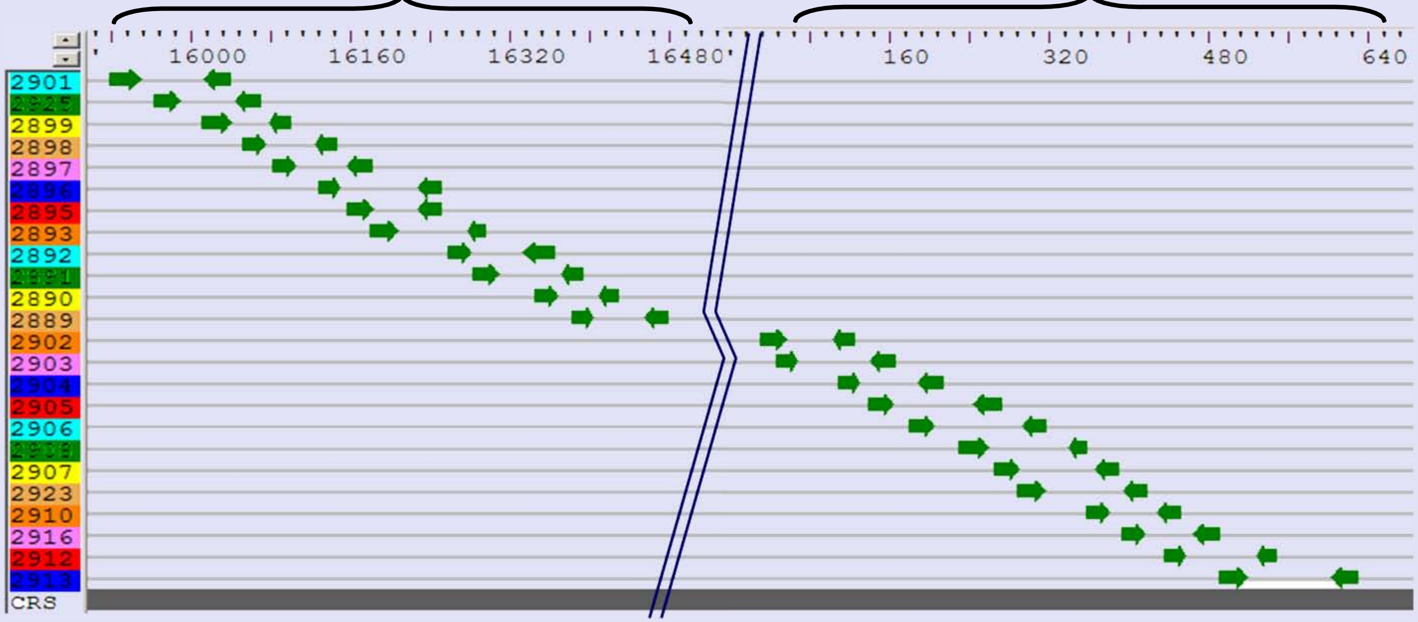
mtDNA Tiling Assay Format

- **24 primer pairs cover amplified coordinates HV1 15924..16428 and HV2 31..576**
- **Target most highly-conserved positions on 3′ ends of primers**
- **Grouped into 8 triplexed sets (colored grouping) by maximum spatial separation and suitable mass separation of products**

HV1 15893..16451
(15924..16428 amplified)

HV2 5..603
(31..576 amplified)



(middle of mtDNA hidden)

IMAGE COURTESY OF TOM HALL, PH.D.

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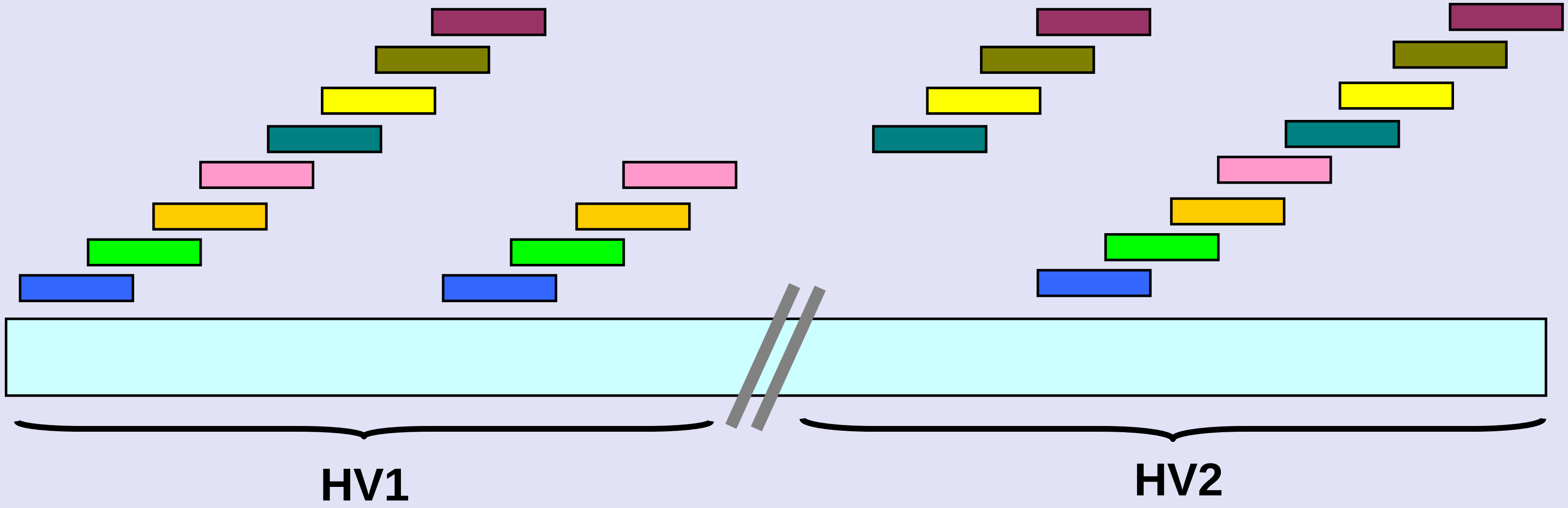
mtDNA Tiling Assay Format



- ☐ **Primers grouped to maximize target site separation**
- ☐ **PCR reactions performed with short extension cycle (5 seconds)**
- ☐ **Product masses resolve from each other in triplex groupings**
- ☐ **Product sizes range from 85 to 140 bp. All but 3 are <130 bp**
- ☐ **Relative primer pair concentrations in triplexes have been adjusted to favor simultaneous amplification of all products**

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Base Composition Analysis

1. **PCR**
2. **Desalting**
3. **ESI-TOF mass spectrometry**
4. **Raw spectrum processing / deconvolution**
5. **Base composition assignment / profile development**

PCR: 3 Primer Pairs Per Reaction

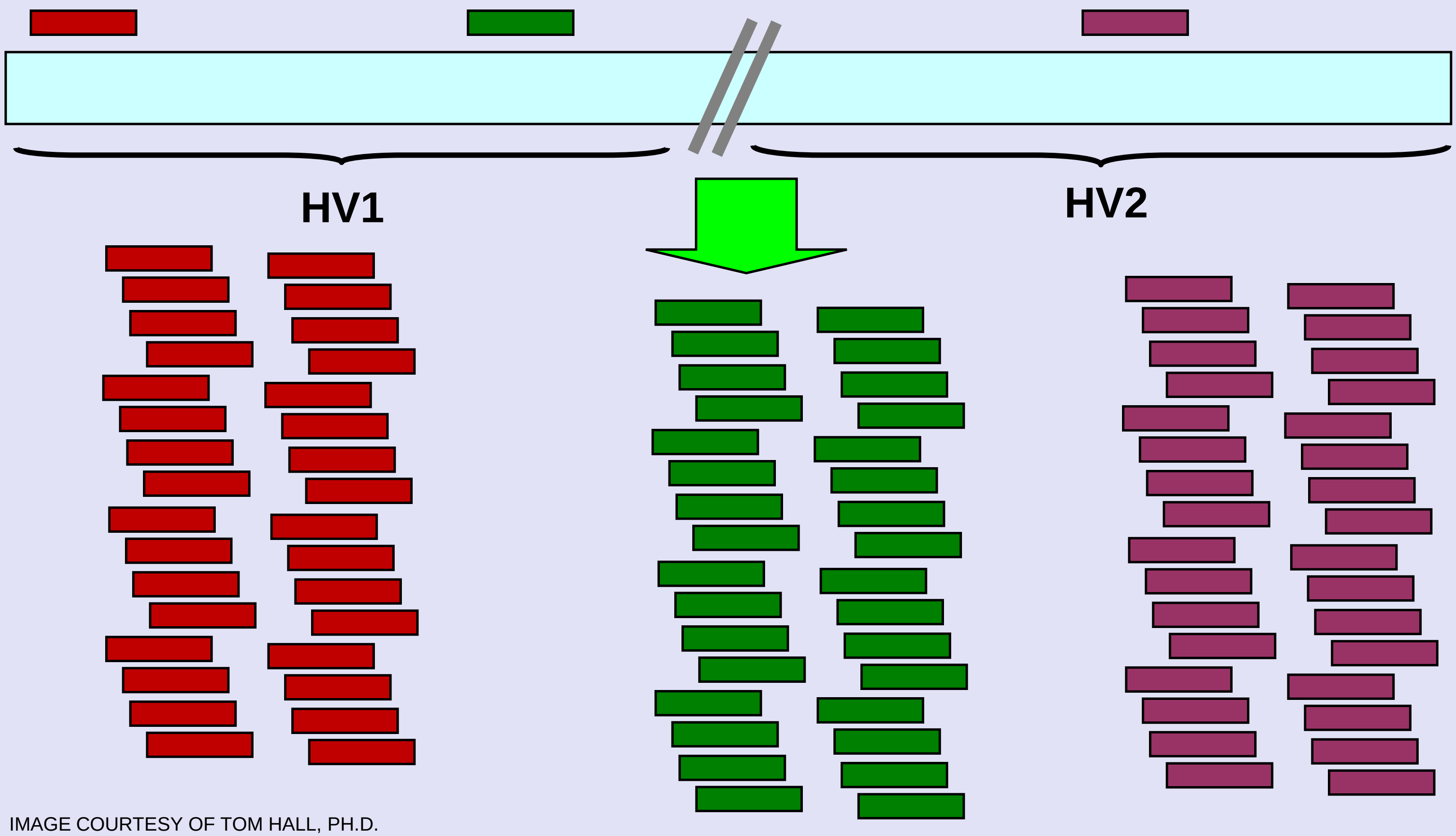


IMAGE COURTESY OF TOM HALL, PH.D.

Desalting of PCR Reactions

Magnetic bead anion exchange

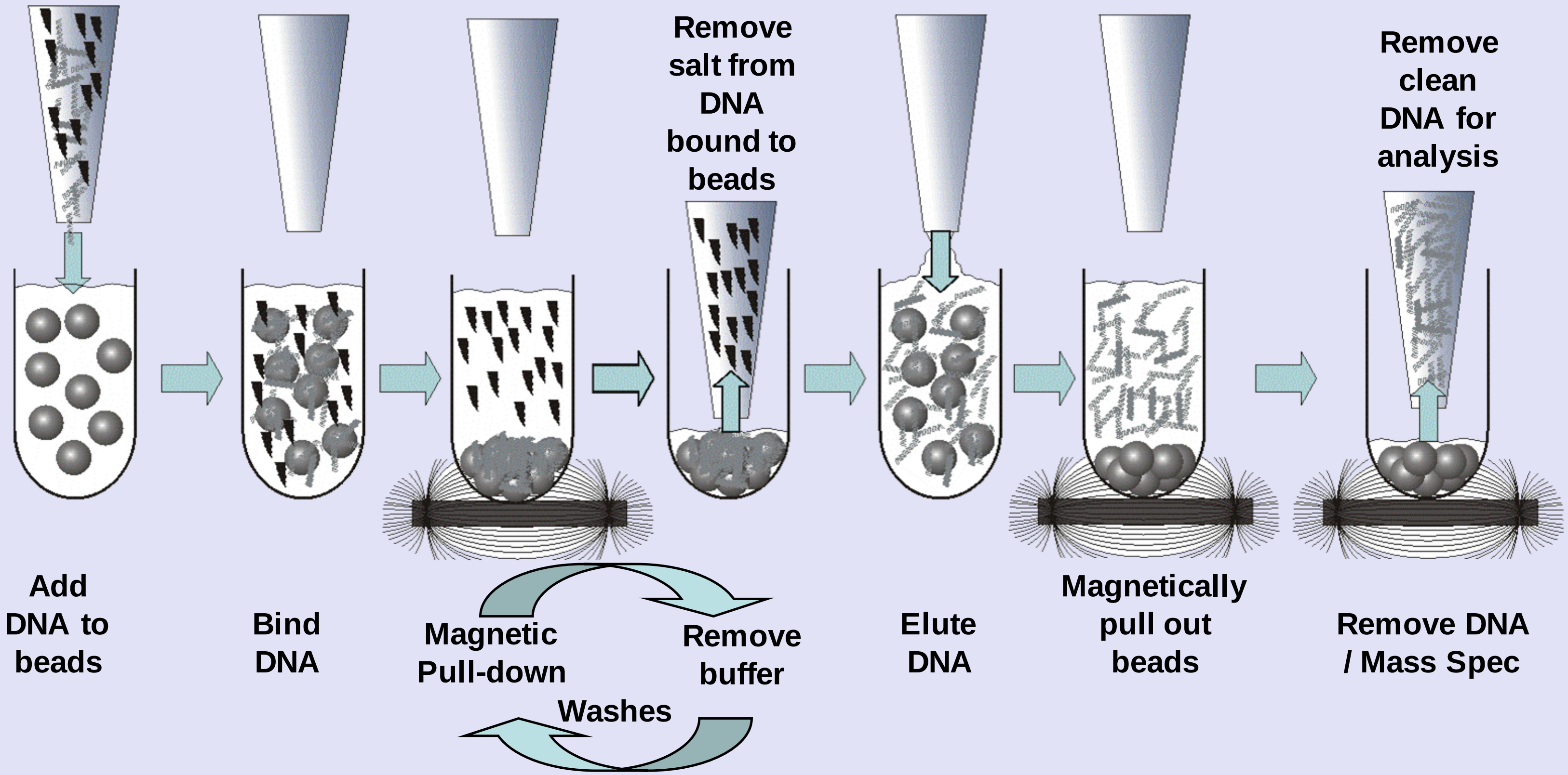


IMAGE COURTESY OF TOM HALL, PH.D.

