



Technology Transition Workshop | *Thomas Hall, Ph.D.*

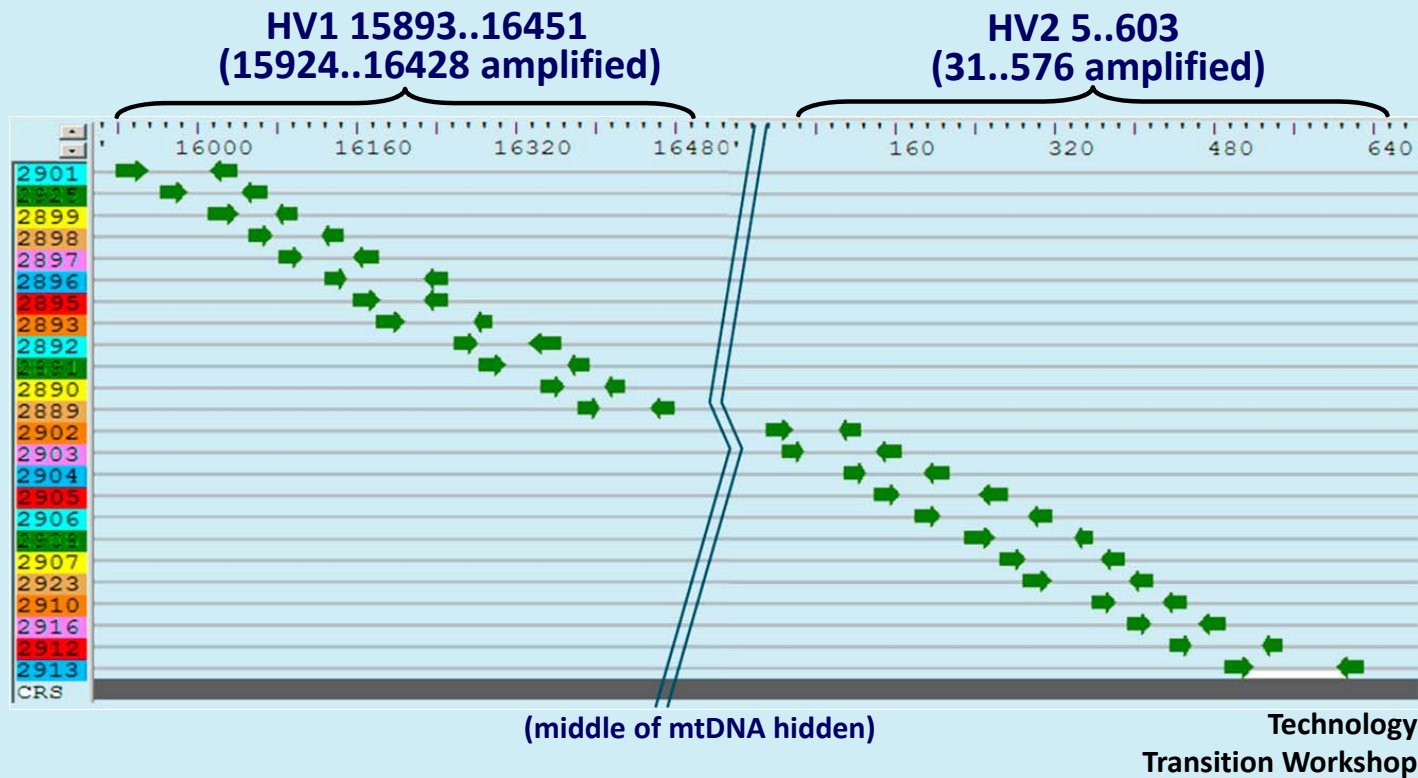
# ***Overview of the Ibis™ mtDNA Tiling Assay***

# ***Outline***

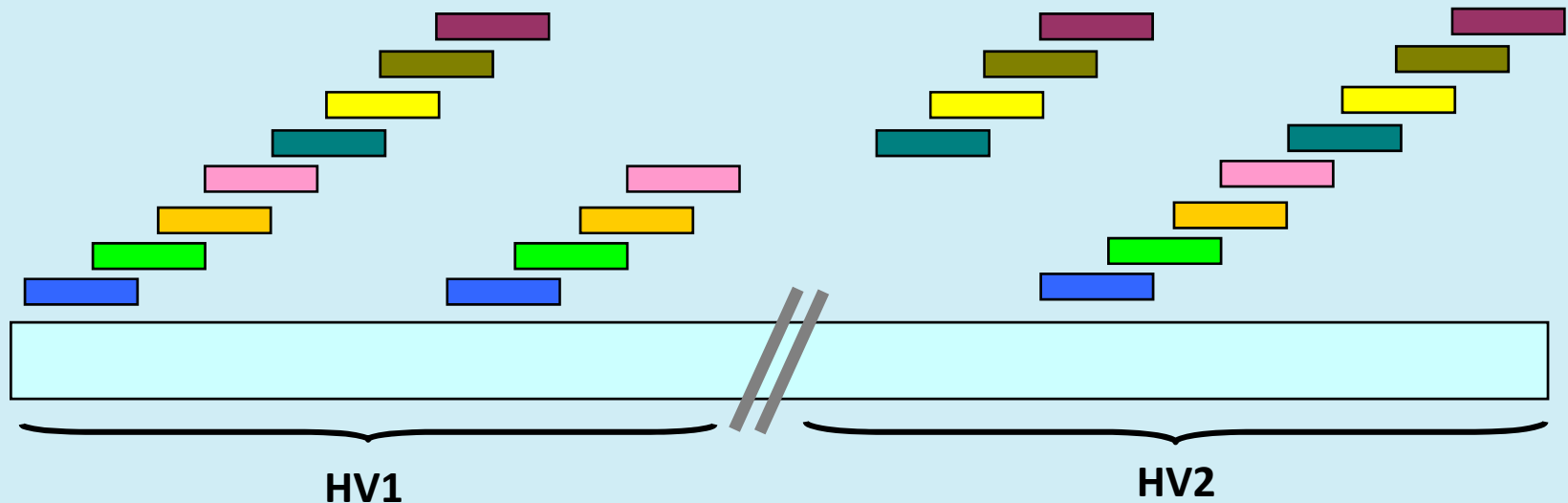
- **Ibis™ mtDNA assay format**
- **Base composition analysis of mtDNA samples**
  - **Data processing and analysis**
  - **Blinded samples**
- **Information content relative to sequencing**
- **Heteroplasmy detection**
- **Sensitivity**
- **Reproducibility**

# 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 eight triplexed sets (colored grouping) by maximum spatial separation and suitable mass separation of products



# *mtDNA Tiling Assay Format*

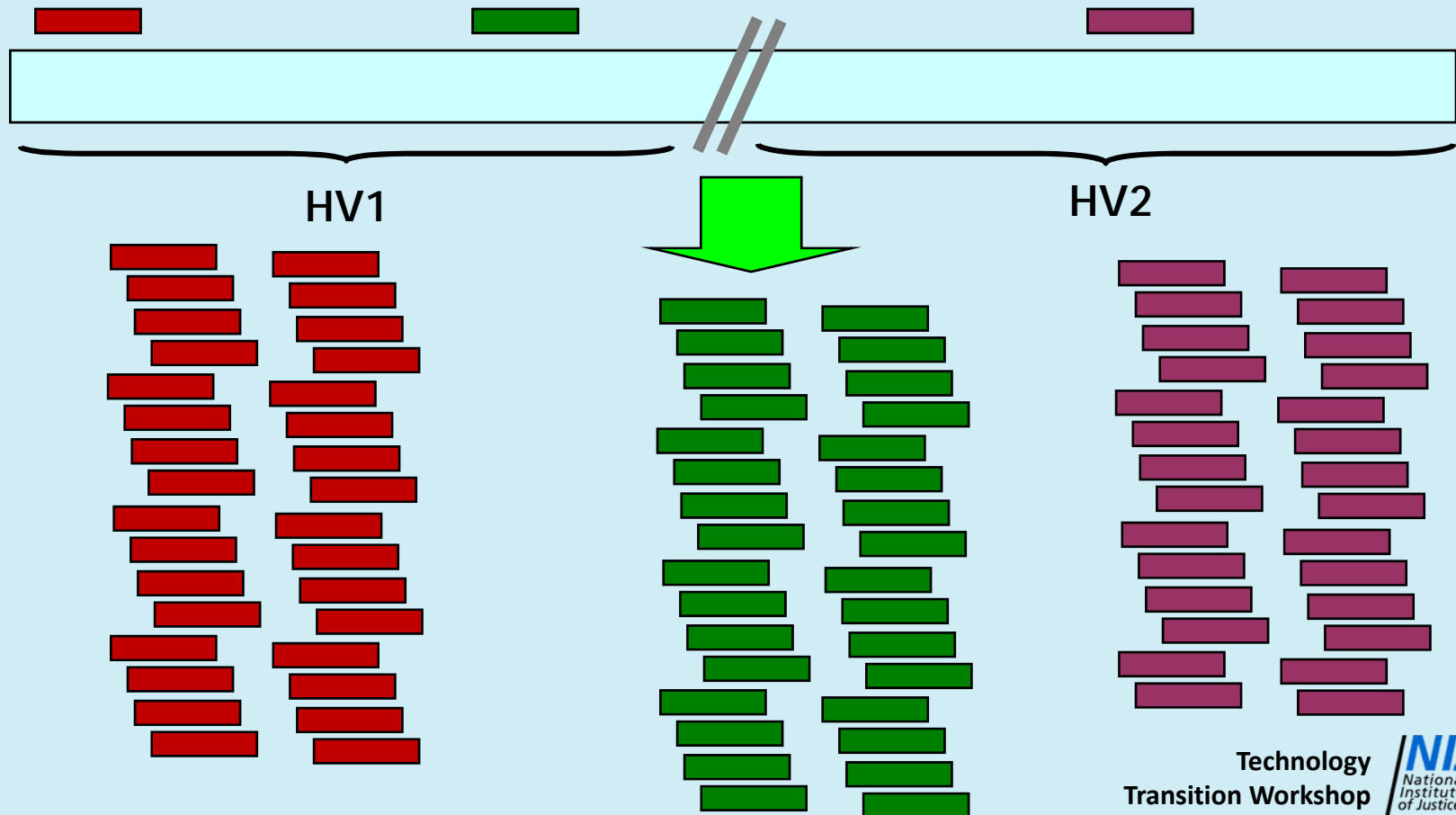


- Primers grouped to maximize target site separation
- PCR reactions performed with short extension cycle (five seconds)
- Product masses resolve from each other in triplex groupings
- Product sizes range from 85 to 140 bp; all but three are <130 bp
- Relative primer pair concentrations in triplexes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

# ***Base Composition Analysis***

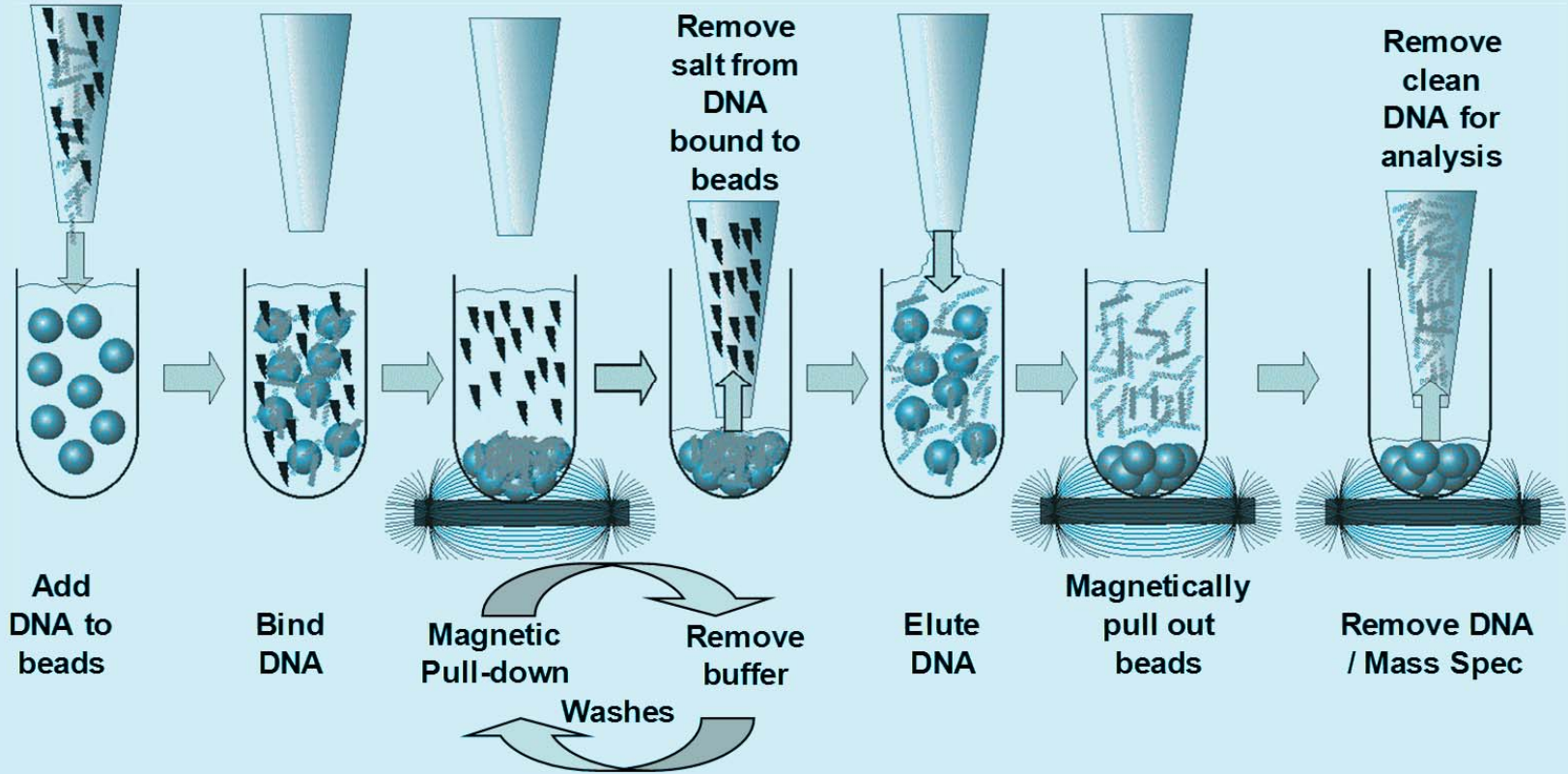
- 1. PCR**
- 2. Desalting**
- 3. ESI-TOF mass spectrometry**
- 4. Raw spectrum processing / deconvolution**
- 5. Base composition assignment / profile development**

# PCR: Three Primer Pairs Per Reaction



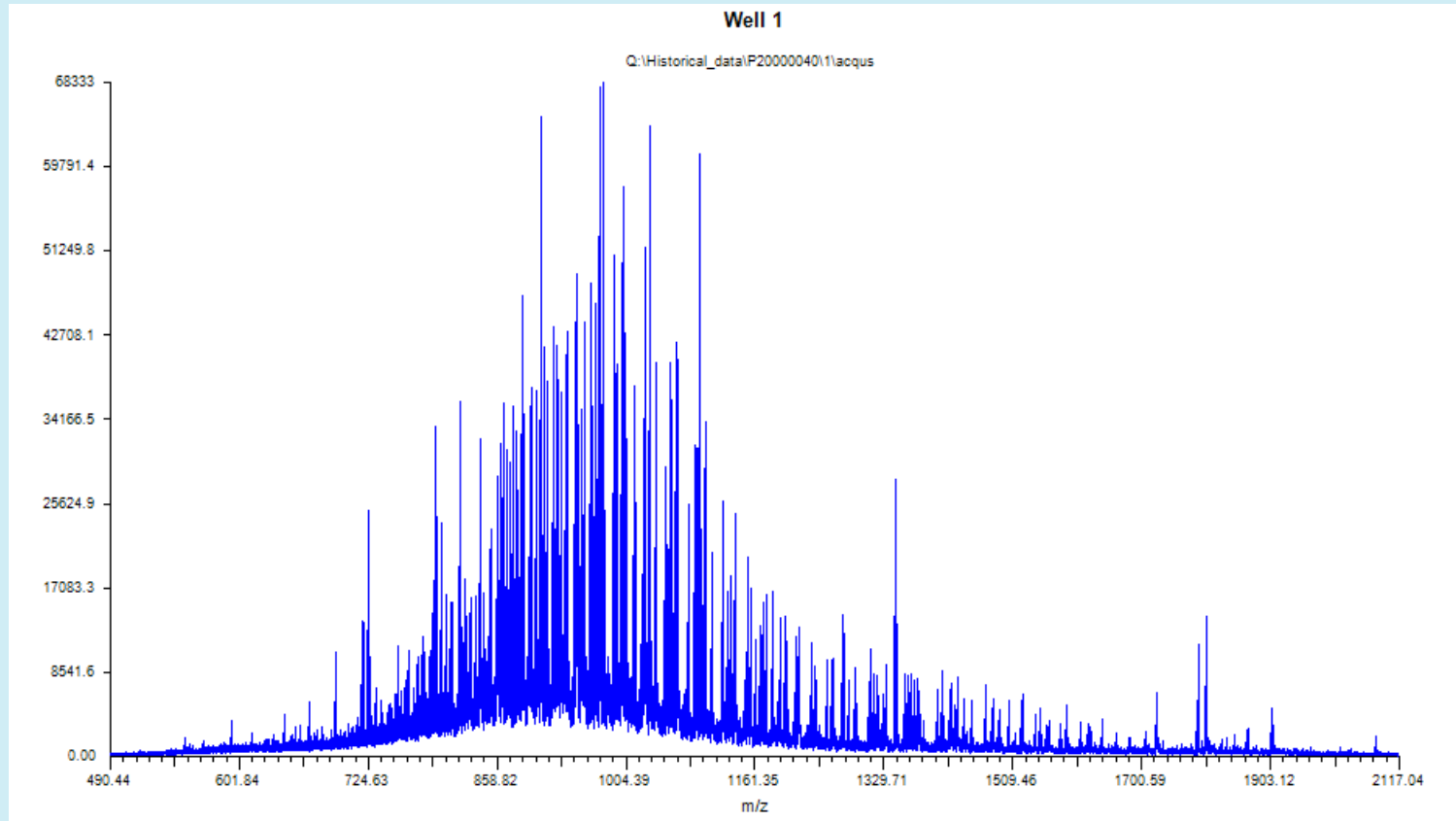
# Desalting of PCR Reactions

## Magnetic bead anion exchange



# ESI-TOF Mass Spectrometry

- Three primer pairs per reaction
- Complex raw spectrum



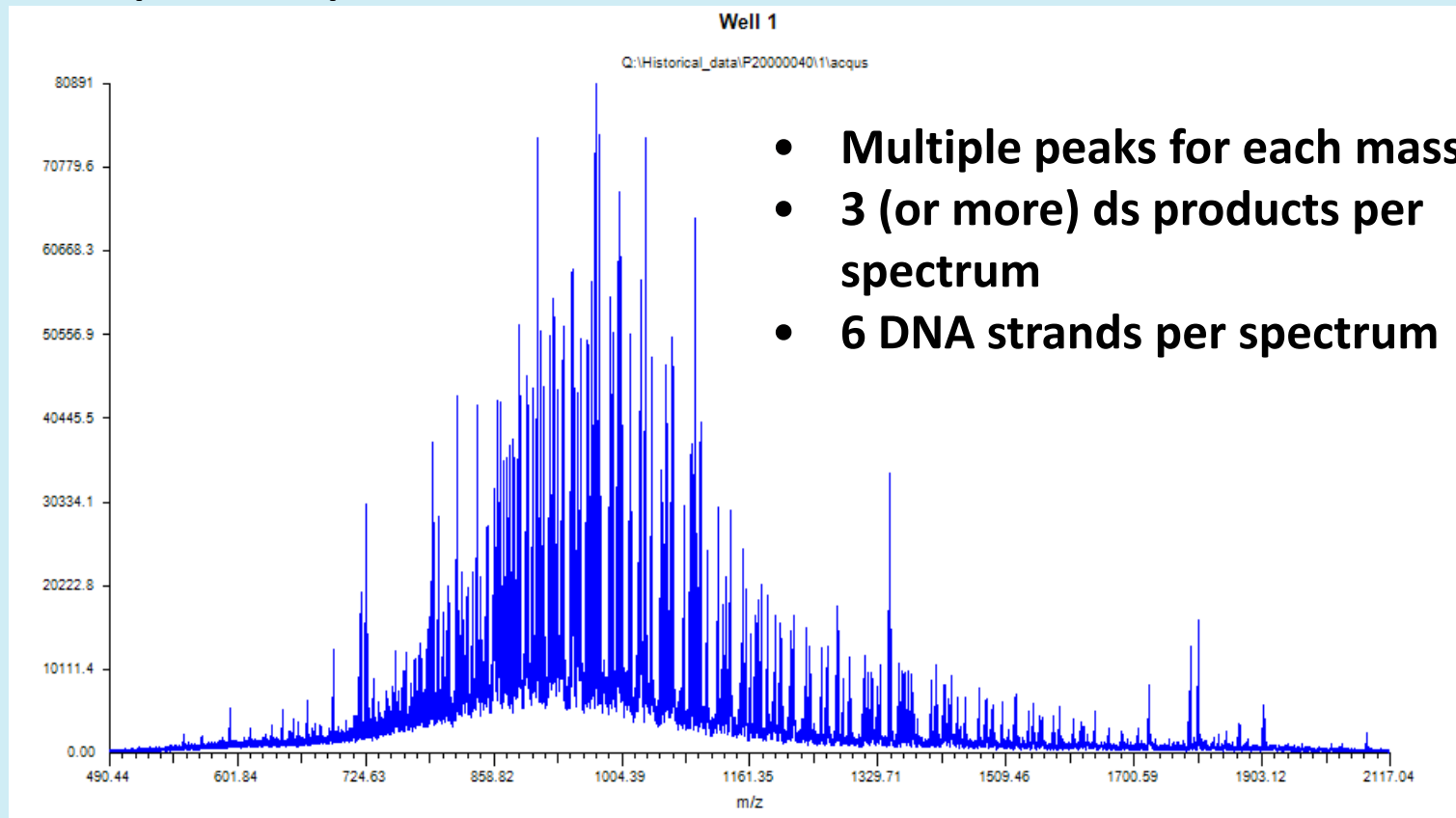
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# ESI-TOF Mass Spectrometry