ESI-TOF Mass Spectromery

Three primer pairs per reaction

Complex raw spectrum

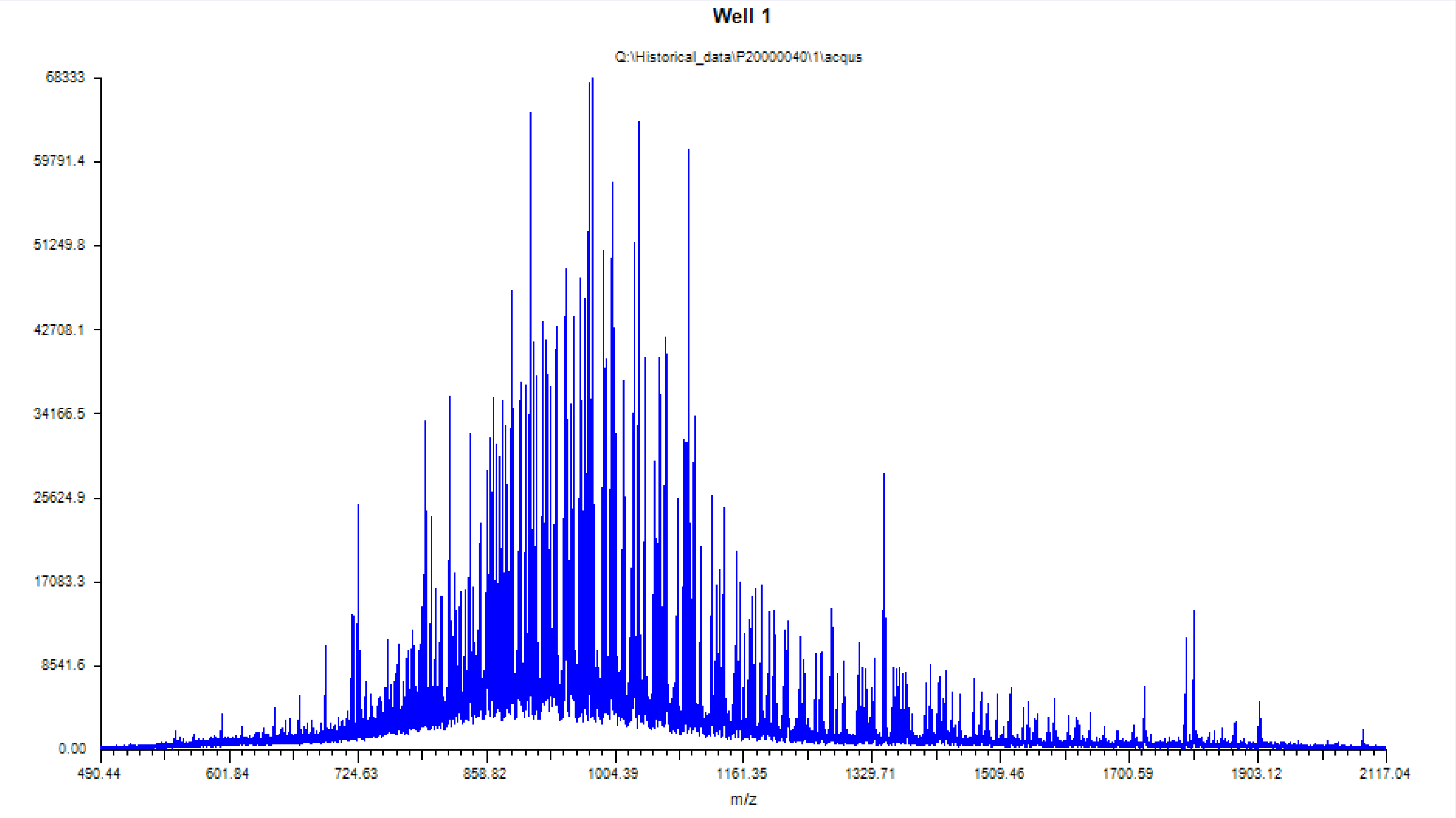
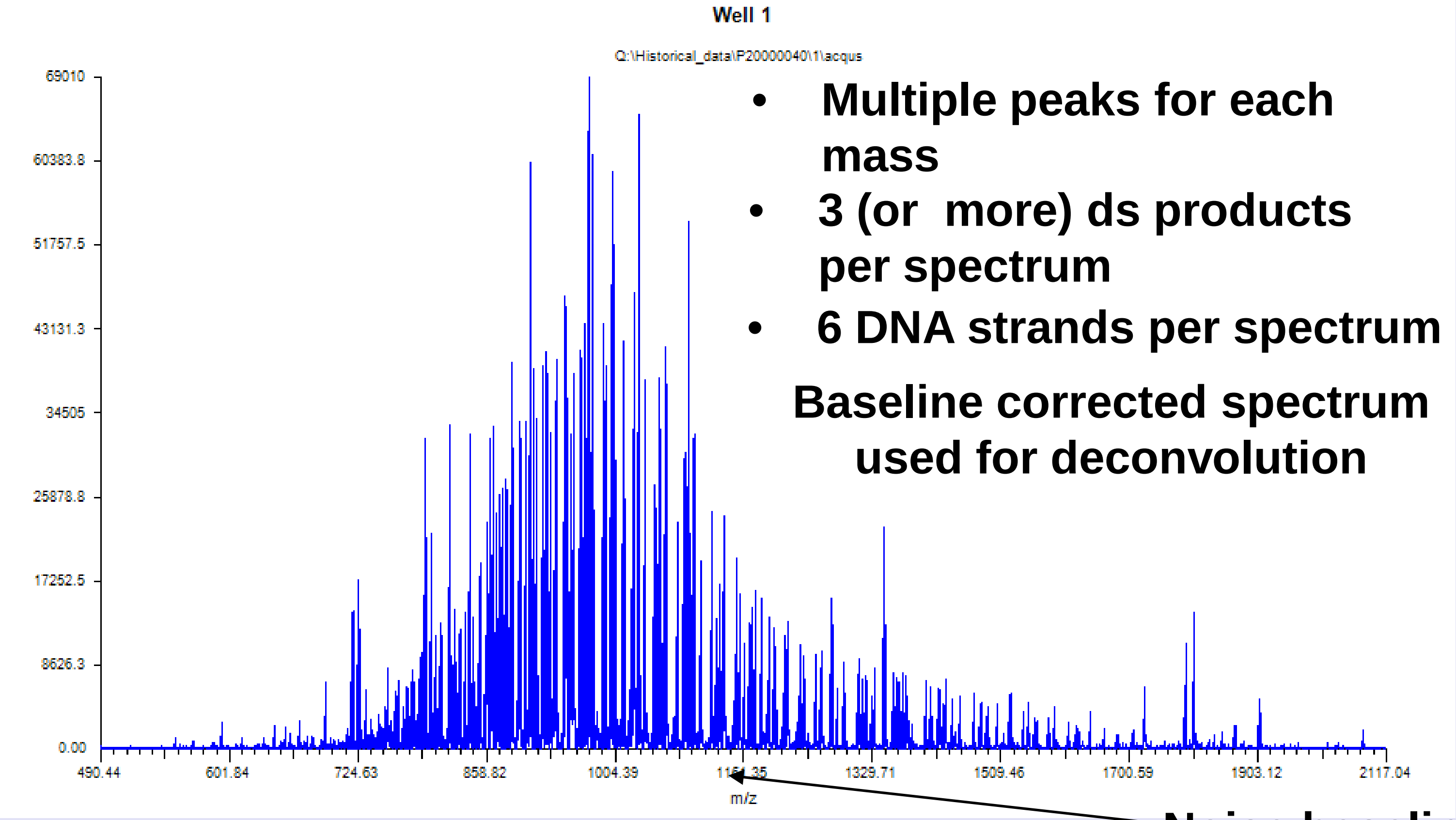


IMAGE COURTESY OF TOM HALL, PH.D.

ESI TOF Mass Spectromery

Three primer pairs per reaction

Complex raw spectrum



- **Multiple peaks for each mass**
- **3 (or more) ds products per spectrum**
- **6 DNA strands per spectrum**

Baseline corrected spectrum used for deconvolution

Noise baseline

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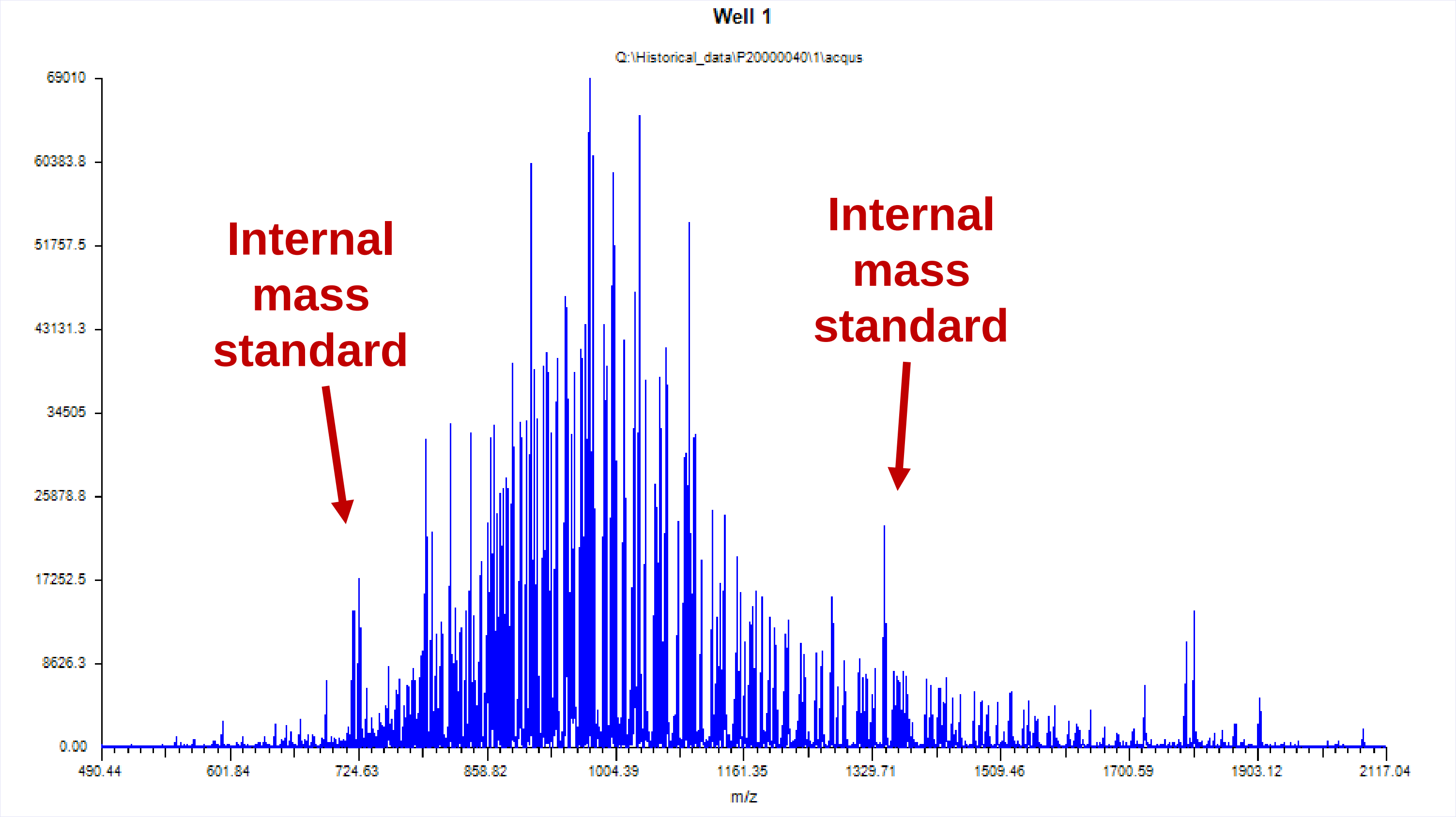
Raw Spectrum Processing

Internal mass standards bracket the spectrum for accurate calibration of the measurements before deconvolution



Internal mass standard

Internal mass standard

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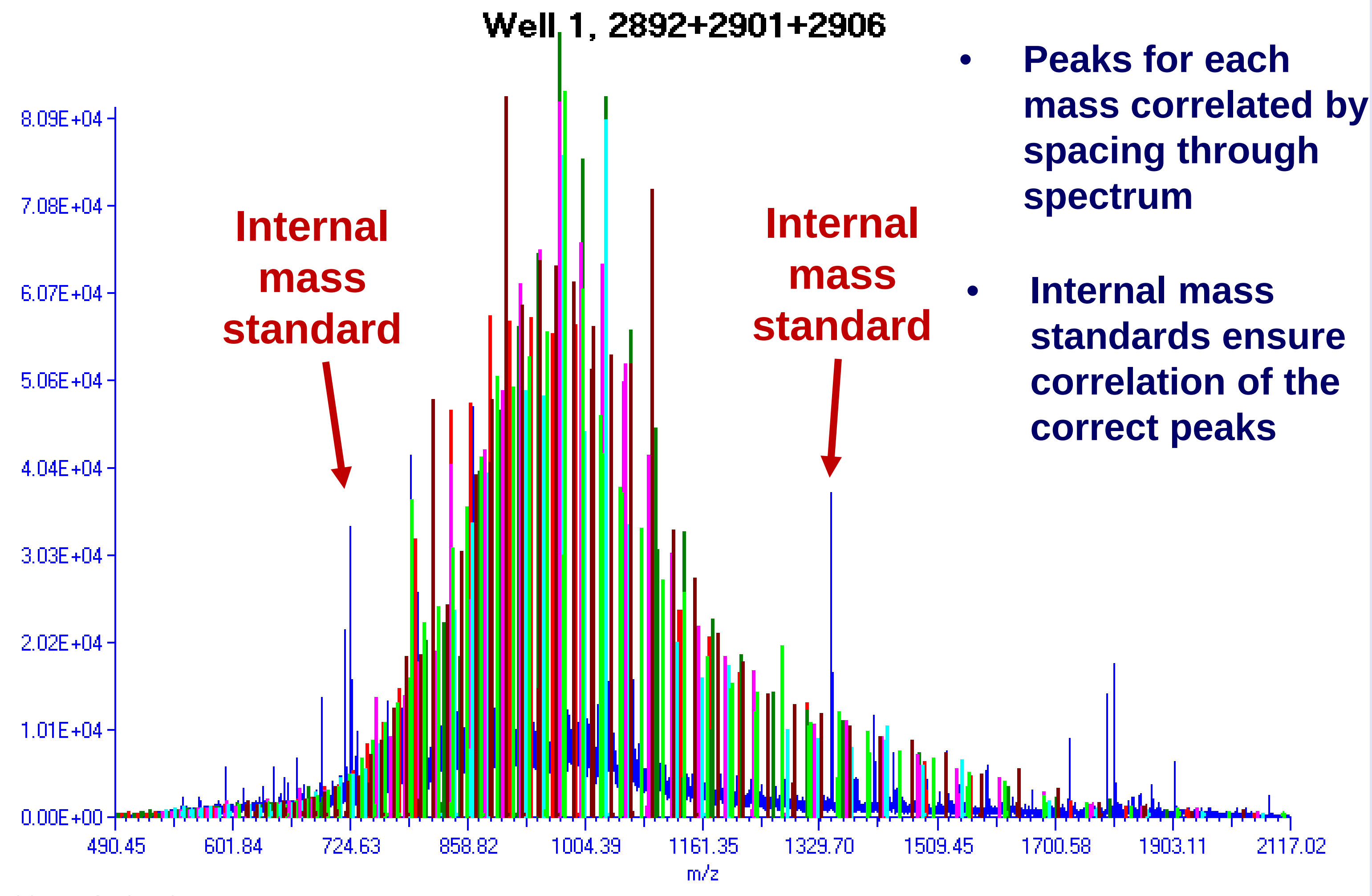
Spectral Deconvolution



- **Peaks for each mass correlated by spacing through spectrum**
- **Internal mass standards ensure correlation of the correct peaks**

IMAGE COURTESY OF TOM HALL, PH.D.