- Three primer pairs per reaction
- Complex raw spectrum



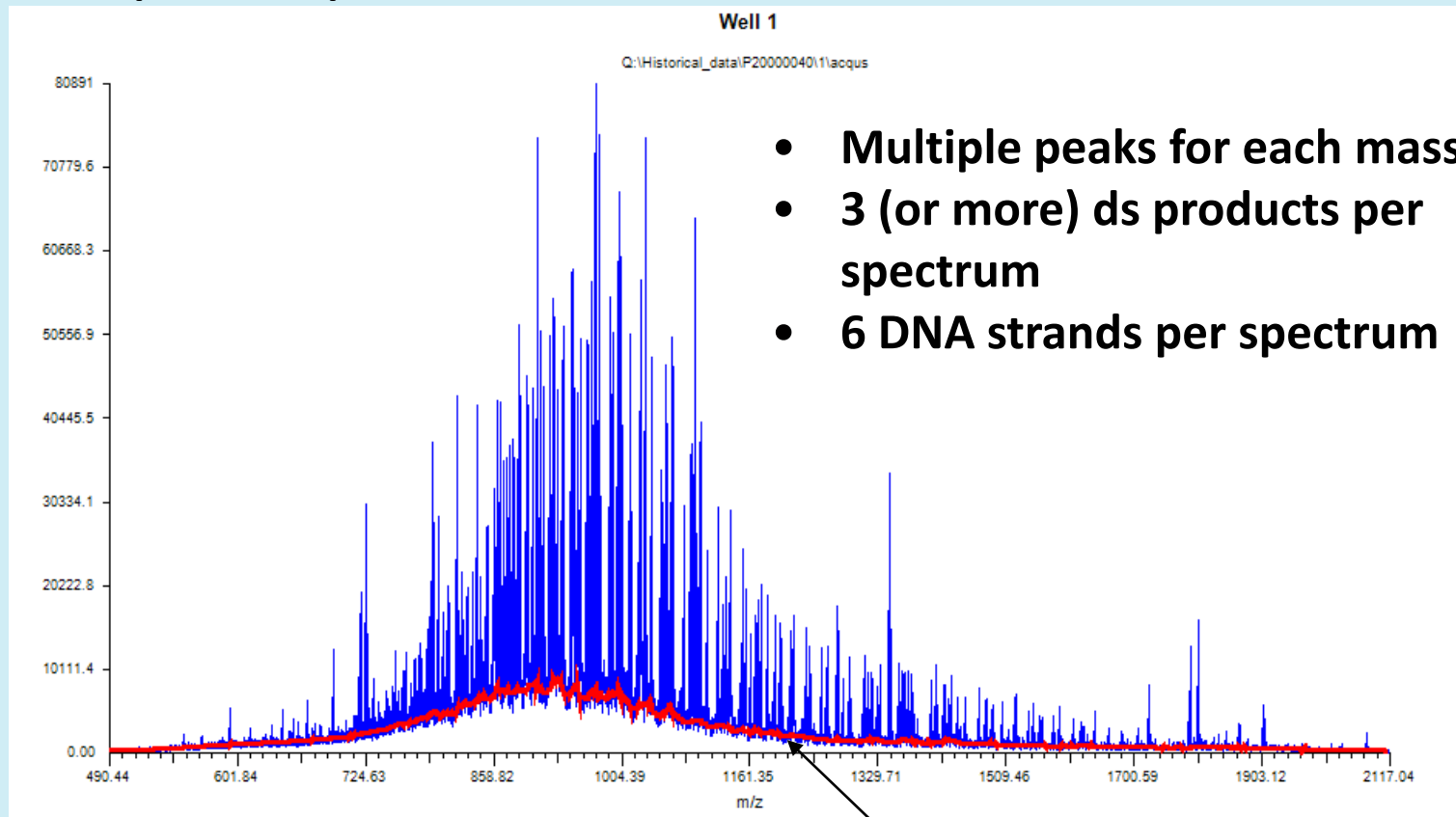
- Multiple peaks for each mass
- 3 (or more) ds products per spectrum
- 6 DNA strands per spectrum

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# ESI-TOF Mass Spectrometry

- 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

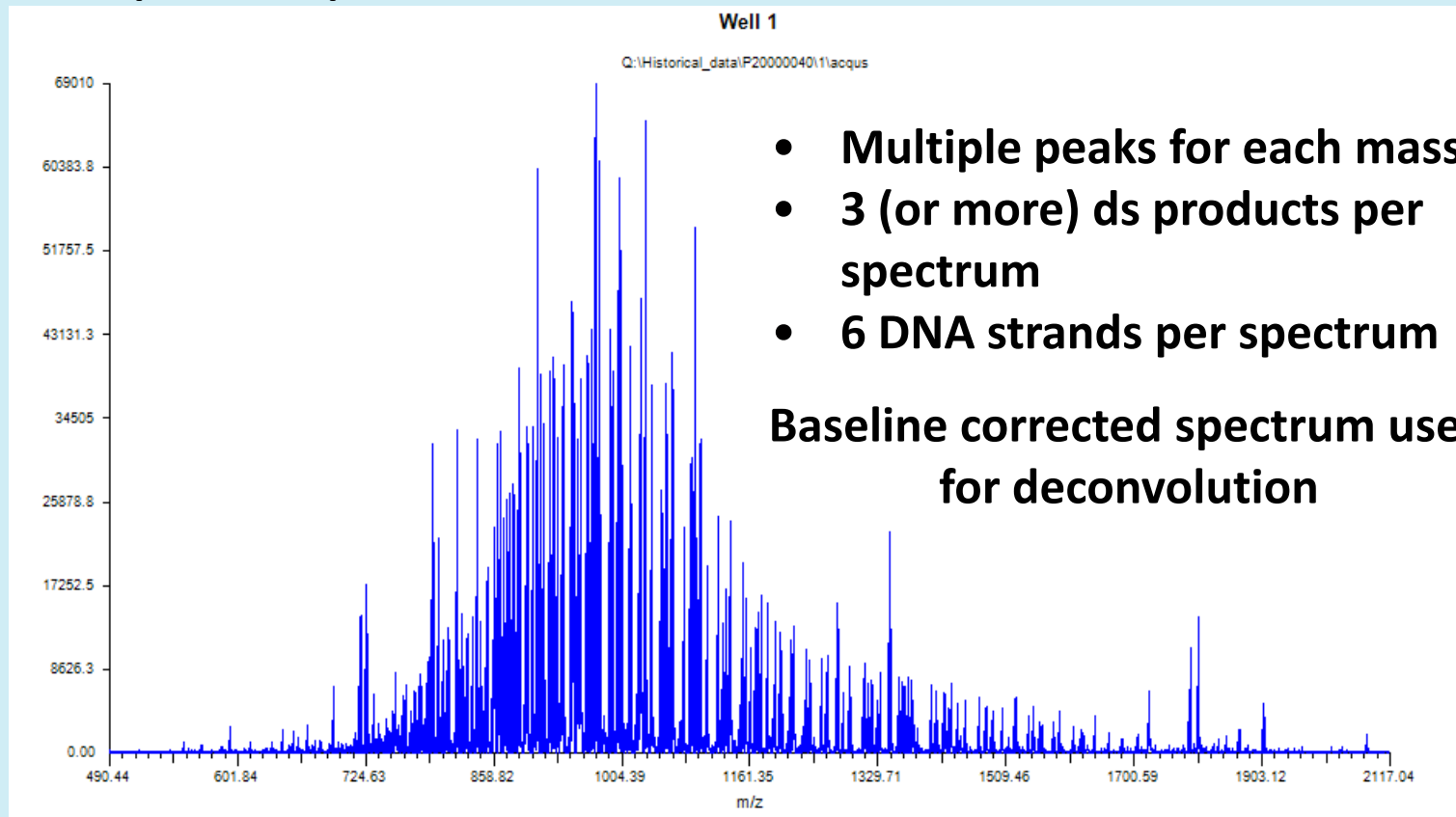
Noise baseline

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# ESI-TOF Mass Spectrometry

- 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

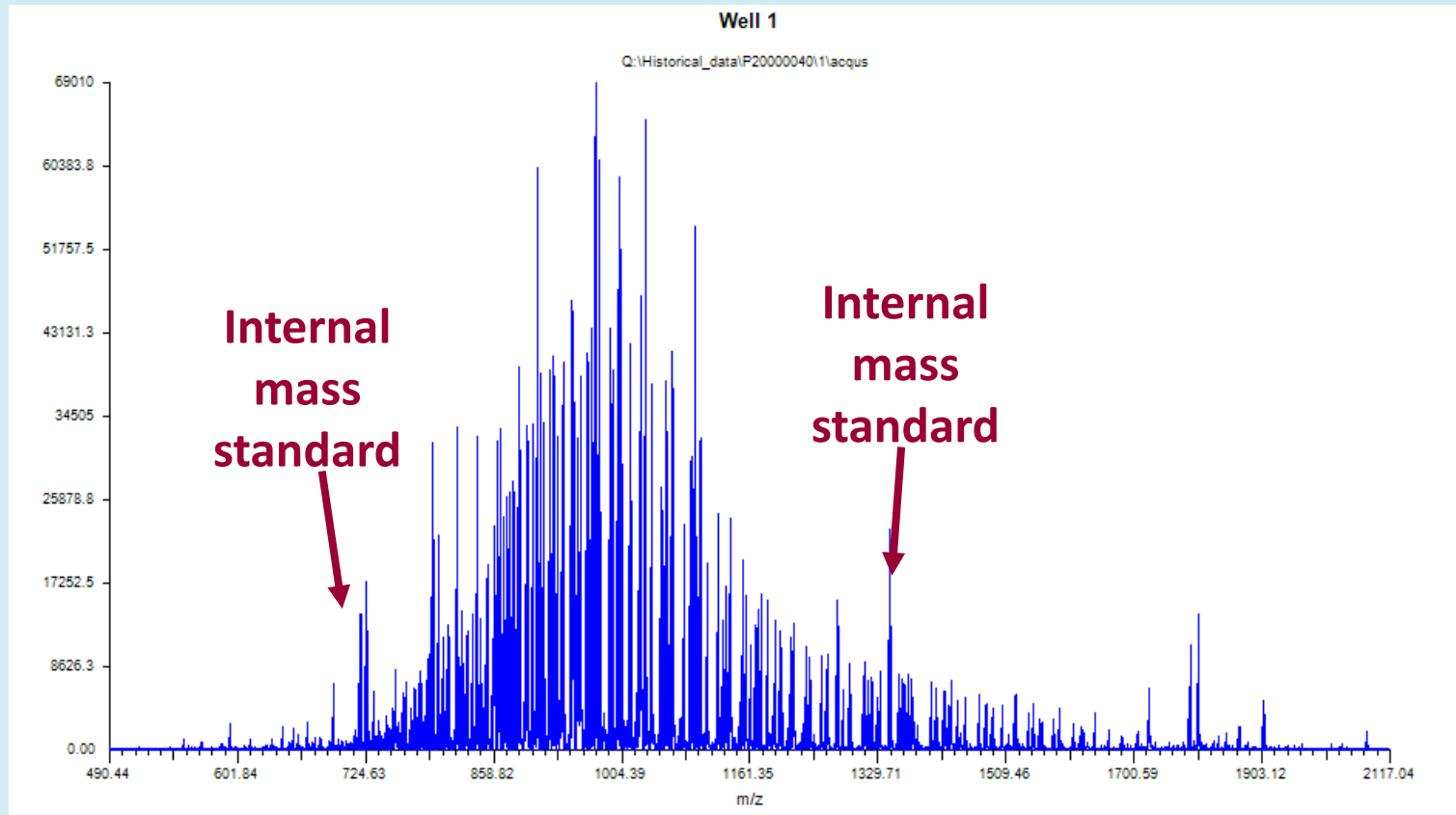
**Baseline corrected spectrum used for deconvolution**

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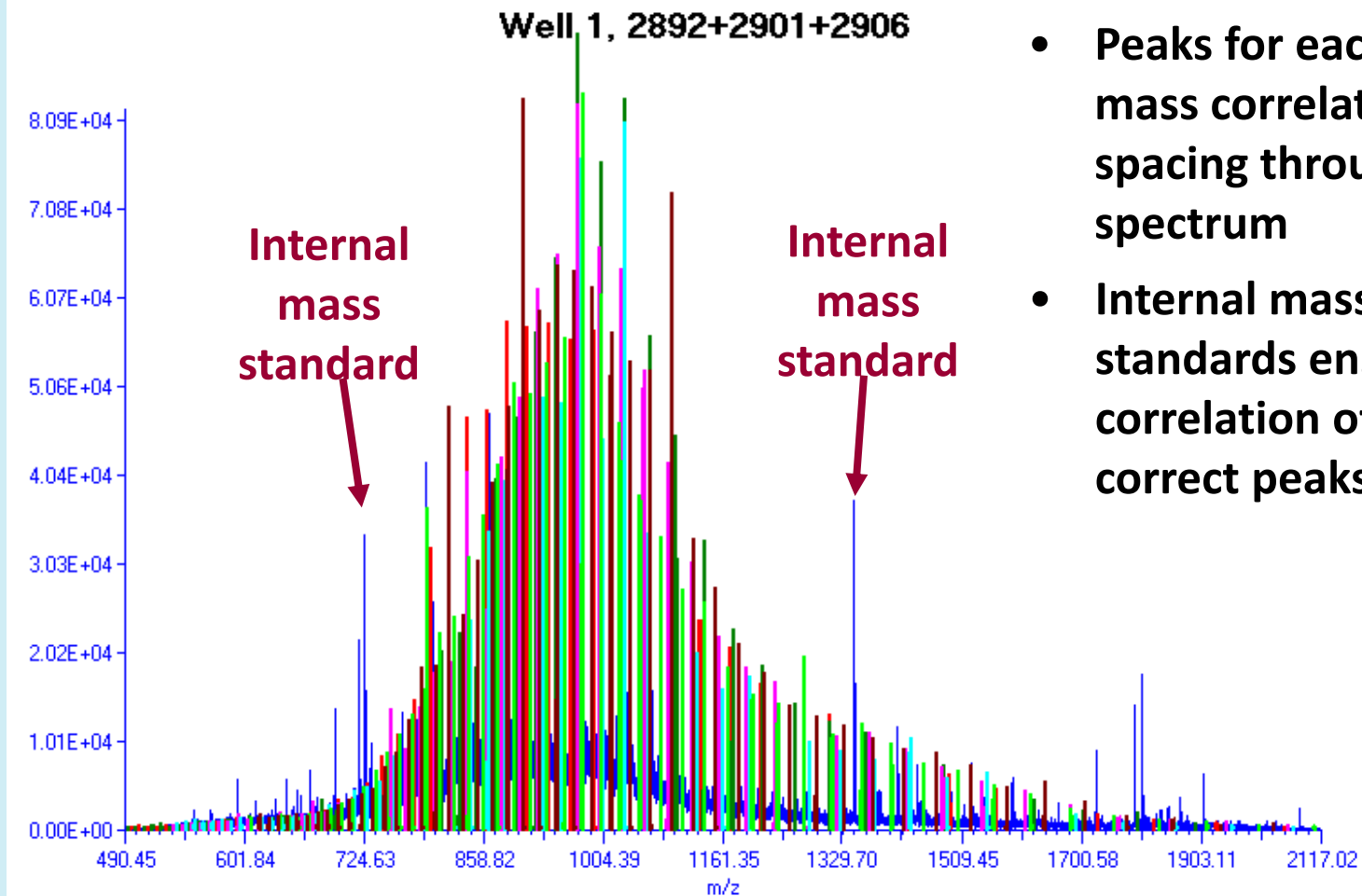


# Raw Spectrum Processing

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



# Spectral Deconvolution



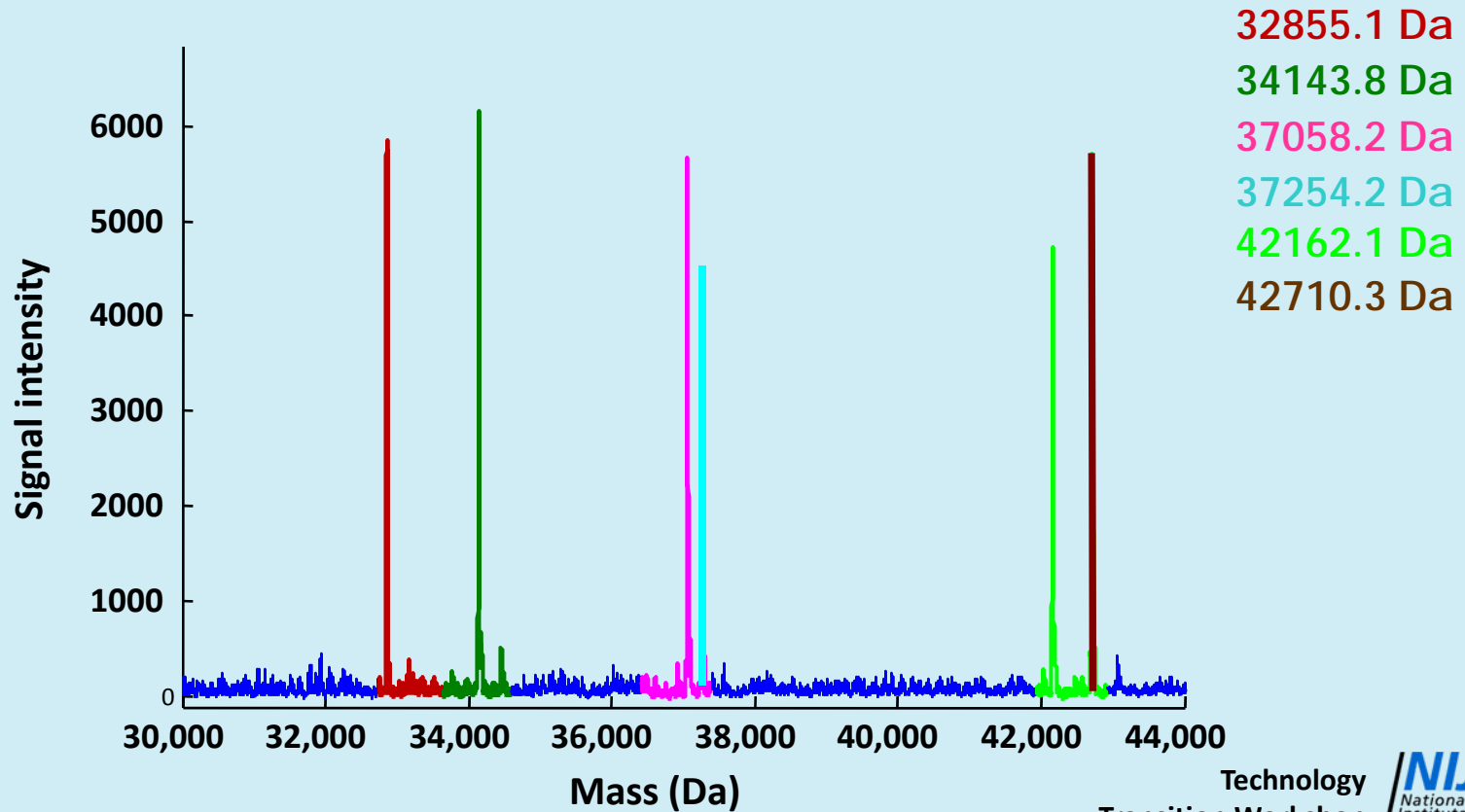
- Peaks for each mass correlated by spacing through spectrum
- Internal mass standards ensure correlation of the correct peaks

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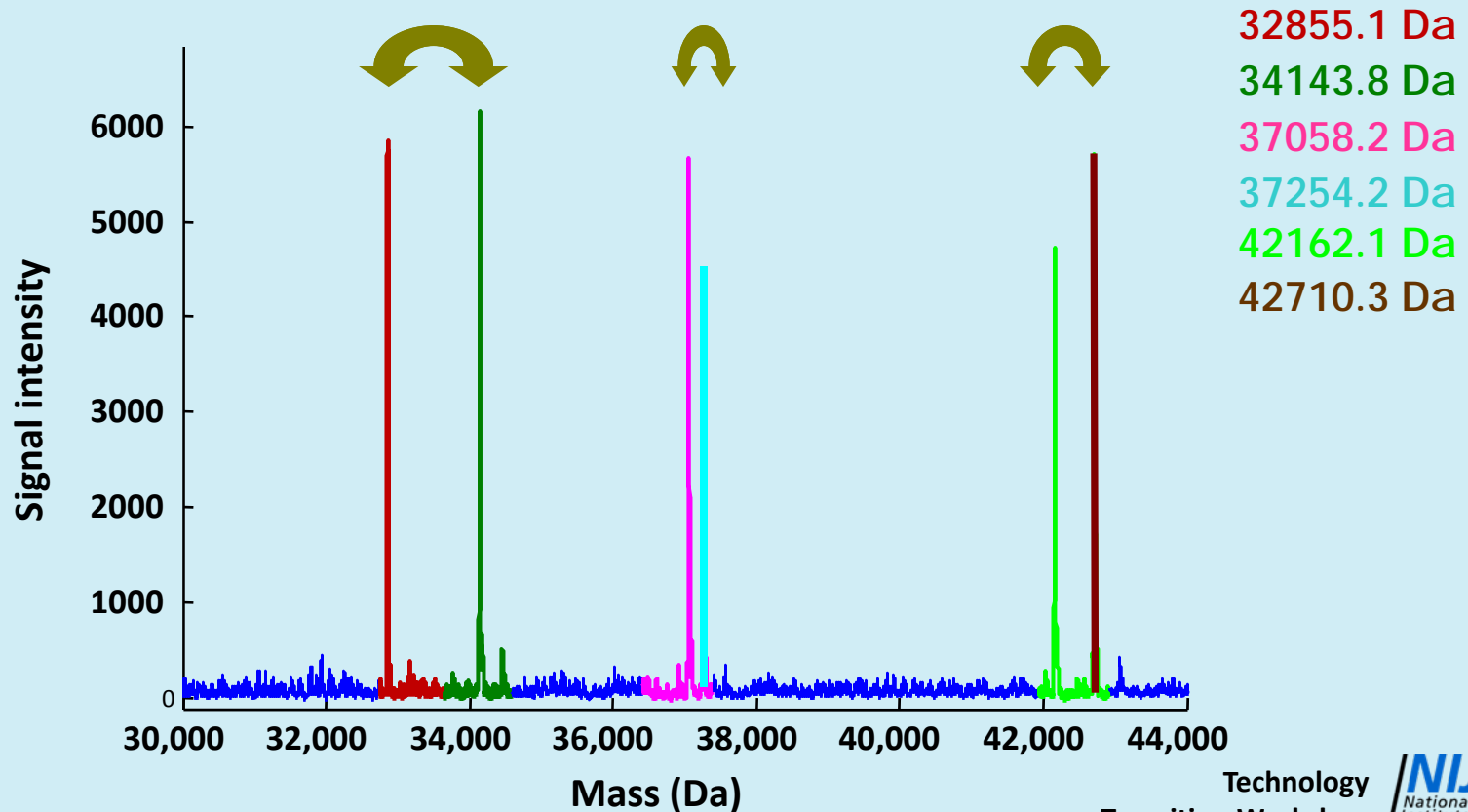
# Deconvolution to Masses