Deconvolution to Masses

Deconvolution results in one final measurement per molecular species

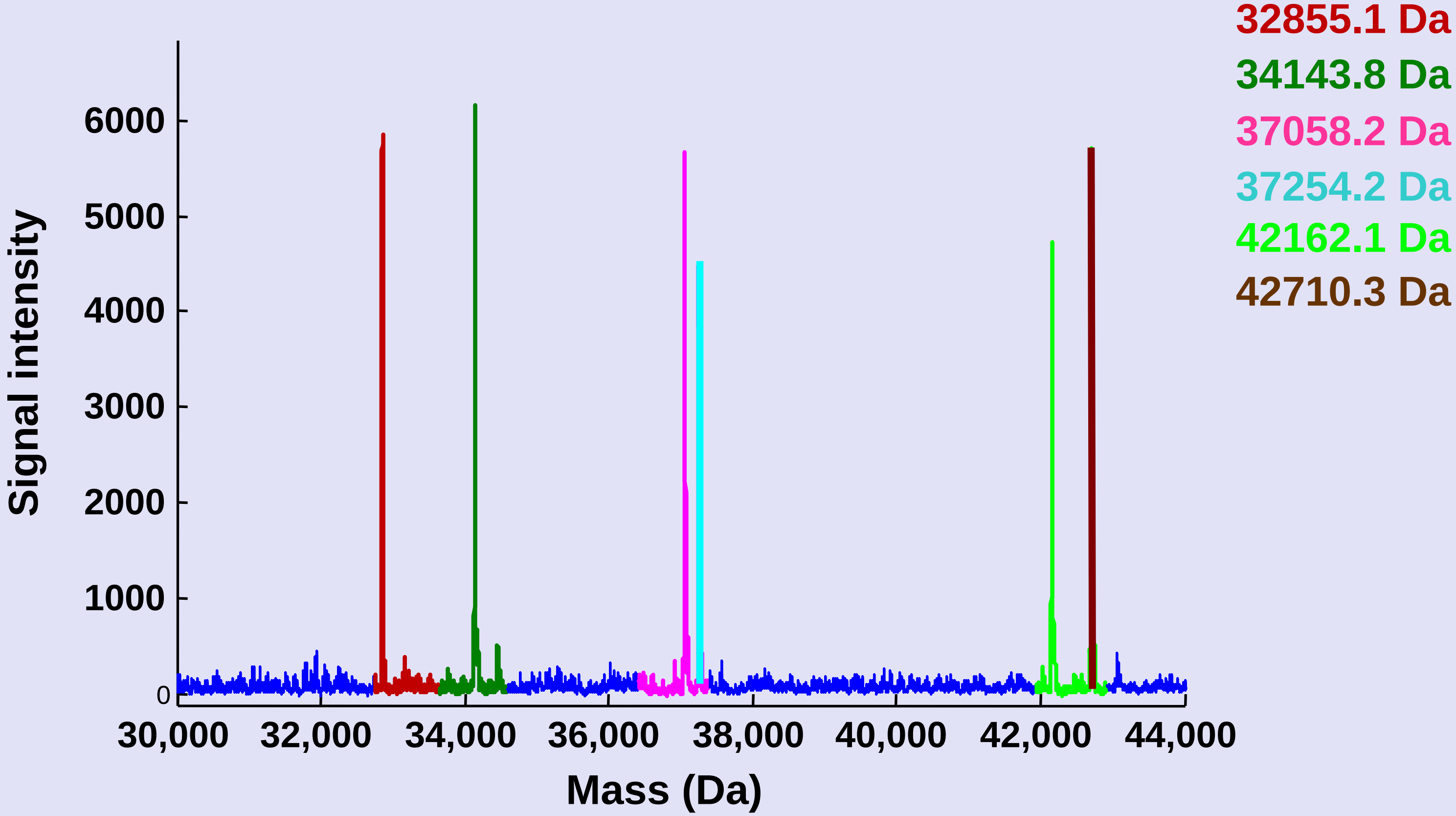


IMAGE COURTESY OF TOM HALL, PH.D.

DS DNA Strand Association

Forward and reverse strands of a double stranded DNA can be associated by mass

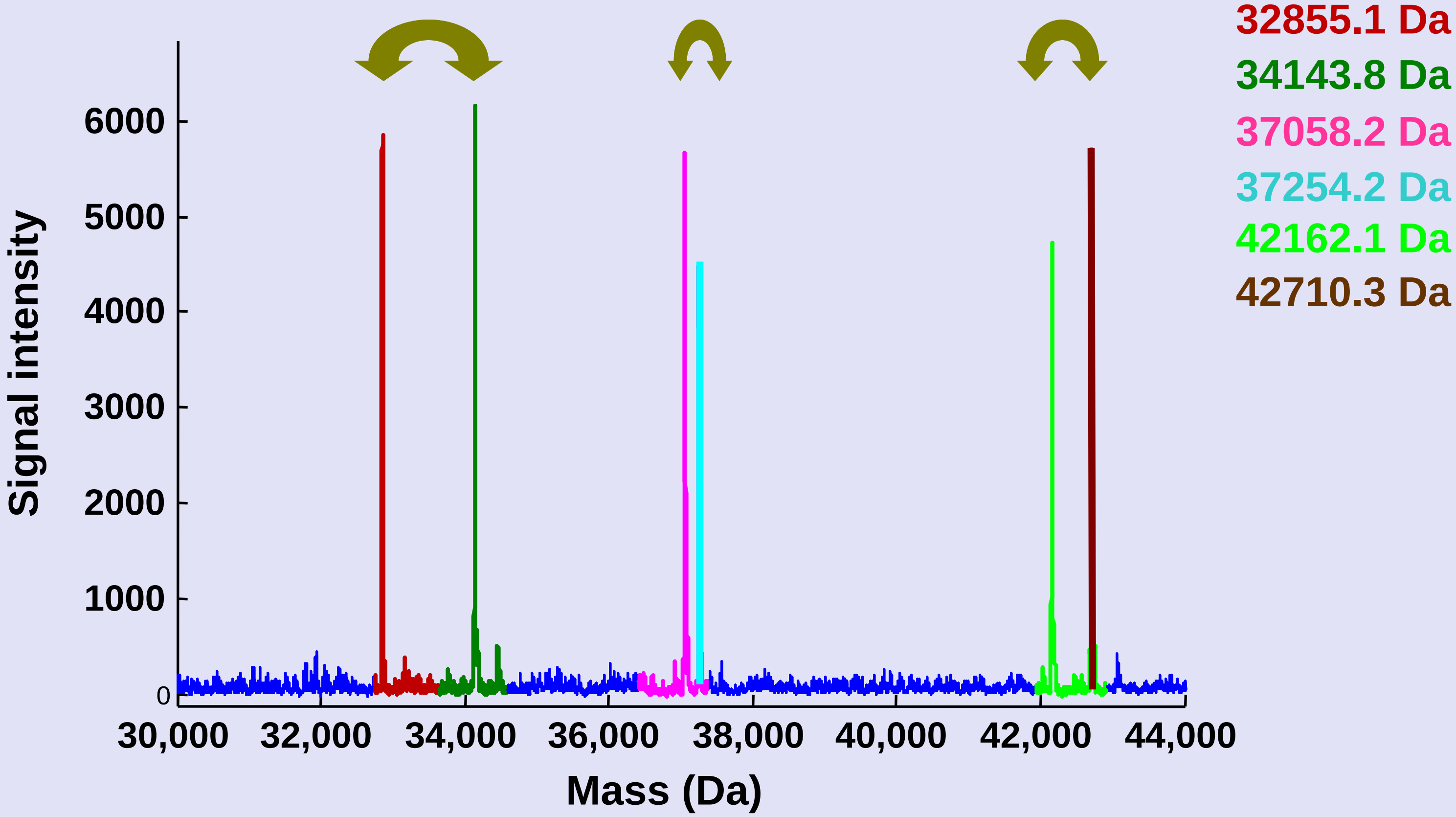


IMAGE COURTESY OF TOM HALL, PH.D.

DS DNA Strand Association

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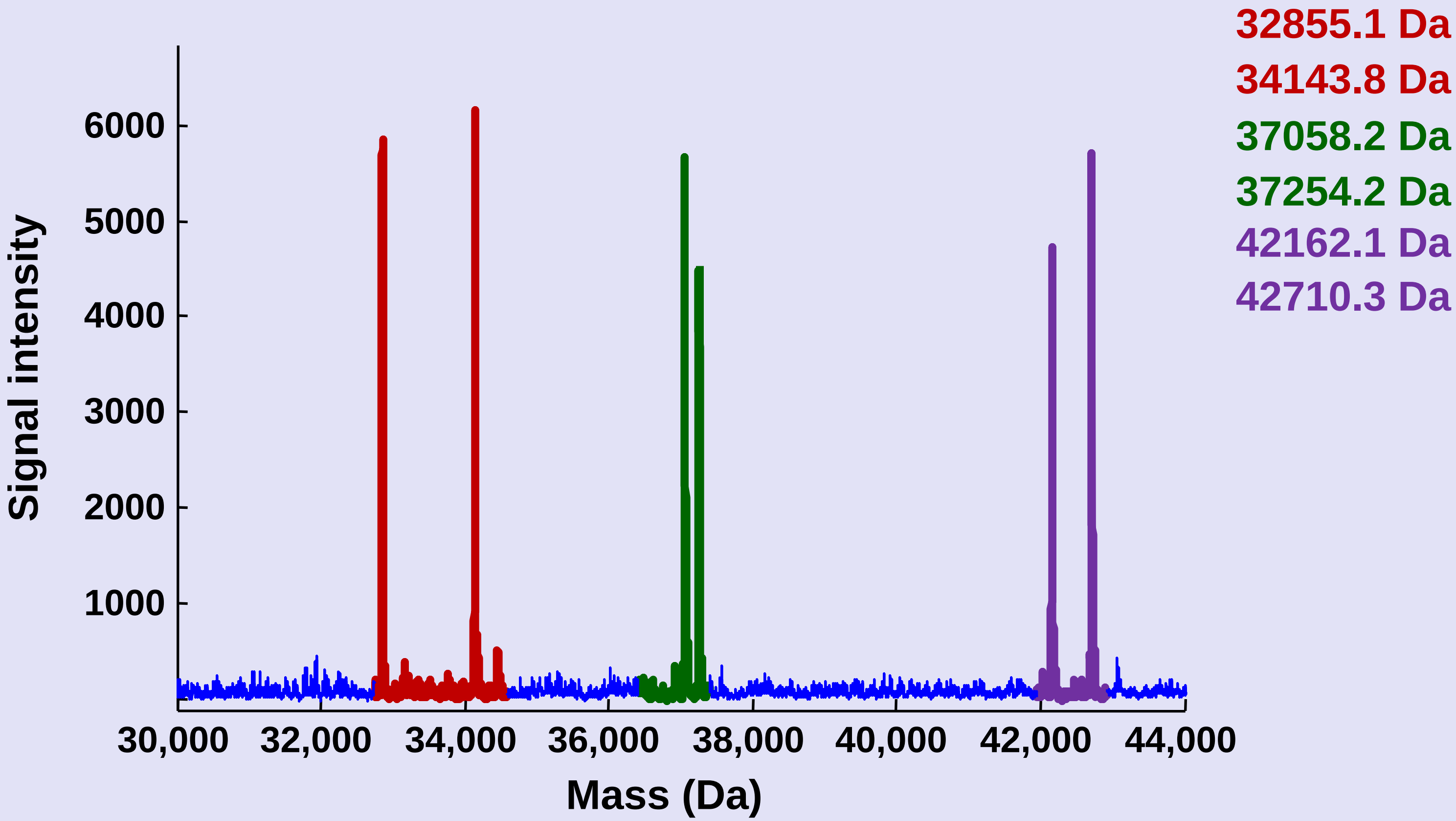
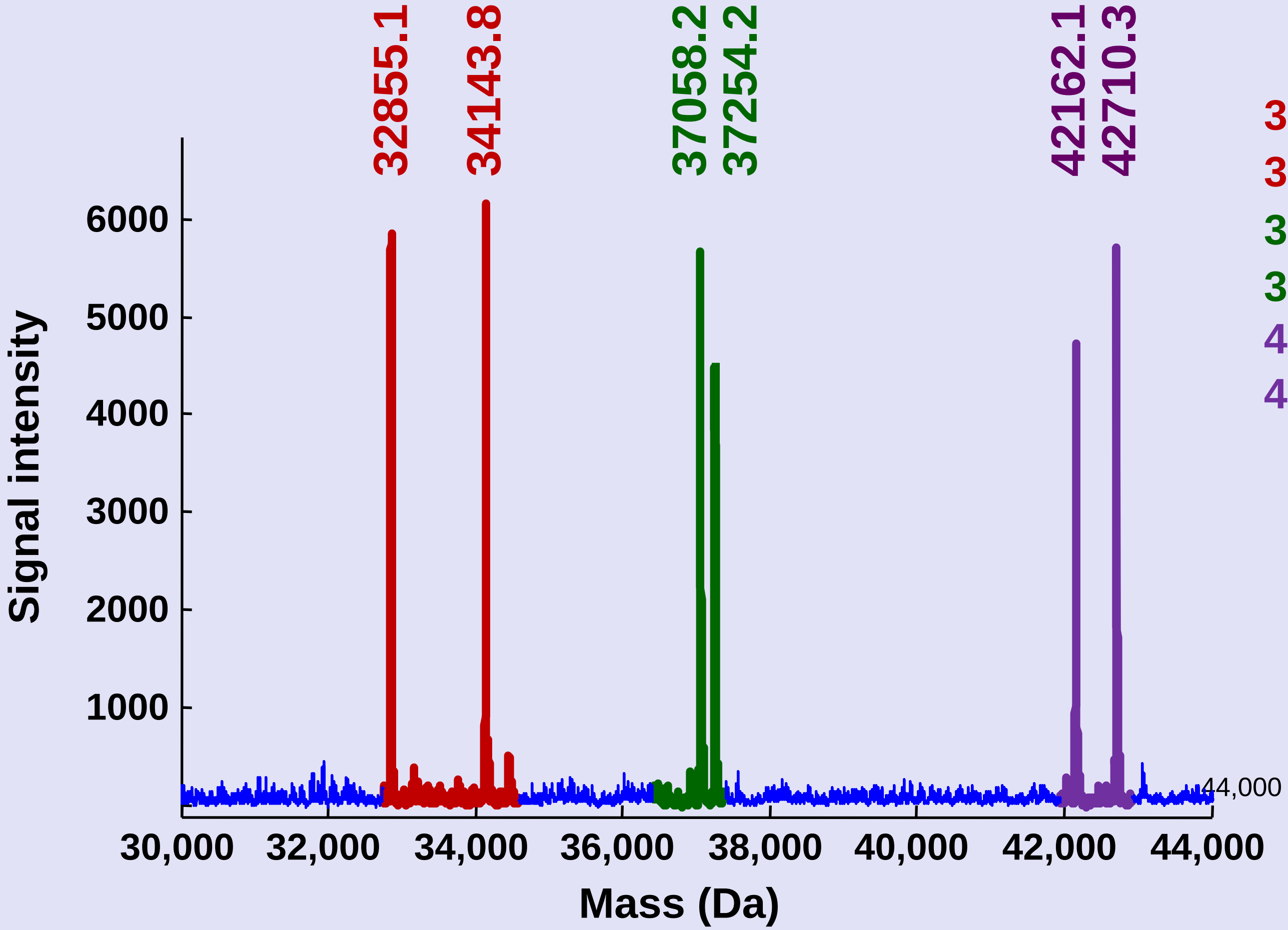