Deconvolution results in one final measurement per molecular species



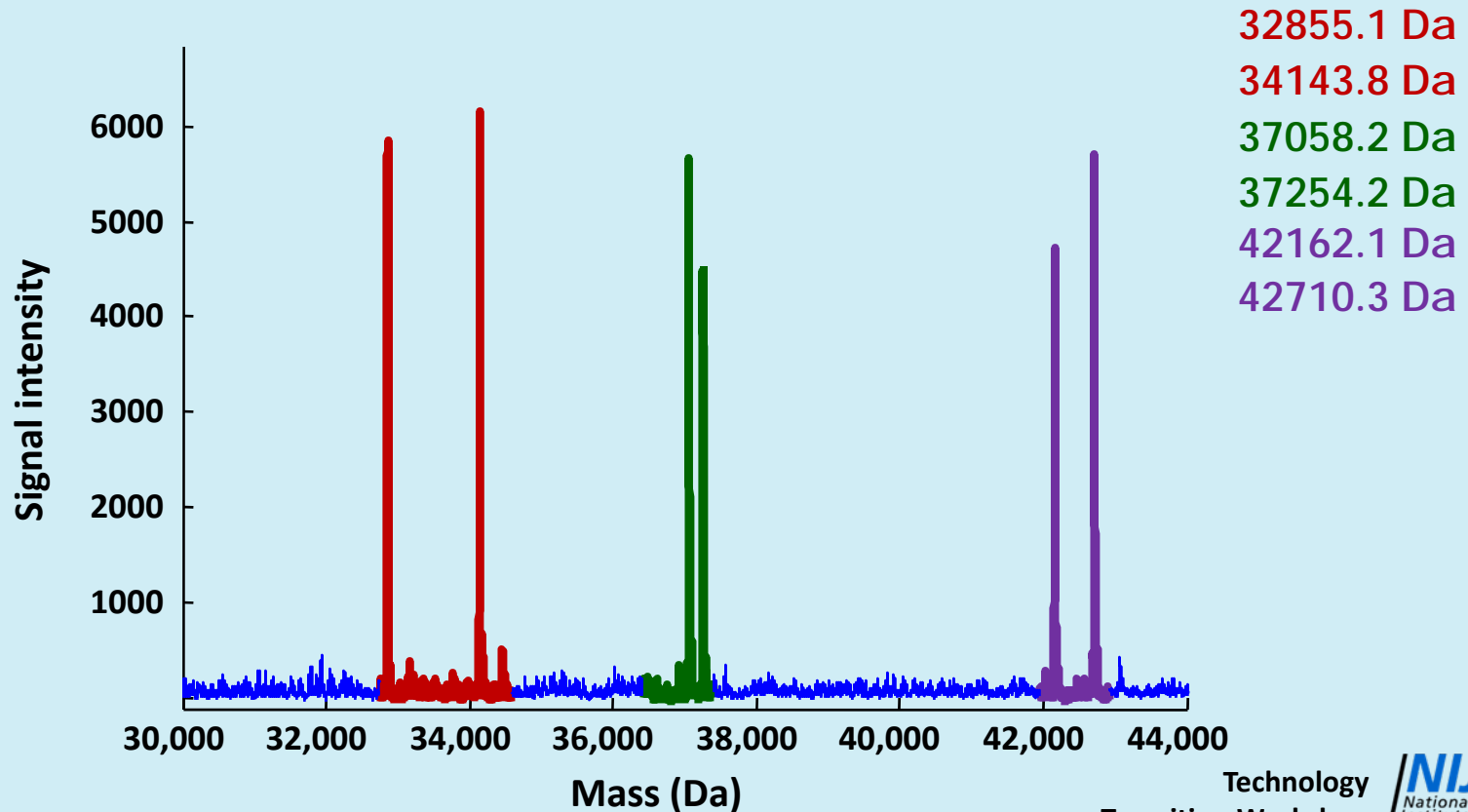
# DS DNA Strand Association

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



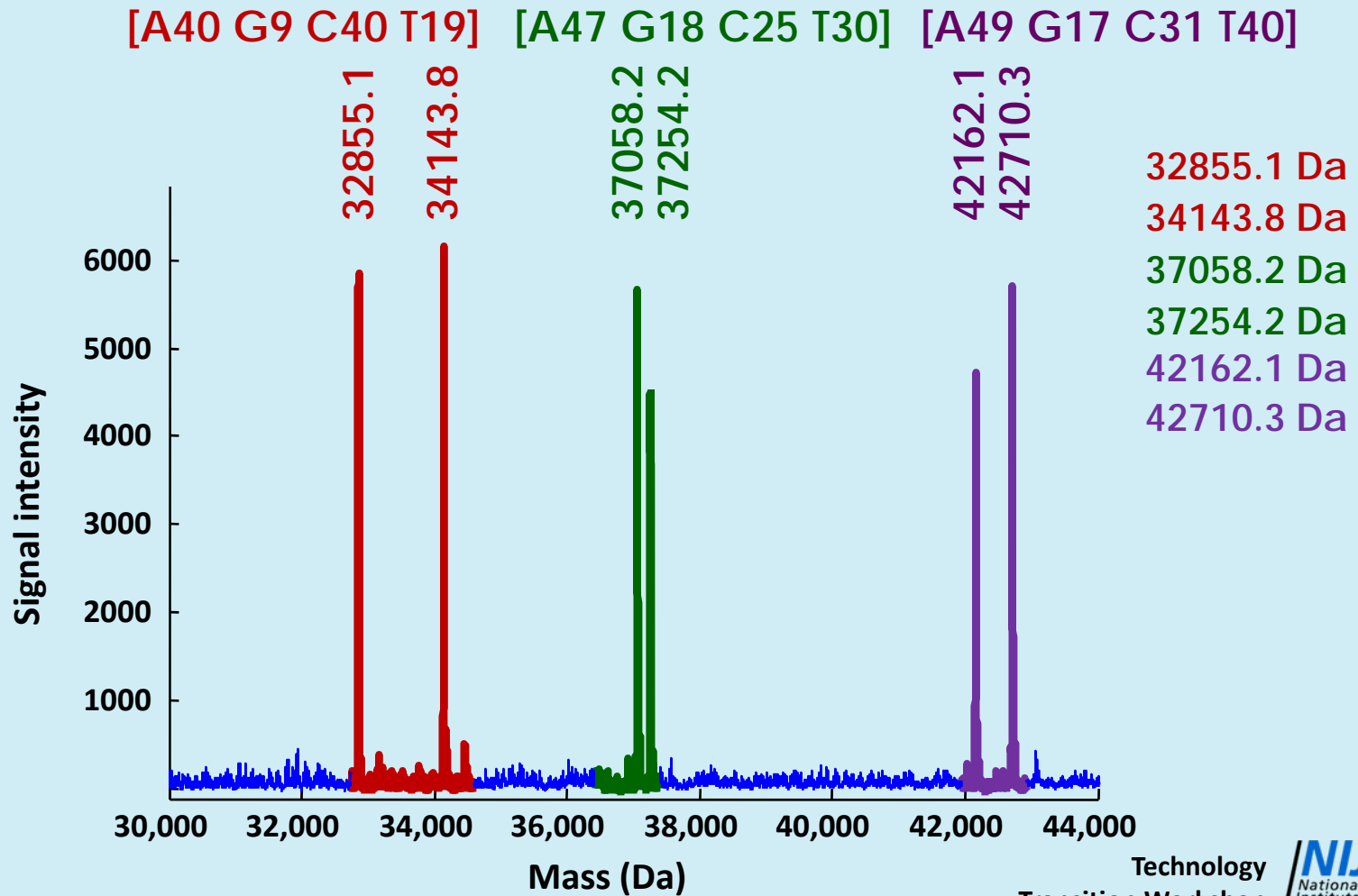
# *DS DNA Strand Association*

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

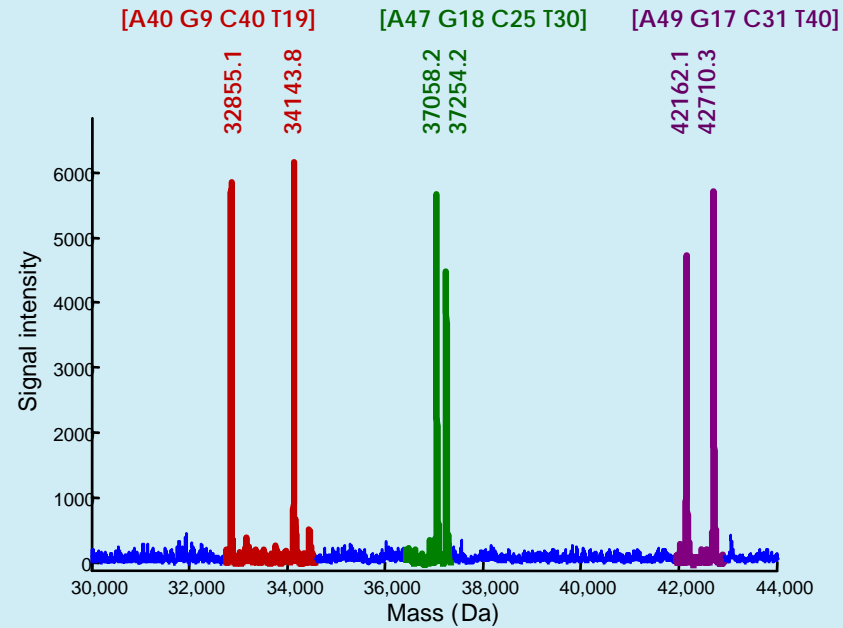




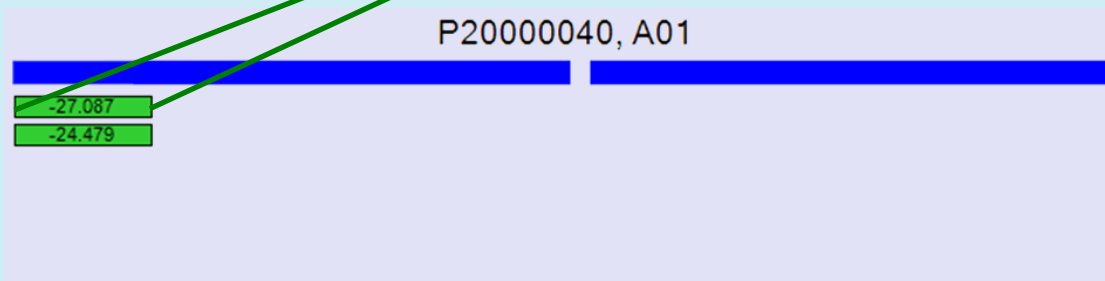
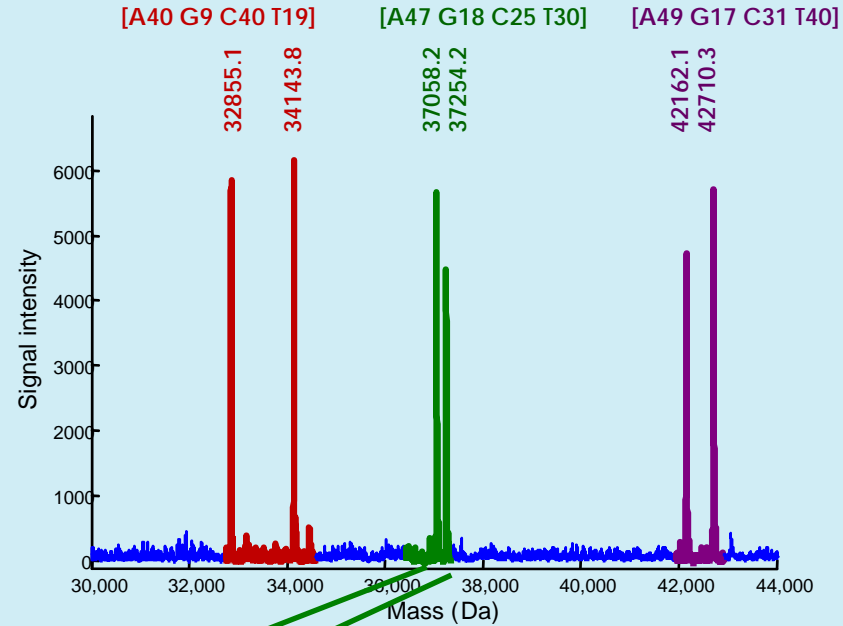
# Base Composition Assignment



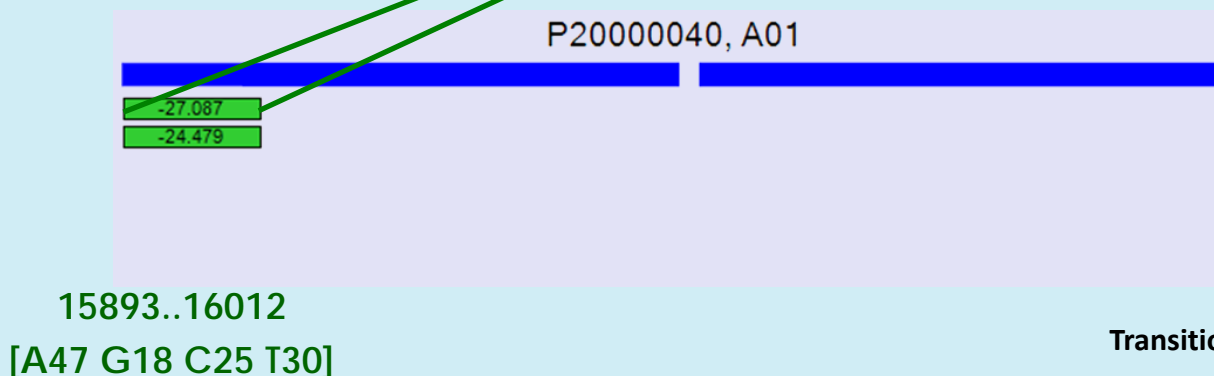
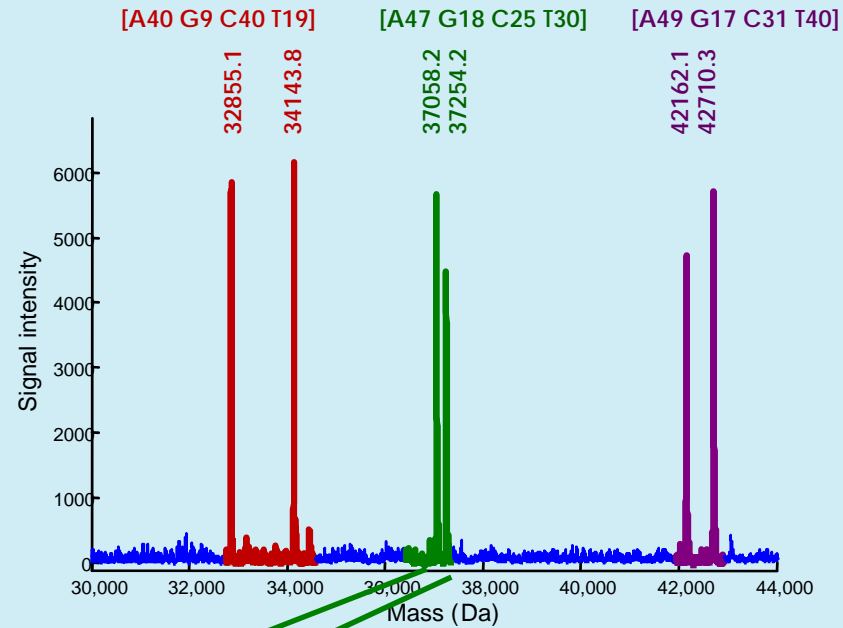
# Base Composition Assignment



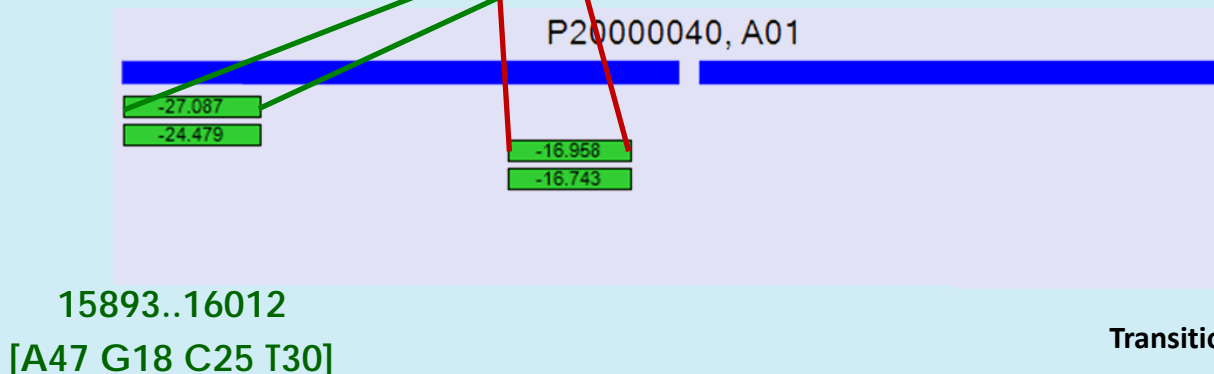
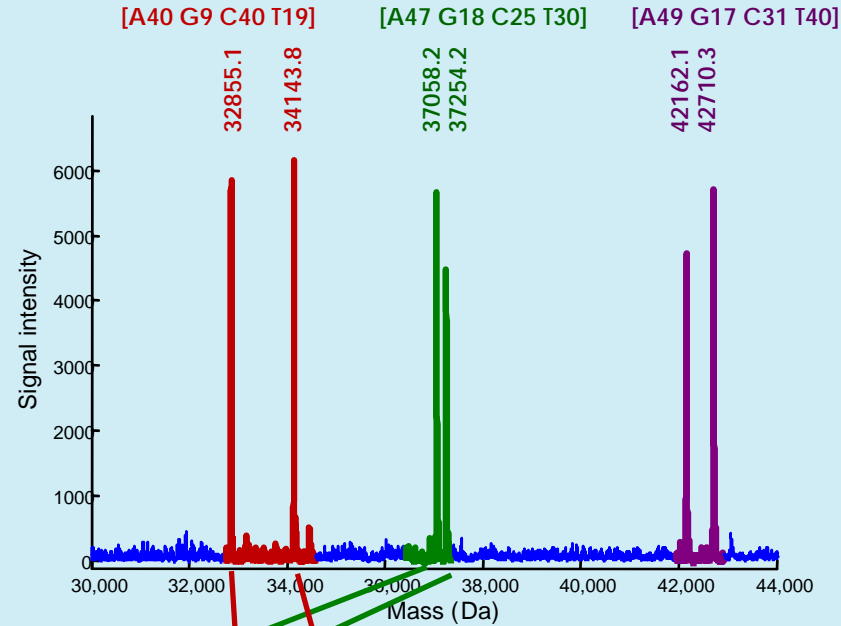
# Final Product Assignment



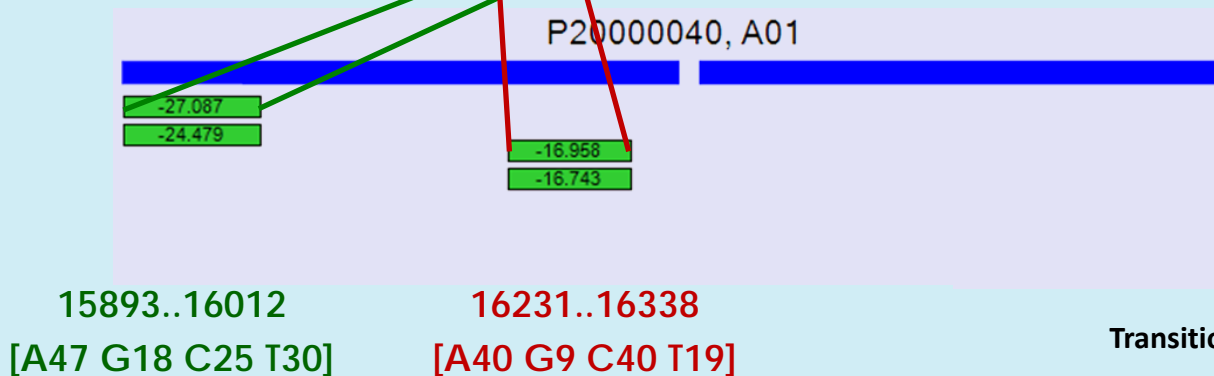
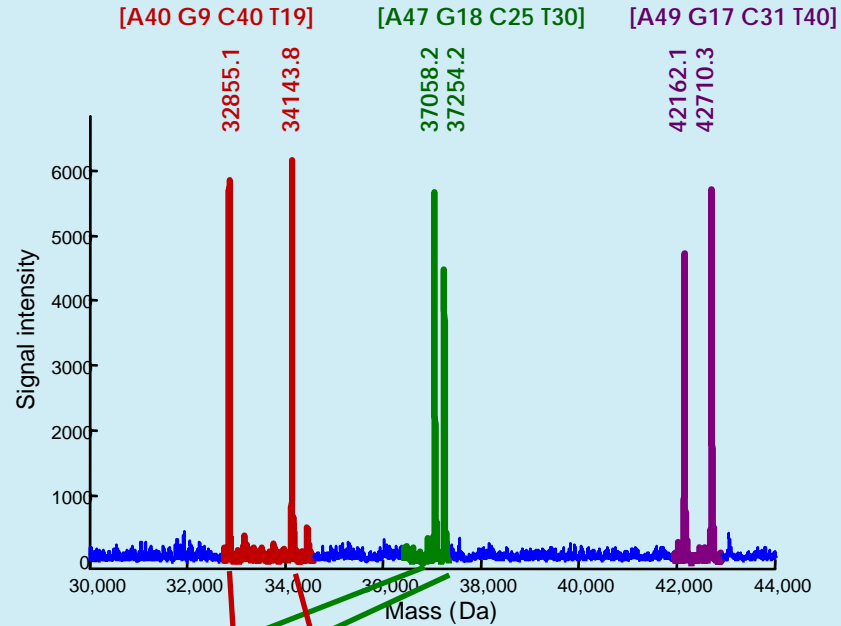
# Final Product Assignment



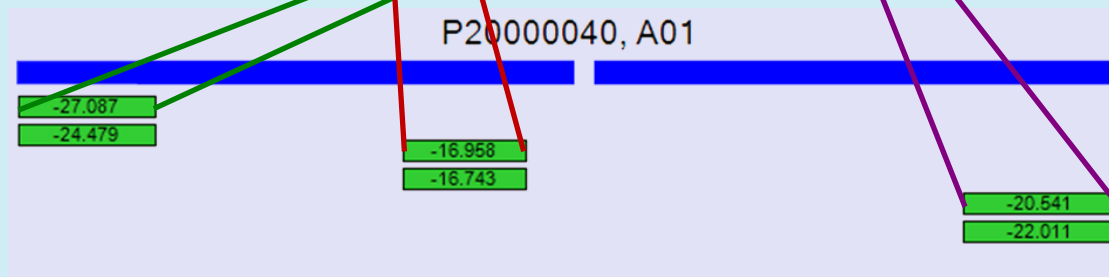
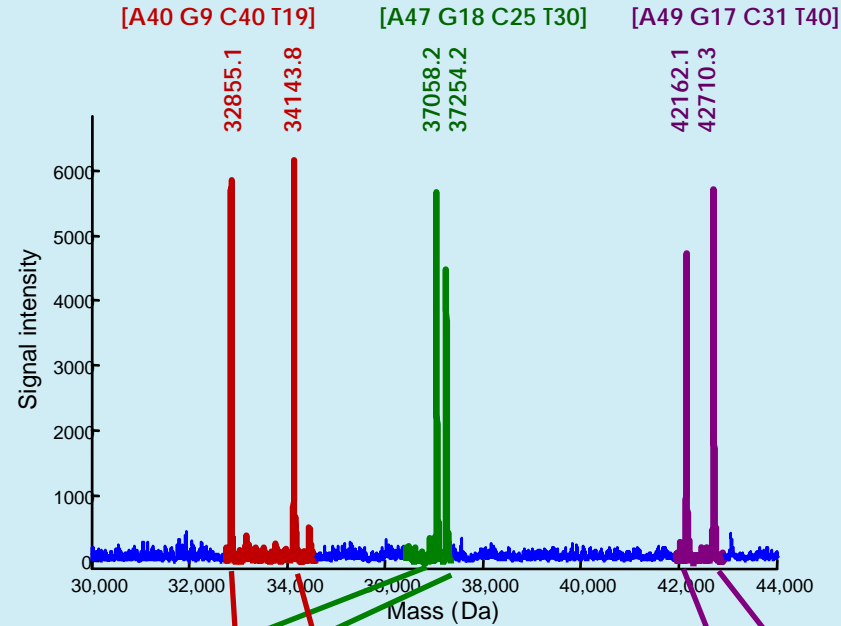
# Final Product Assignment