IMAGE COURTESY OF TOM HALL, PH.D.

Base Composition Assignment

[A40 G9 C40 T19] [A47 G18 C25 T30] [A49 G17 C31 T40]



32855.1 Da
34143.8 Da
37058.2 Da
37254.2 Da
42162.1 Da
42710.3 Da

IMAGE COURTESY OF TOM HALL, PH.D.

Base Composition Assignment

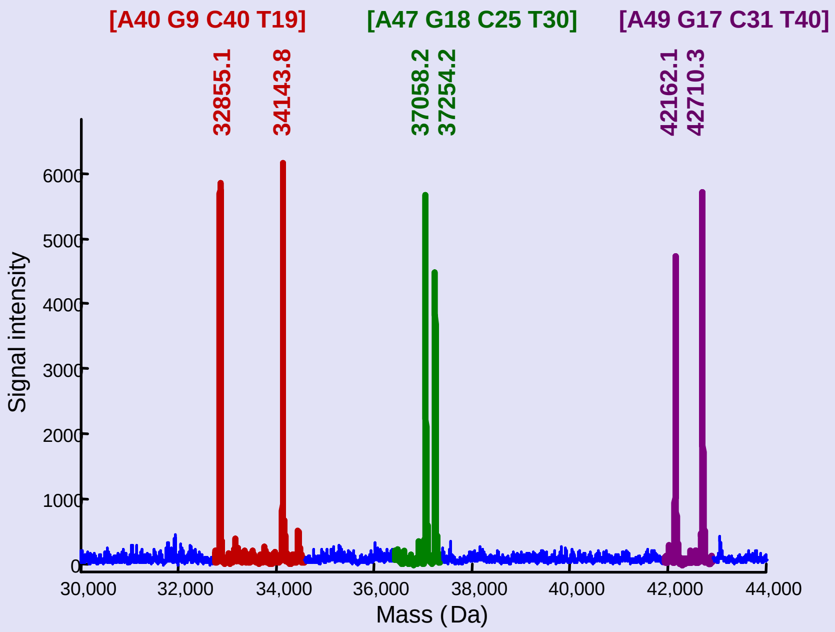


IMAGE COURTESY OF TOM HALL, PH.D.

Final Product Assignment

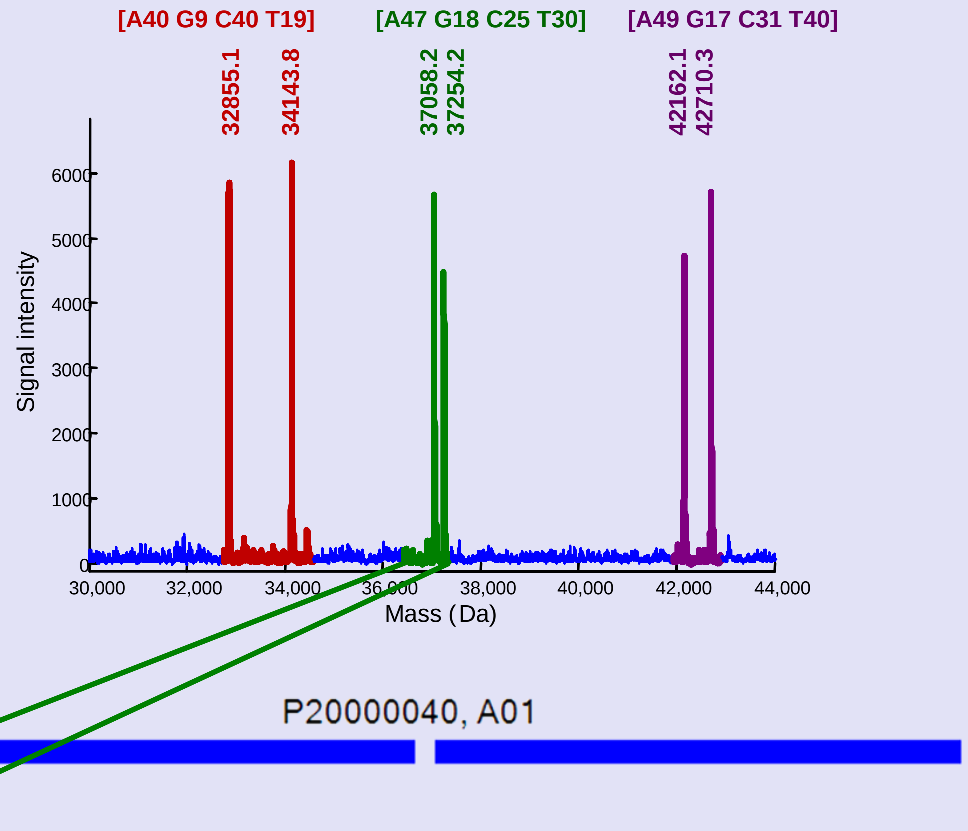
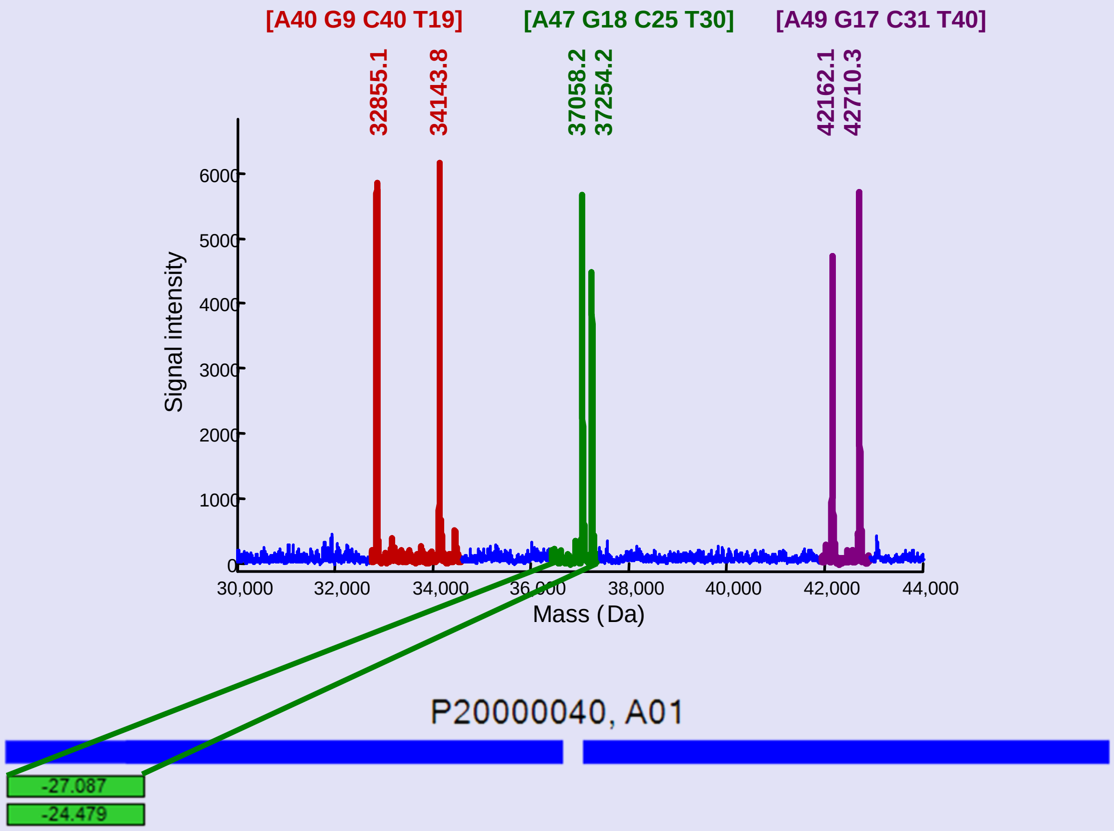


IMAGE COURTESY OF TOM HALL, PH.D.

Final Product Assignment

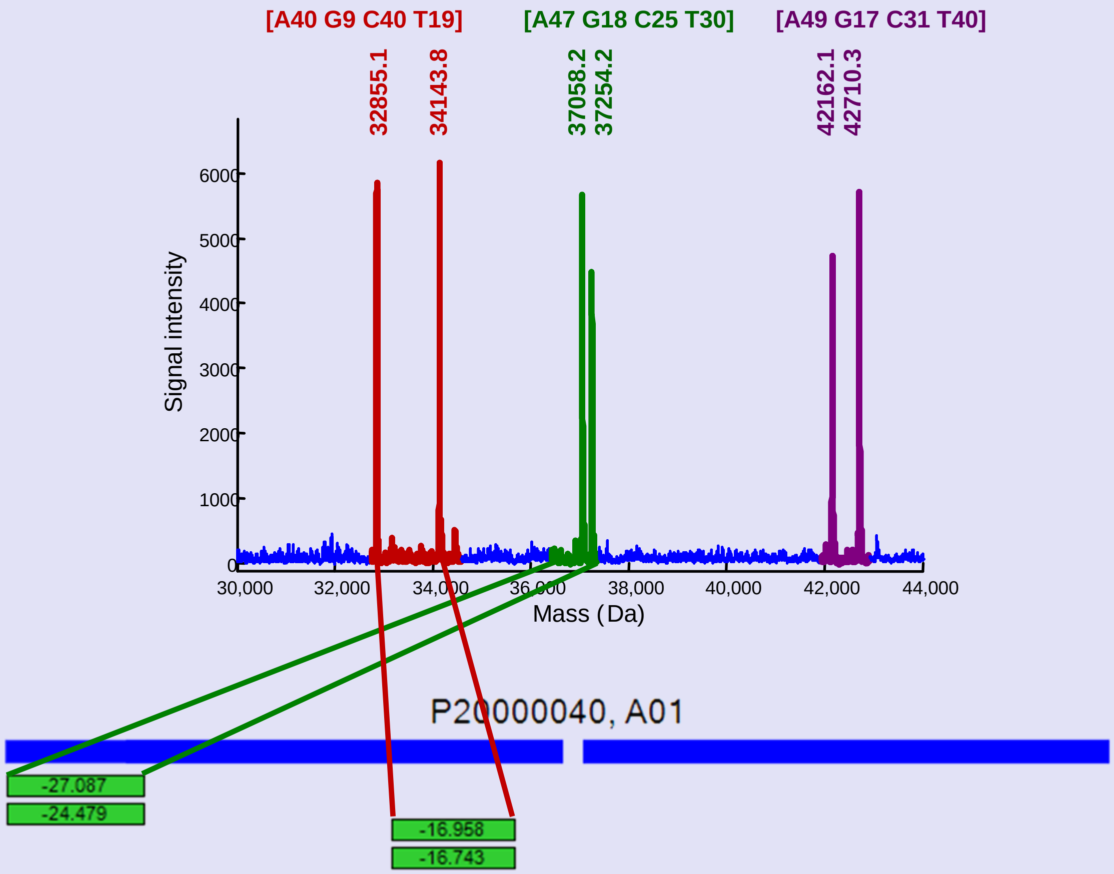


15893..16012

[A47 G18 C25 T30]

IMAGE COURTESY OF TOM HALL, PH.D.