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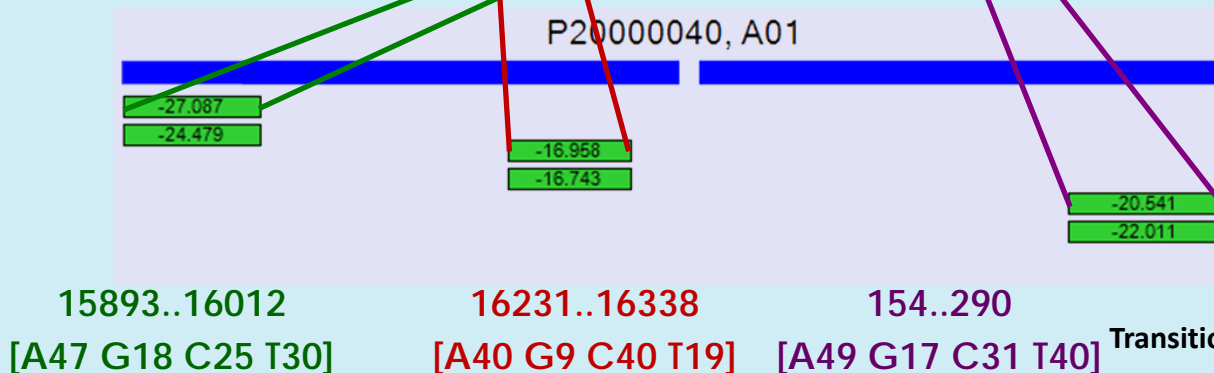
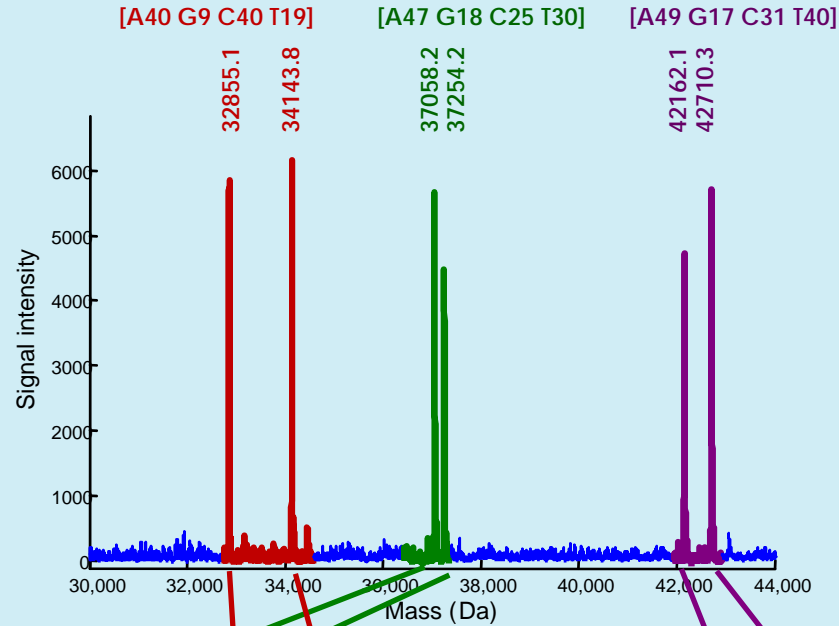
# Final Product Assignment



15893..16012  
[A47 G18 C25 T30]

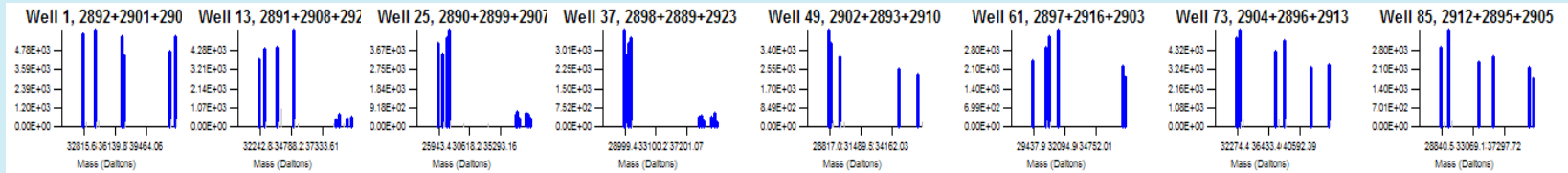
16231..16338  
[A40 G9 C40 T19]

# Final Product Assignment

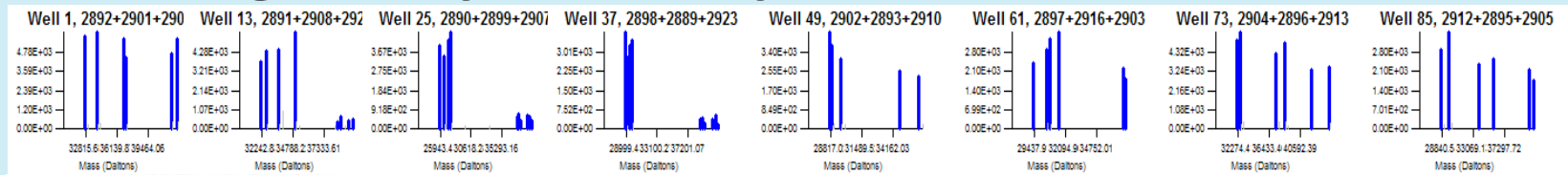




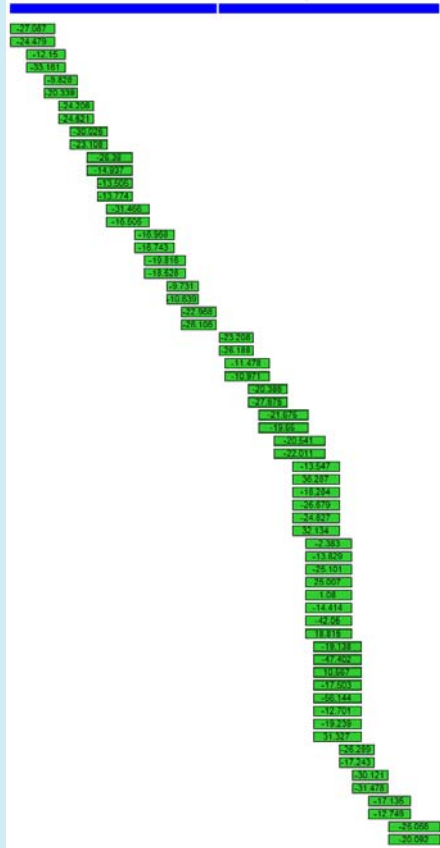
# Eight Spectra Per Sample



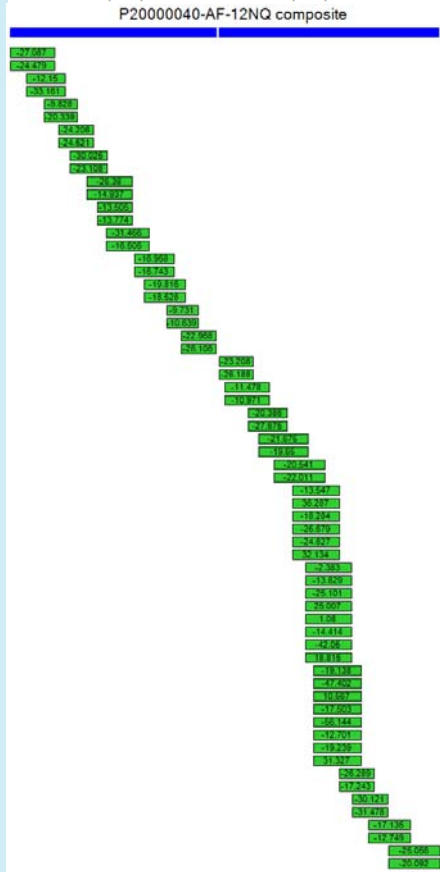
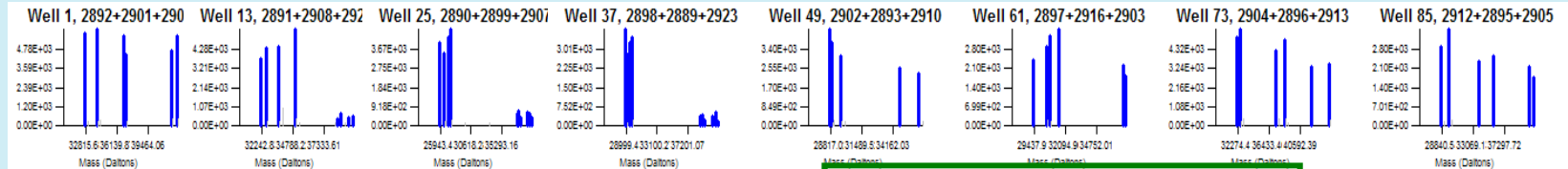
# Coverage Map Development



P20000040-AF-12NQ composite



# Base Composition Profile



Base  
composition  
profile

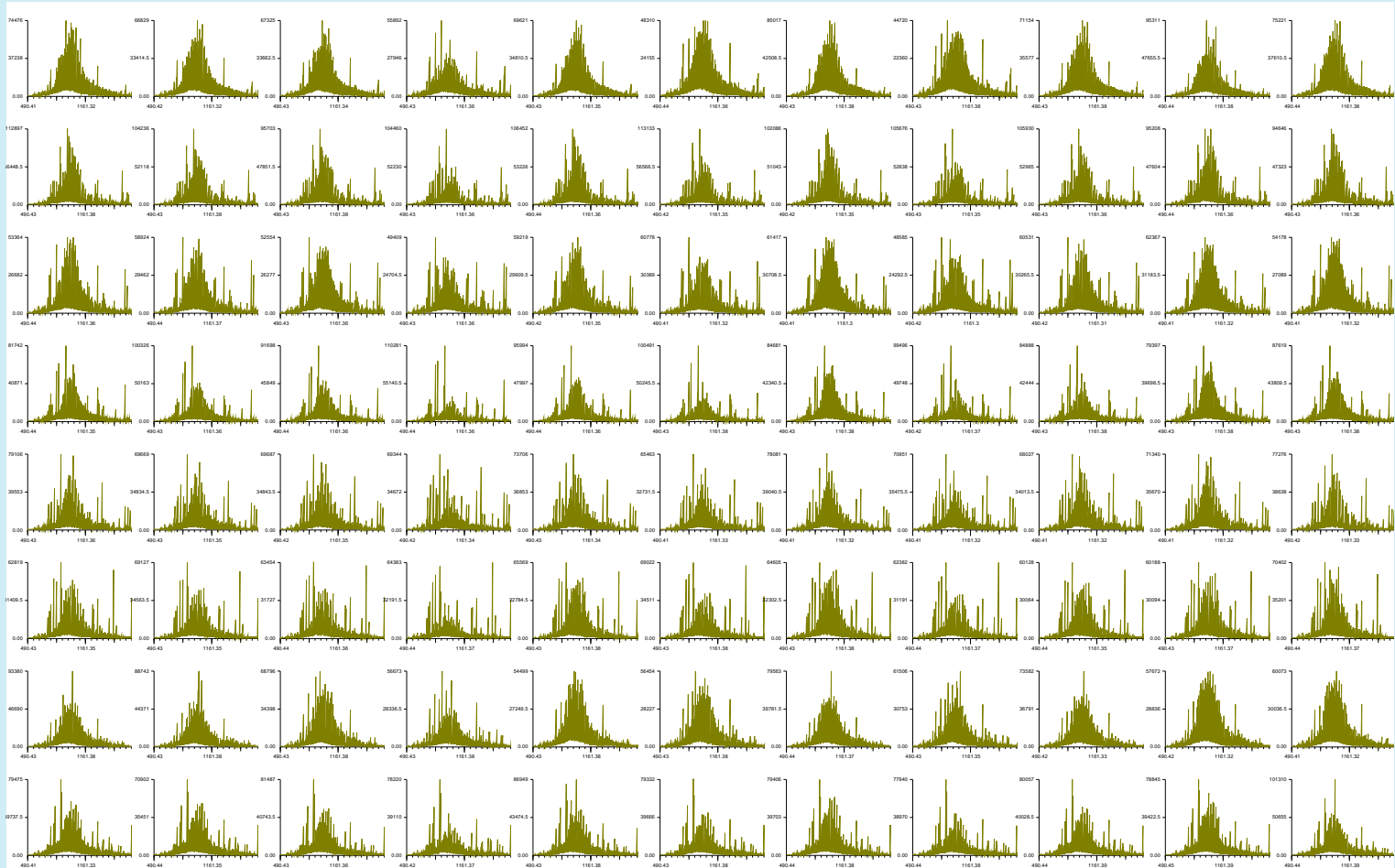
Base composition  
rCRS coordinates  
Primer pair