Final Product Assignment



15893..16012

[A47 G18 C25 T30]

IMAGE COURTESY OF TOM HALL, PH.D.

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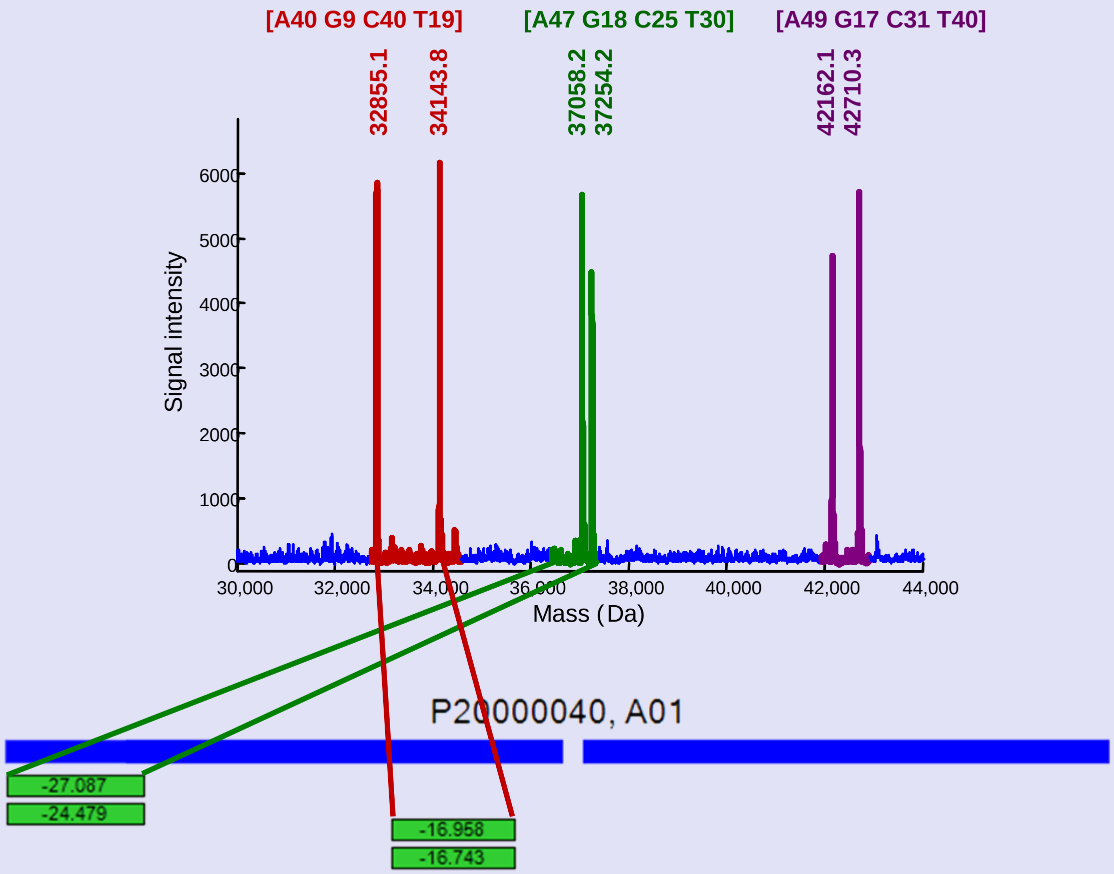
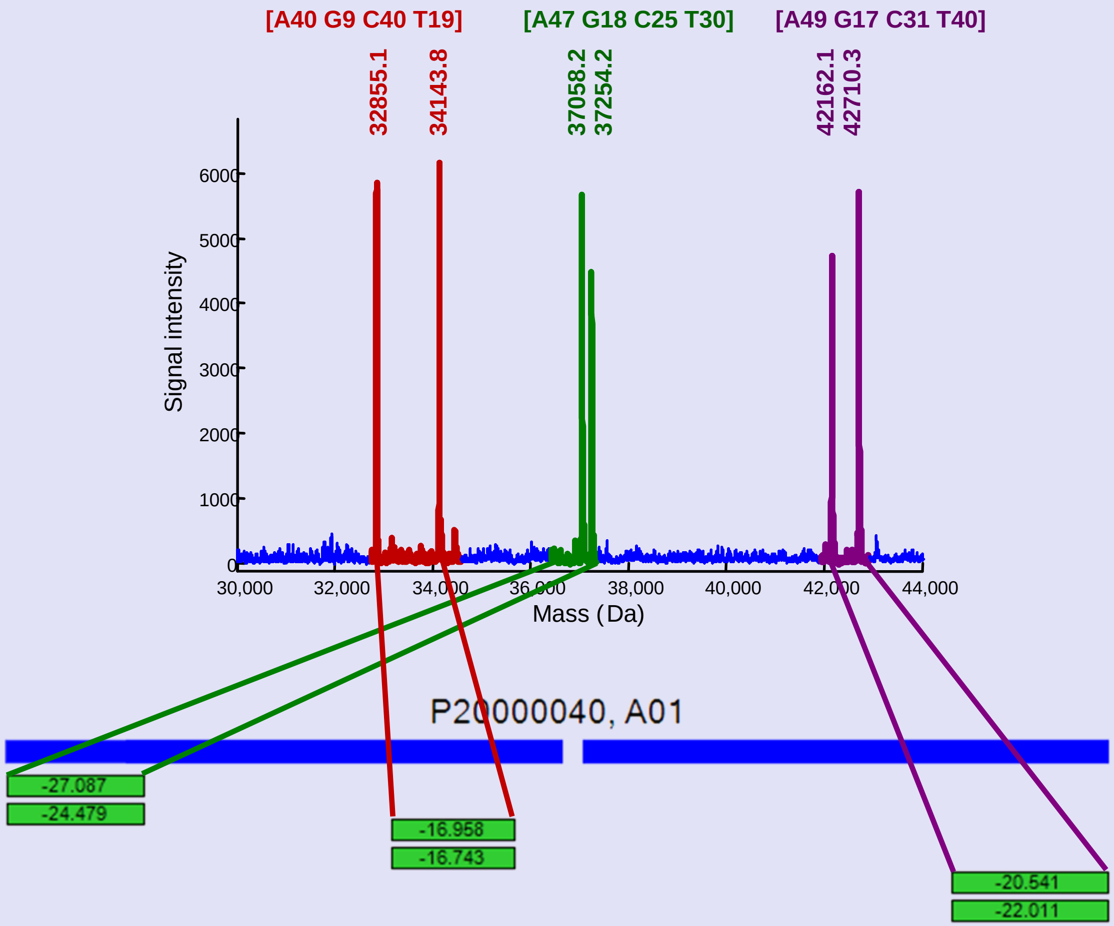


15893..16012
[A47 G18 C25 T30]

16231..16338
[A40 G9 C40 T19]

IMAGE COURTESY OF TOM HALL, PH.D.

Final Product Assignment



15893..16012
[A47 G18 C25 T30]

16231..16338
[A40 G9 C40 T19]

IMAGE COURTESY OF TOM HALL, PH.D.

Final Product Assignment

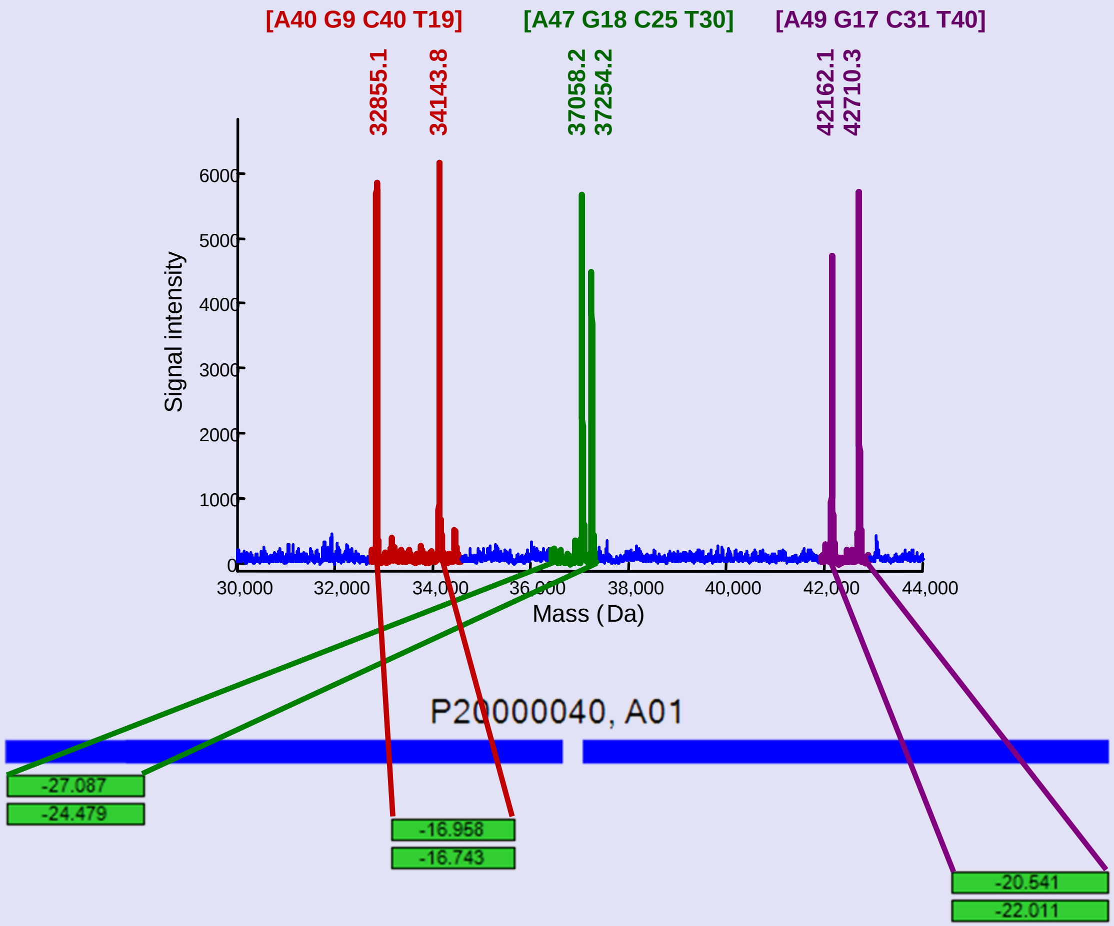


IMAGE COURTESY
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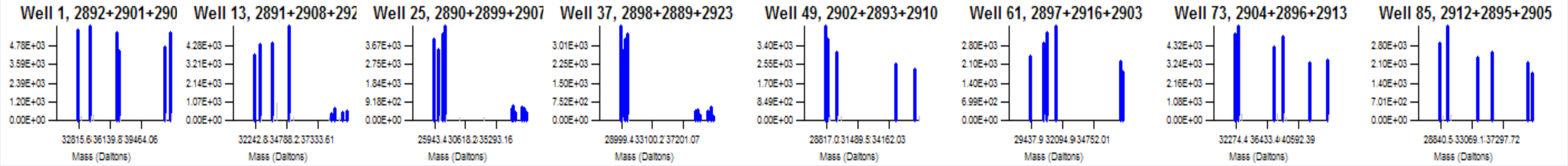
15893..16012
[A47 G18 C25 T30]

16231..16338
[A40 G9 C40 T19]

154..290
[A49 G17 C31 T40]

Eight Spectra Per Sample



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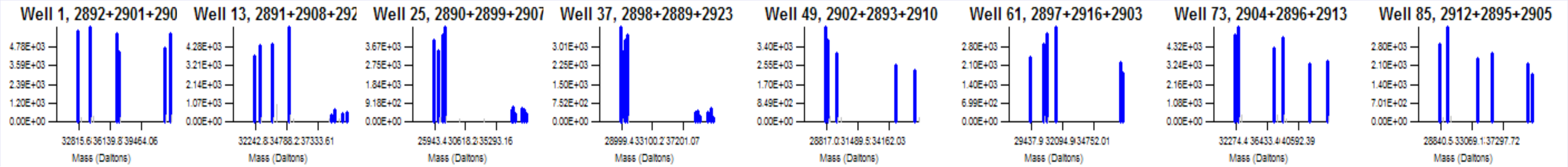
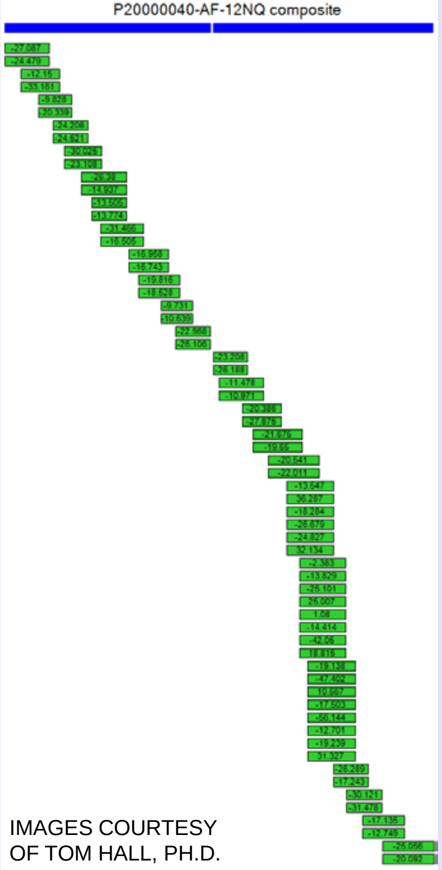
Coverage Map Development





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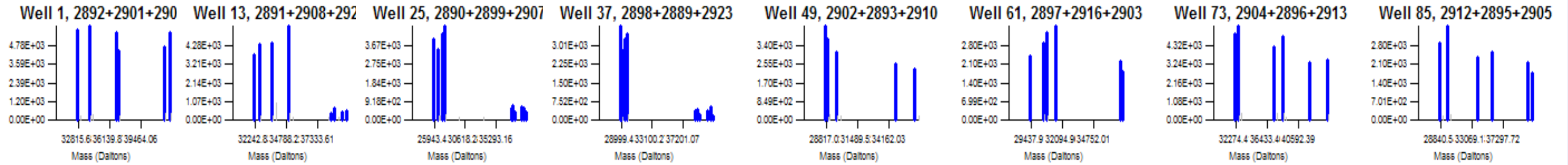
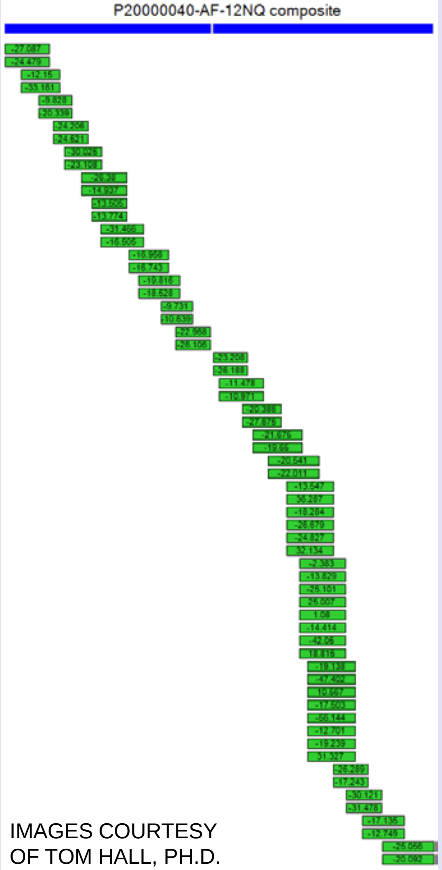
Base Composition Profile





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Base composition profile

2901: 15893..16012: A47 G18 C25 T30
2925: 15937..16041: A35 G14 C24 T32
2899: 15985..16073: A26 G15 C21 T27
2898: 16025..16119: A26 G17 C26 T26
2897: 16055..16155: A31 G13 C30 T27
2896: 16102..16224: A45 G13 C42 T23
2895: 16130..16224: A36 G7 C33 T19
2893: 16154..16268: A44 G7 C46 T18
2892: 16231..16338: A40 G9 C40 T19
2891: 16256..16366: A37 G9 C41 T24
2890: 16318..16402: A20 G14 C30 T21
2889: 16357..16451: A21 G17 C36 T21
2902: 5..97: A19 G24 C24 T26
2903: 20..139: A24 G34 C29 T33
2904: 83..187: A23 G21 C29 T32
2905: 113..245: A39 G18 C28 T48
2906: 154..290: A49 G17 C31 T40
2908: 204..330: A42 G16 C35 T32
2908: 204..330: A42 G16 C36 T32
2908: 204..330: A42 G16 C37 T32
2908: 204..330: A42 G16 C38 T32
2907: 239..363: A43 G11 C46 T23
2907: 239..363: A43 G11 C47 T23
2907: 239..363: A43 G11 C48 T23
2907: 239..363: A43 G11 C49 T23
2923: 262..390: A47 G10 C50 T20
2923: 262..390: A47 G10 C51 T20
2923: 262..390: A47 G10 C52 T20
2923: 262..390: A47 G10 C53 T20
2910: 331..425: A33 G9 C27 T26
2916: 367..463: A27 G8 C32 T30
2912: 409..521: A32 G7 C48 T26
2913: 464..603: A44 G10 C63 T23

Base composition

rCRS coordinates

Primer pair

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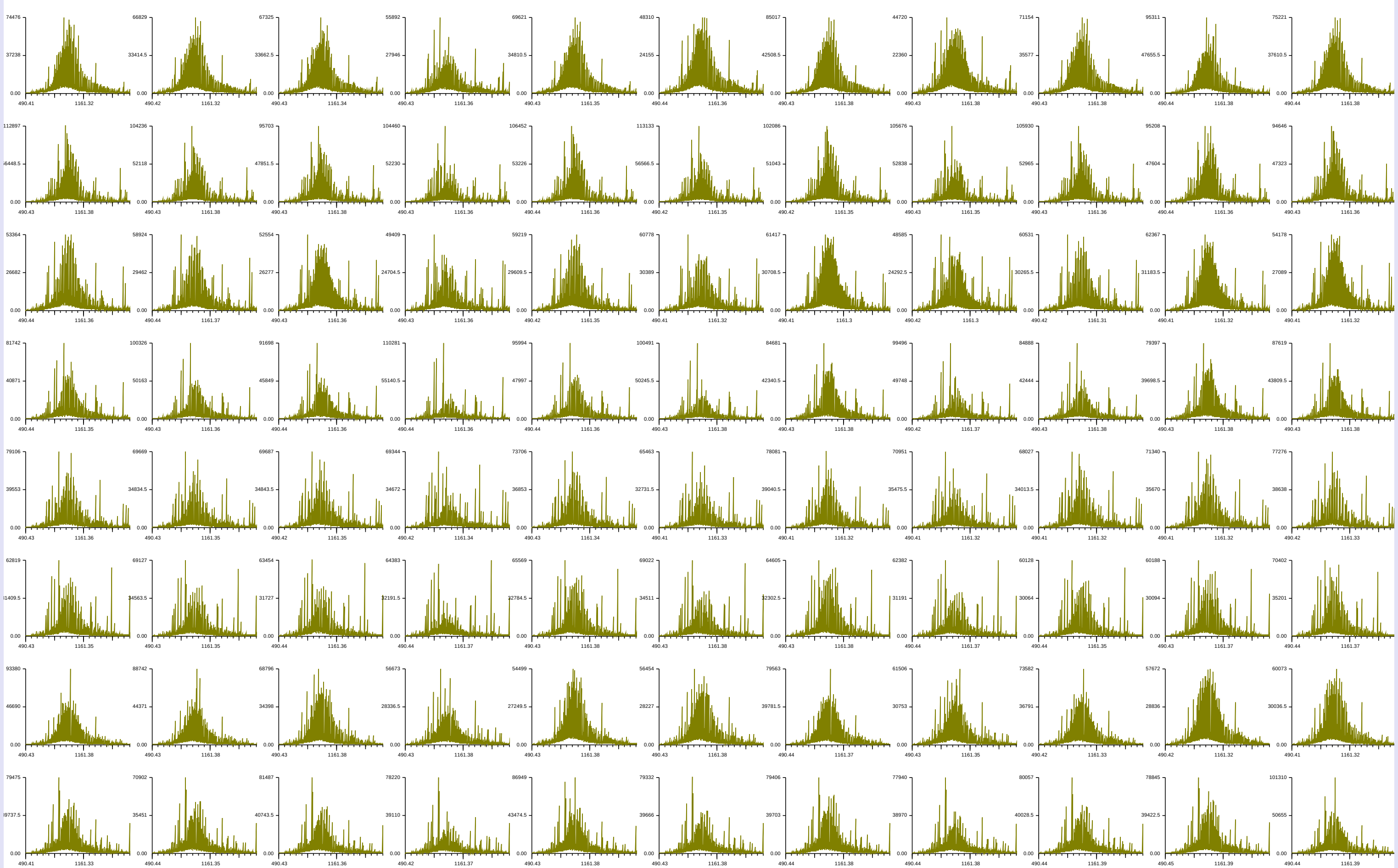
Multiple Assays Per Plate



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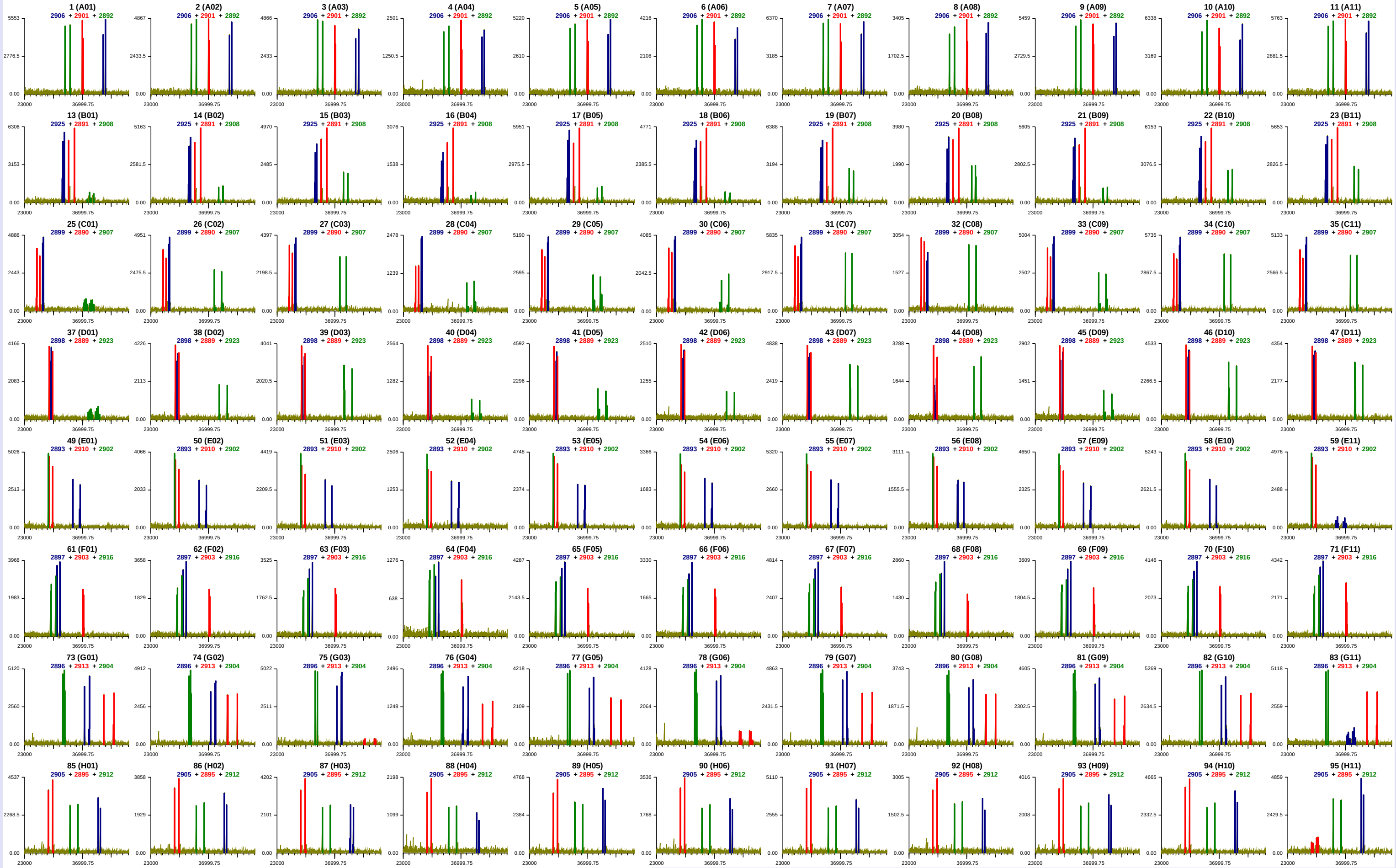
Multiple Mass Assignments



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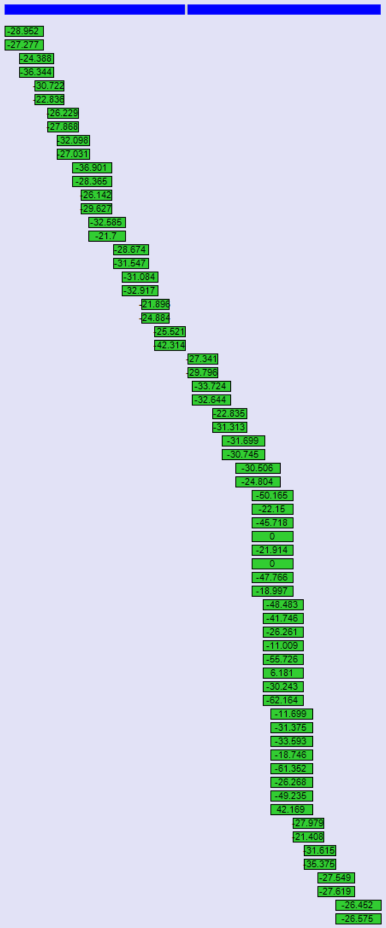
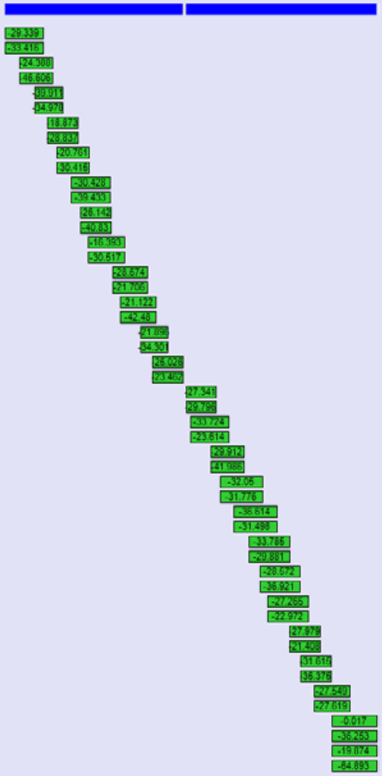
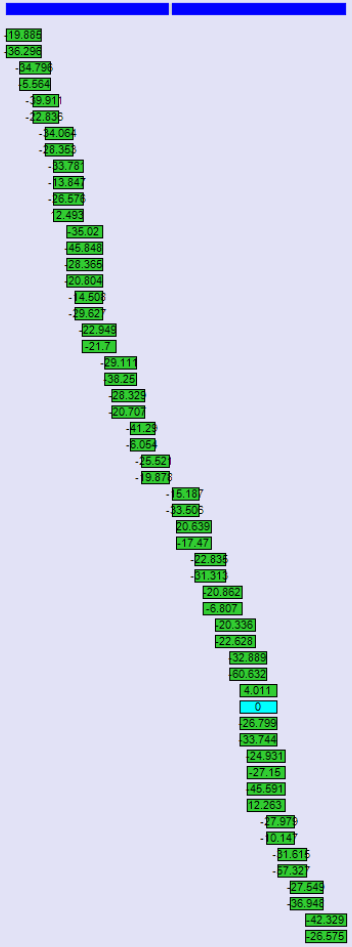
Multiple Samples Per Plate







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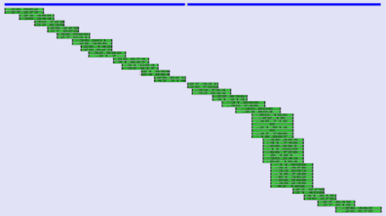
Multiple Profiles Per Plate



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2898: 16025..16119: A26 G17 C26 T26
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2895: 16130..16224: A36 G7 C33 T19
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2902: 5..97: A19 G24 C24 T26
2903: 20..139: A24 G34 C29 T33
2904: 83..187: A23 G21 C29 T32
2905: 113..245: A39 G18 C28 T48
2906: 154..290: A49 G17 C31 T40
2908: 204..330: A42 G16 C35 T32
2908: 204..330: A42 G16 C36 T32
2908: 204..330: A42 G16 C37 T32
2908: 204..330: A42 G16 C38 T32
2907: 239..363: A43 G11 C46 T23
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2907: 239..363: A43 G11 C48 T23
2907: 239..363: A43 G11 C49 T23
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2923: 262..390: A47 G10 C51 T20
2923: 262..390: A47 G10 C52 T20
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2910: 331..425: A33 G9 C27 T26
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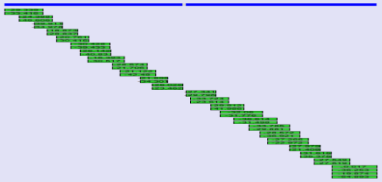

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2899: 15985..16073: A26 G15 C21 T27
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2897: 16055..16155: A32 G12 C30 T27
2896: 16102..16224: A46 G12 C41 T24
2895: 16130..16224: A36 G7 C33 T19
2893: 16154..16268: A44 G7 C45 T19
2892: 16231..16338: A40 G9 C40 T19
2891: 16256..16366: A37 G9 C41 T24
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2903: 20..139: A24 G34 C29 T33
2904: 83..187: A23 G21 C30 T31
2905: 113..245: A39 G18 C31 T45
2906: 154..290: A48 G18 C35 T36
2908: 204..330: A42 G16 C40 T30
2907: 239..363: A43 G11 C49 T23
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2910: 331..425: A33 G9 C27 T26
2916: 367..463: A27 G8 C32 T30
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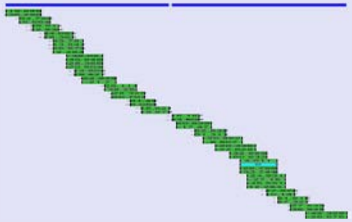

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2899: 15985..16073: A26 G15 C21 T27
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2892: 16231..16338: A39 G10 C40 T19
2891: 16256..16366: A36 G10 C42 T23
2890: 16318..16402: A20 G14 C30 T21
2889: 16357..16451: A21 G17 C36 T21
2902: 5..97: A20 G23 C24 T26
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2916: 367..463: A27 G8 C32 T30
2912: 409..521: A32 G7 C48 T26
2913: 464..603: A44 G10 C63 T23

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Blinded Samples

- ☐ **AFDIL**
  - ➢ **25 dried blood spots on filter paper**
- ☐ **FBI**
  - ➢ **25 buccal swabs**
  - ➢ **Samples chosen to test system because each had mismatches in at least one primer pair**
- ☐ **NIST**
  - ➢ **95 population reference samples**
- ☐ **All reactions were run on pre-fabricated, frozen PCR plates (just add template)**
- ☐ **Batch of plates had been through a QC test prior to use**
- ☐ **Each assay has a least one positive control**
- ☐ **Each plate has at least one negative control**

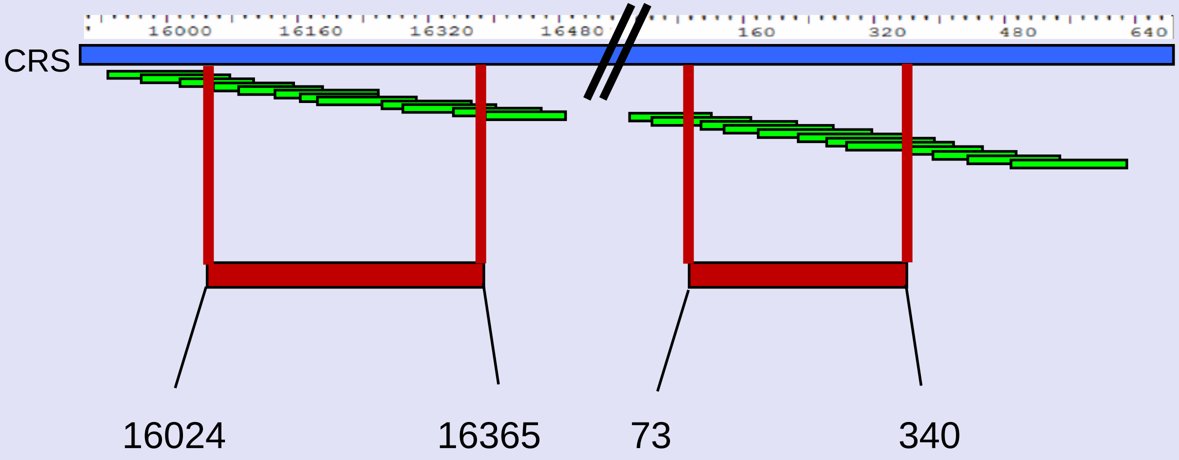
Blinded Samples

- **All samples gave complete profiles**
- **3 FBI samples were not pure single-contributor templates**
  - **2 FBI samples were mixed**
    - **Both were consistent with one sample in primary set mixed with another sample not in the set**
    - **FBI confirmed correct identification of mixtures**
    - **Approximate ratios of contributing templates were quantified**
  - **1 FBI sample was a blank swab**
- **Several heteroplasmies, both C-length and non-C-length were noted in all sample sets and were consistent with sequence profile data**

Tiling Compared to Sequencing

- 1266 unique tiling region sequences were selected from GenBank genomes
  - Each sequence differed from all others by at least one base
  - C-stretch length differences ignored
- Sequences converted to tiling base compositions
- Cross-compared for minimum differences using mtDNA search algorithm
  - Ignores C-stretch length differences
  - Corrects for primer pair overlaps
- 94.2% of unique tiling region sequences were uniquely discriminated by tiling assay

Tiling Compared to Sequencing



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Tiling assay amplifies 15924-16428 and 31-576

Minimum HVI +HVII sequences 16024-16365 and 73-340

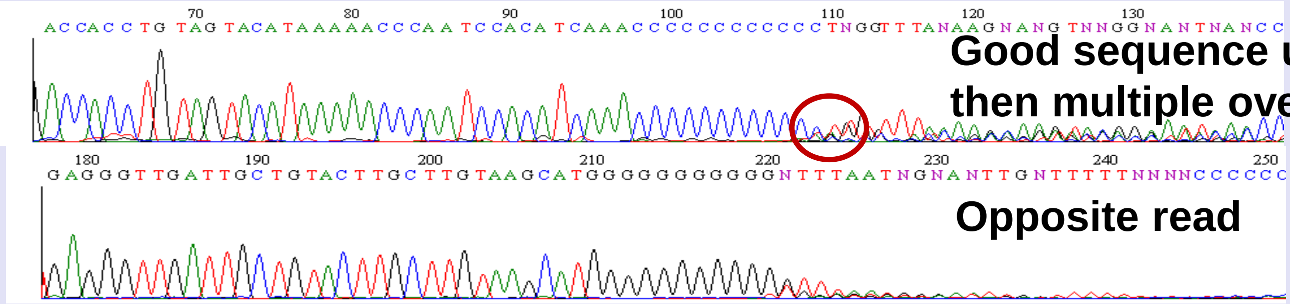
For the same set of 1266 unique sequences spanning mtDNA tiling coordinates:

- ☐ **94.2% can be differentiated with the tiling assay**
- ☐ **90.2% can be differentiated by sequencing HVI 16024-16365 and HV2 73-340**

NIJ National Institute of Justice *Technology Transition Workshop*

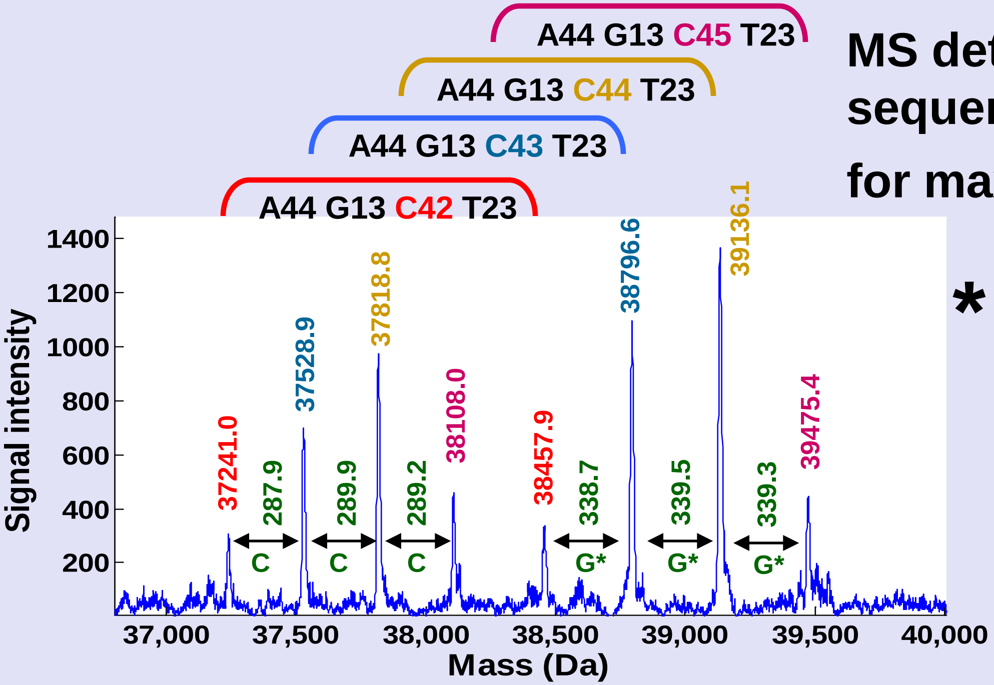
Length Heteroplasmy Detection

Example = USA.FBI.000009, region 16102..16224 *



Good sequence up to C stretch, then multiple overlapping reads

Opposite read



MS detects multiple products while sequencing requires a pure sample for maximum information content

*** Happened to be same sample given as a blinded sample by Bruce Budowle in 2003. This was suggested by our assay in the FBI validation, then confirmed.**

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Length Heteroplasmy Example

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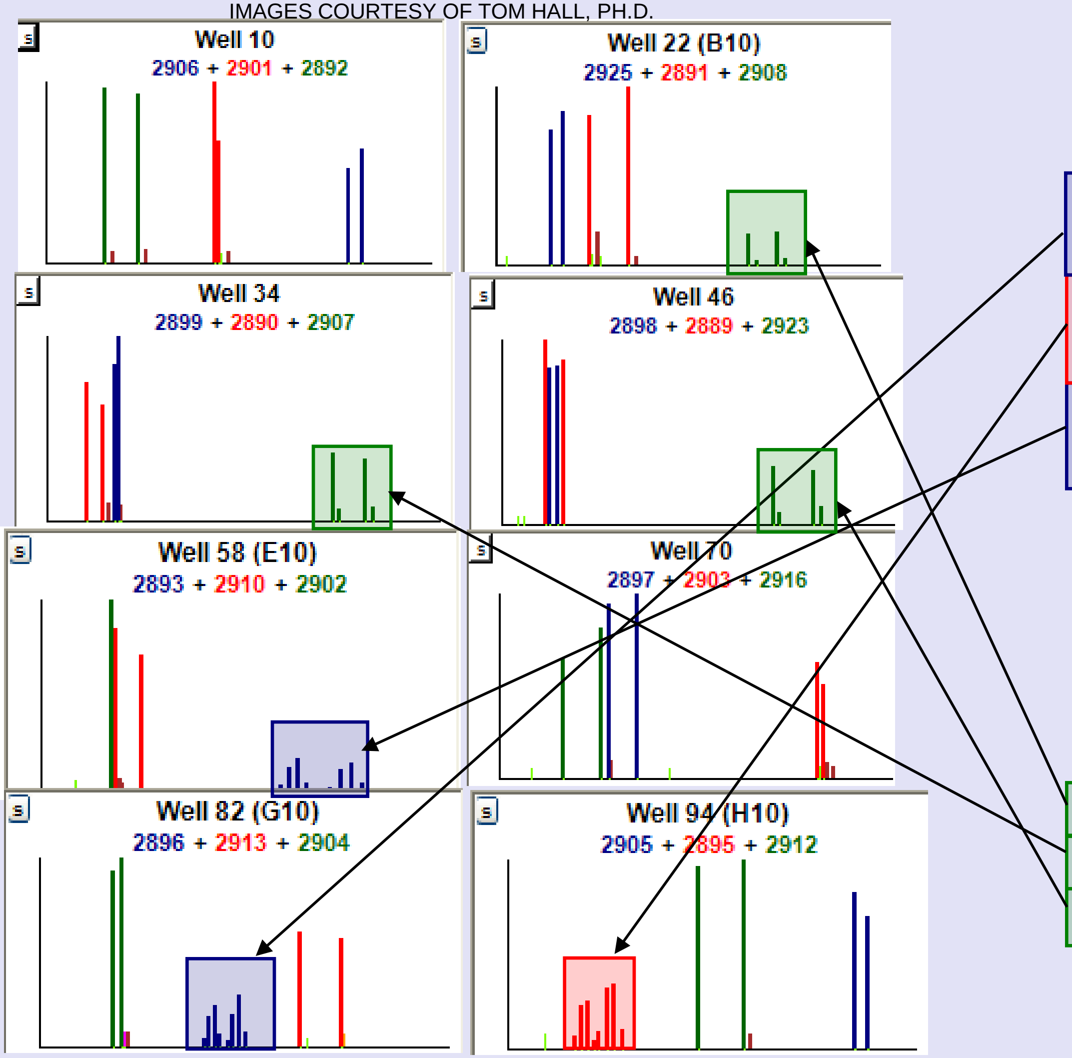

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2899: 15985..16073: A25 G16 C21 T27
2898: 16025..16119: A25 G18 C27 T25
2897: 16055..16155: A31 G12 C30 T28
2896: 16102..16224: A44 G12 C42 T24
2896: 16102..16224: A44 G12 C43 T24
2896: 16102..16224: A44 G12 C44 T24
2896: 16102..16224: A44 G12 C45 T24
2895: 16130..16224: A35 G7 C33 T19
2895: 16130..16224: A35 G7 C34 T19
2895: 16130..16224: A35 G7 C35 T19
2895: 16130..16224: A35 G7 C36 T19
2893: 16154..16268: A43 G7 C47 T17
2893: 16154..16268: A43 G7 C48 T17
2893: 16154..16268: A43 G7 C49 T17
2893: 16154..16268: A43 G7 C50 T17
2892: 16231..16338: A40 G9 C39 T20
2891: 16256..16366: A37 G9 C41 T24
2890: 16318..16402: A20 G14 C31 T20
2889: 16357..16451: A21 G17 C36 T21
2902: 5..97: A19 G24 C24 T26
2903: 20..139: A24 G34 C29 T33
2904: 83..187: A23 G21 C30 T31
2905: 113..245: A39 G18 C30 T46
2905: 113..245: A39 G18 C29 T47
2906: 154..290: A48 G18 C32 T39
2906: 154..290: A48 G18 C31 T40
2908: 204..330: A42 G16 C39 T32
2908: 204..330: A42 G16 C40 T32
2907: 239..363: A43 G11 C49 T24
2907: 239..363: A43 G11 C50 T24
2923: 262..390: A47 G10 C53 T21
2923: 262..390: A47 G10 C54 T21
2910: 331..425: A33 G9 C27 T26
2916: 367..463: A27 G8 C32 T30
2912: 409..521: A32 G7 C48 T26
2913: 464..603: A43 G11 C63 T23

NIJ National Institute of Justice

Technology Transition Workshop

SNP Heteroplasmy Detection

From sequence profile

AF-4: 16024-16365
C 16176 N
T 16362 C

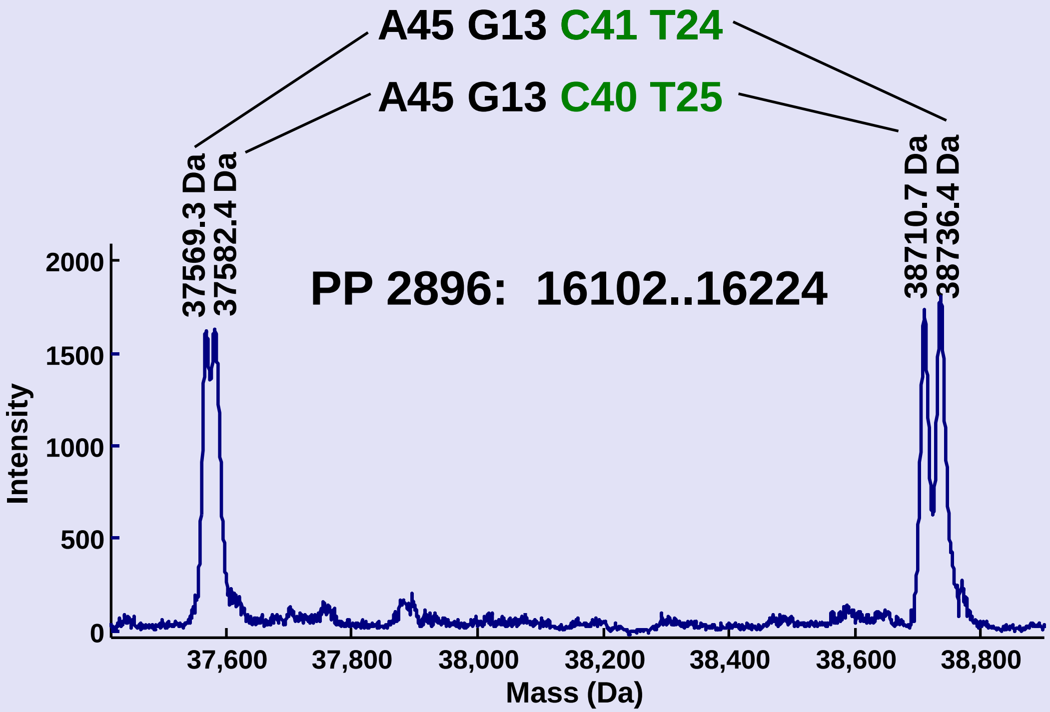
Observed profile

C/T heteroplasmy
16102..16224: A45 G13 C41 T24
16102..16224: A45 G13 C40 T25

C/T heteroplasmy
16130..16224: A36 G7 C33 T19
16130..16224: A36 G7 C32 T20

Calculated from truth key

16102..16224: A45 G13 C40 T24 N
16130..16224: A36 G7 C32 T19 N



From blinded sample AF-4

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SNP Heteroplasmy Detection

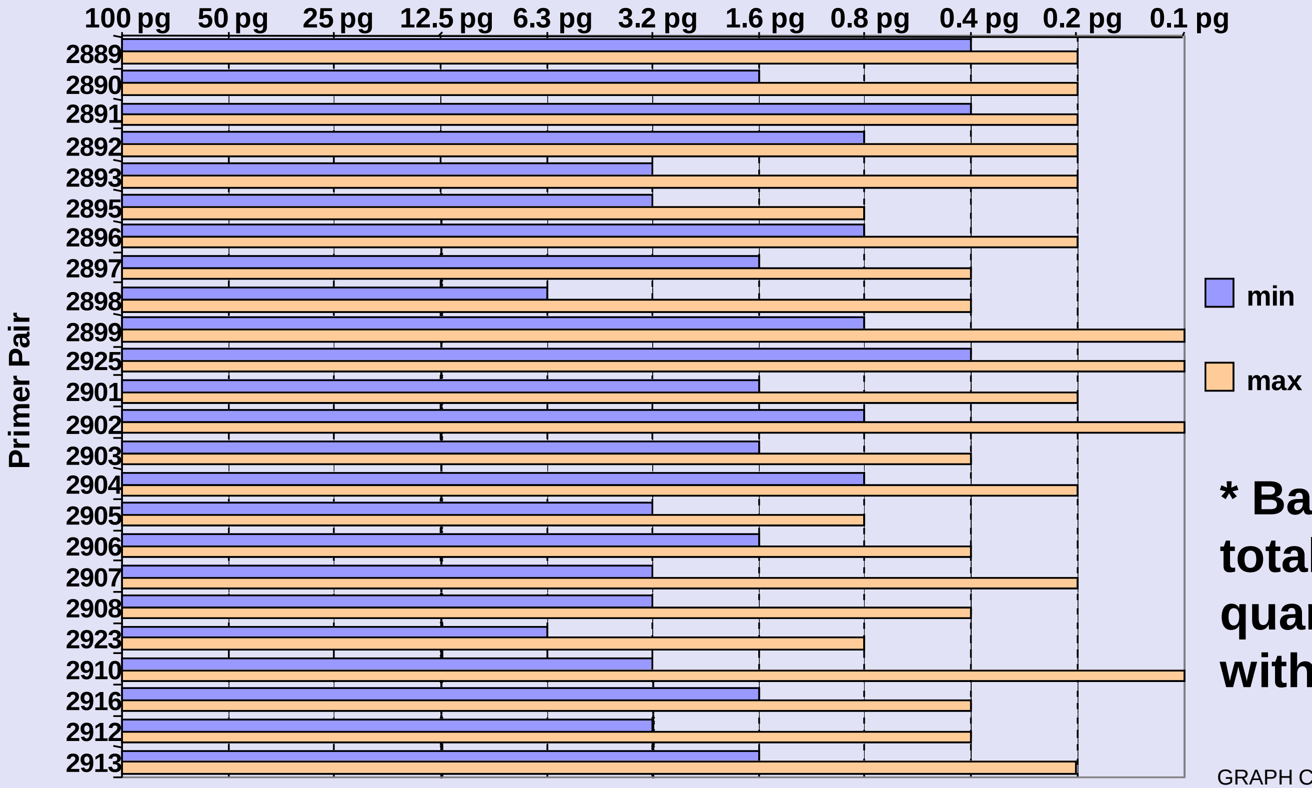
SNP heteroplasmies observed in blinded samples plus 21 in-house samples used for comparison to sequence data

Contributor	Sample	Hetero-plasmy	Replicates in observed region	Var. 1	Percentage of variant 1	Var. 2	Percentage of variant 2	Primer pairs	Amplified overlap	Sequence data
In-house	CS0021	C<->T	4	T	14.5 ± 0.6	C	85.5 ± 0.6	2898, 2897	16,078 - 16,098	16,093 Y
	CS0038	C<->T	1	T	25.4	C	74.6	2891	16,283 - 16,344	16,325 Y
	CS0040	C<->T	2	T	35.7 ± 0.3	C	64.3 ± 0.3	2898, 2897	16,078 - 16,098	16,093 Y
AFDIL	AF-2	C<->T	4	T	30.0 ± 2.2	C	70.0 ± 2.2	2892, 2891	16,283 - 16,305	16,293 N
	AF-4	C<->T	4	T	48.6 ± 2.2	C	51.4 ± 2.2	2896, 2895	16,157 - 16,201	16,176 N
	AF-15	A<->G	4	A	21.1 ± 2.0	G	78.9 ± 2.0	2897, 2896	16,124 - 16,129	ND
	AF-19	A<->G	4	G	44.8 ± 1.1	A	55.2 ± 1.1	2899, 2898	16,048 - 16,051	16,051 N
FBI	FBI-37	C<->T	4	T	22.9 ± 0.8	C	77.1 ± 0.8	2892, 2891	16,283 - 16,305	16,298 Y
	FBI-47	C<->T	4	T	17.3 ± 0.6	C	82.7 ± 0.6	2898, 2897	16,078 - 16,098	16,093 Y
	FBI-51	C<->T	2	T	45.9 +- 4.2	C	54.1 +- 4.2	2902, 2903	41 - 76	64 Y
	FBI-66	C<->T	4	C	48.5 ± 1.1	T	51.5 ± 1.1	2905, 2906	178 - 217	195 Y
	FBI-72	C<->T	4	T	39.8 ± 4.1	C	60.2 ± 4.1	2905, 2906	178 - 217	217 Y
NIST	NIST-JT51499	C<->T	4	T	41.4 ± 0.4	C	58.6 ± 0.4	2905, 2906	178 - 217	198 Y
	NIST-JT52076	C<->T	2	C	34.9 ± 0.1	T	65.1 ± 0.1	2892	16,254 - 16,305	16,260 Y
	NIST-PT84224	C<->T	4	T	48.3 ± 0.8	C	51.7 ± 0.8	2898, 2897	16,078 - 16,098	ND
	NIST-PT84231	C<->T	4	T	19.1 ± 0.5	C	80.9 ± 0.5	2905, 2906	178 - 217	204 Y

- **21 in-house samples had full control-region sequence data**
- **Other samples had sequence covering 12 to 22 of 24 primer pairs**
- **All profiles were consistent with sequence data**

Sensitivity

- ☐ **Analysis of 5 templates in dilution-to-extinction**
- ☐ **Sensitivity ranged from 0.1 pg to 6.3 pg template / primer pair ***
- ☐ **All templates had full profile at 6.3 pg or below per reaction**
- ☐ **Sensitivity criteria for standard QC plate set at 25 pg / reaction**



*** Based upon total nuclear DNA quantification with Quantifiler.**

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Reproducibility

- ❑ 3,331 trials of one positive control template run over the course of 27 months
- ❑ 500 pg template / reaction
- ❑ Automated data analysis
- ❑ 79,944 expected ds assignments
- ❑ 3,298 (99%) full, correct profiles
- ❑ 33 (1%) samples missing primer pair(s)
- ❑ 85 total missed assignments (0.11%)
- ❑ 113 artifact assignments (0.14%)
  - ➢ All but 10 (0.013%) correctable with manual QC
- ❑ 21 misassignments (0.026%)
  - ➢ All but 12 (0.015%) correctable with manual QC

Distribution of mass measurement deviations for 159,688 DNA strand assignments

Ave error magnitude was 10.12 ± 8.04 ppm

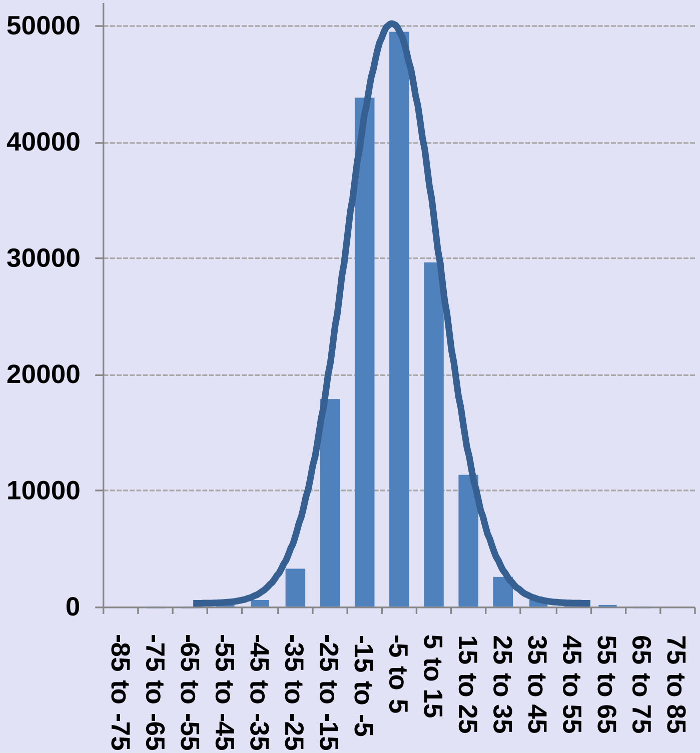

IMAGE COURTESY OF TOM HALL, PH.D.

Summary

- **1051 nucleotide positions covered by 24 primer pairs**
- **Accurate mass measurements and biochemical strategy allow mitochondrial base composition profiles to be developed**
- **Discrimination power is ~94% that of sequencing same region**
- **Discrimination power over tiling region can be greater than sequencing over minimum HV1 and HV2 ranges**
- **Base composition profiles can be compared to each other and to sequence profiles**
- **Databases can be searched and subjected to same type of statistics as a sequence database**
- **Mass spectrometry can resolve heteroplasmy (mixtures)**

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