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
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2912:	509..521:	A32 G7 C48 T26
2913:	464..603:	A44 G10 C63 T23

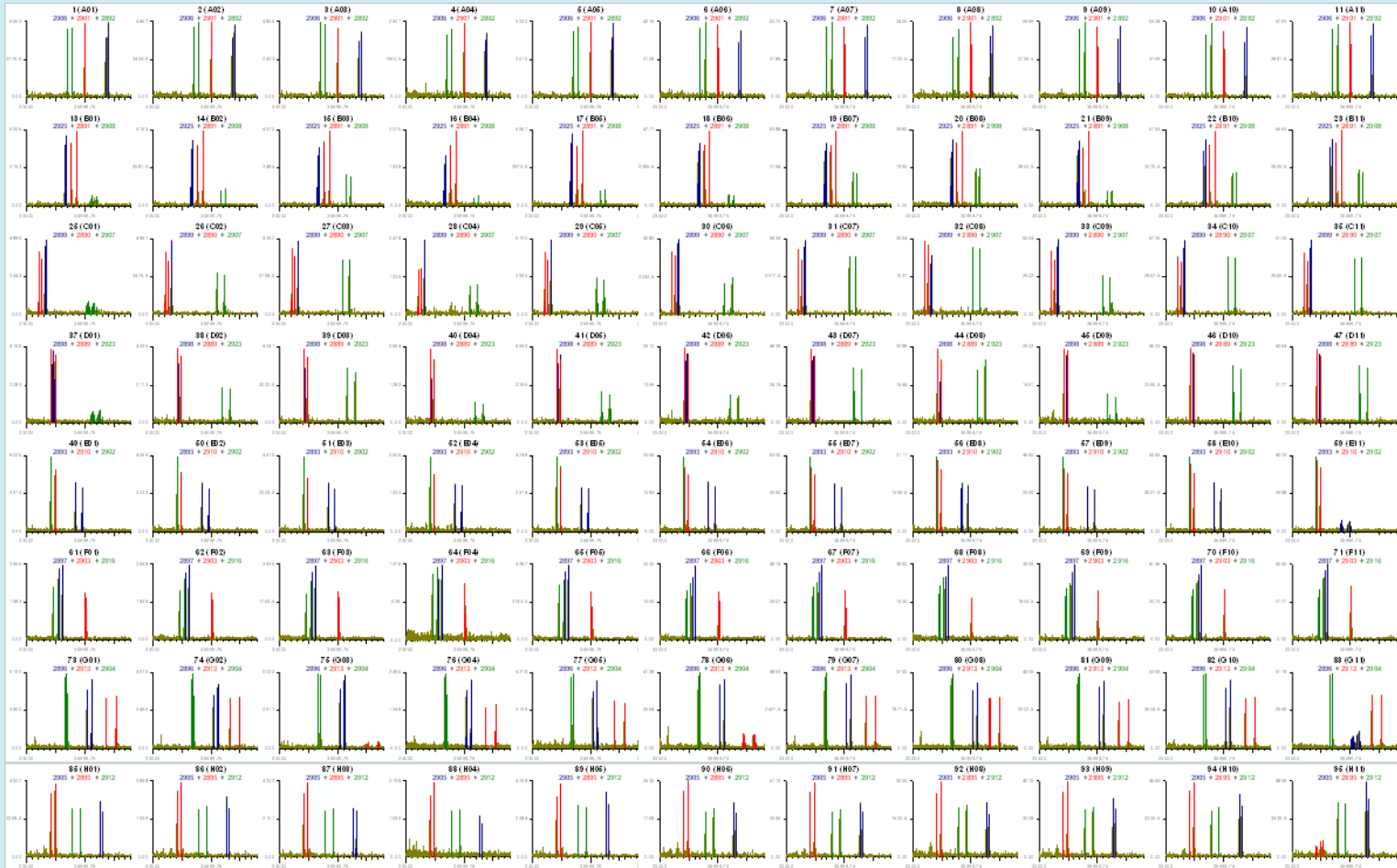
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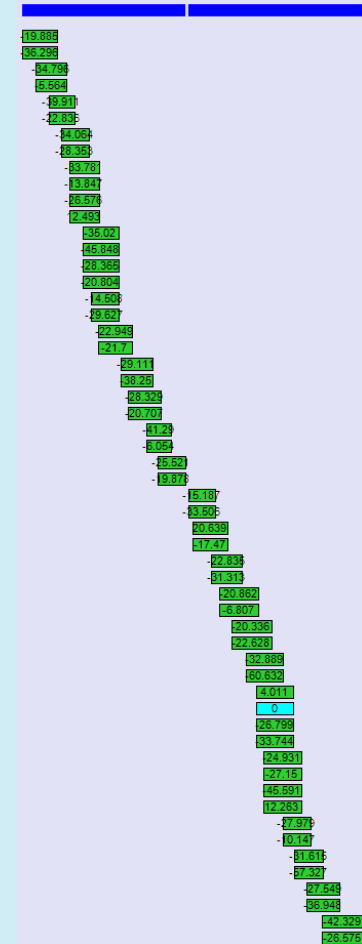
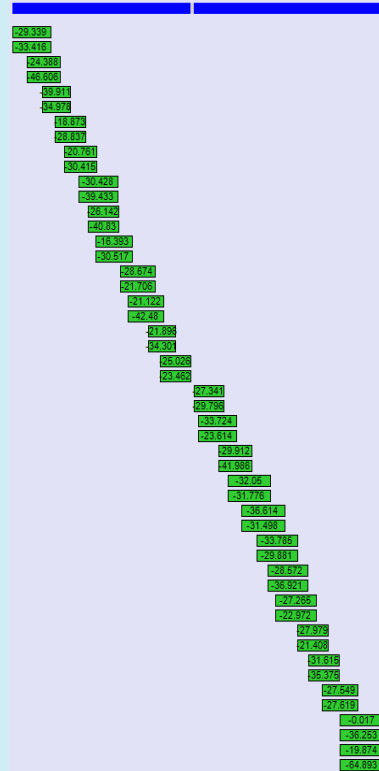
# Multiple Assays Per Plate



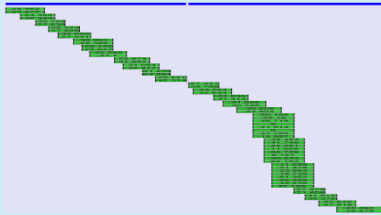
# Multiple Mass Assignments



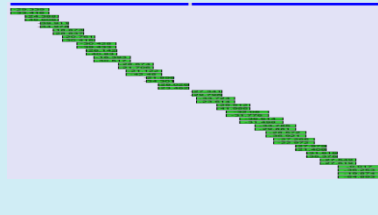
# Multiple Samples Per Plate



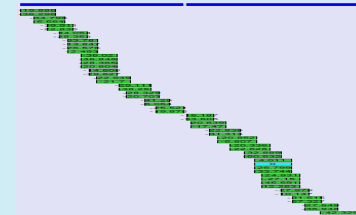
# Multiple Profiles Per Plate



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  
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 2908: 204..330: A42 G16 C37 T32  
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 2916: 367..463: A27 G8 C32 T30  
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 2913: 464..603: A44 G10 C63 T23



2901: 15893..16012: A46 G19 C25 T30  
 2925: 15937..16041: A35 G14 C24 T32  
 2899: 15985..16073: A26 G15 C21 T27  
 2898: 16025..16119: A26 G17 C28 T24  
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 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  
 2890: 16318..16402: A20 G14 C30 T21  
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 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 C31 T45  
 2906: 154..290: A48 G18 C35 T36  
 2908: 204..330: A42 G16 C40 T30  
 2907: 239..363: A43 G11 C49 T23  
 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: A45 G10 C68 T23  
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2901: 15893..16012: A47 G18 C25 T30  
 2925: 15937..16041: A35 G14 C24 T32  
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 2898: 16025..16119: A26 G17 C27 T25  
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 2895: 16130..16224: A36 G7 C33 T19  
 2893: 16154..16268: A44 G7 C46 T18  
 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  
 2903: 20..139: A25 G33 C29 T33  
 2904: 83..187: A23 G21 C29 T32  
 2905: 113..245: A39 G18 C29 T47  
 2906: 154..290: A48 G18 C32 T39  
 2908: 204..330: A42 G16 C39 T32  
 2907: 239..363: A43 G11 C50 T23  
 2907: 239..363: A43 G11 C51 T23  
 2923: 262..390: A47 G10 C54 T20  
 2923: 262..390: A47 G10 C55 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

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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 had a least one positive control**
- **Each plate had at least one negative control**



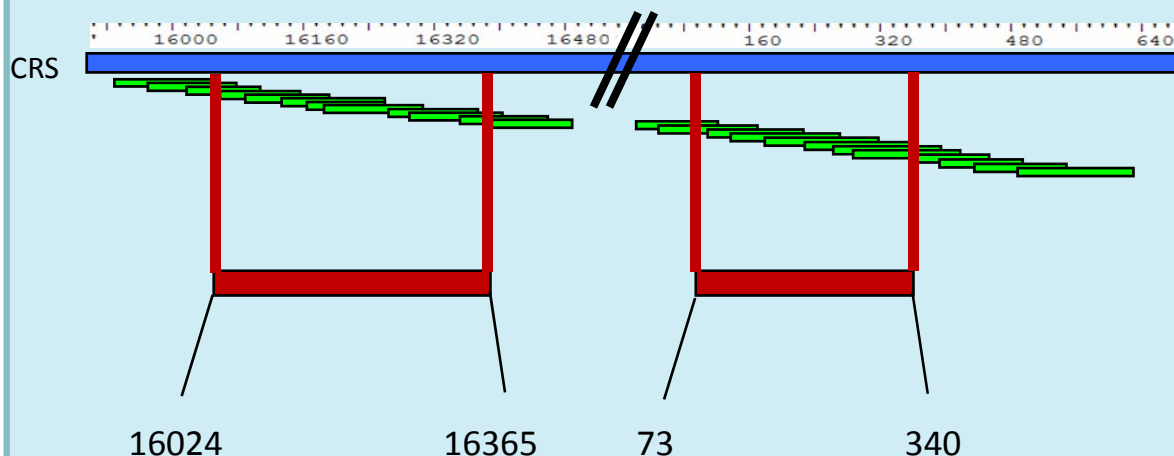
# ***Blinded Samples***

- All samples gave complete profiles
- Three FBI samples were not pure single-contributor templates
  - Two 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
  - One FBI sample was a blank swab
- Several heteroplasmies, both C-length and non-C-length were noted in all sample sets
  - 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



Tiling assay amplifies  
15924-16428 and  
31-576

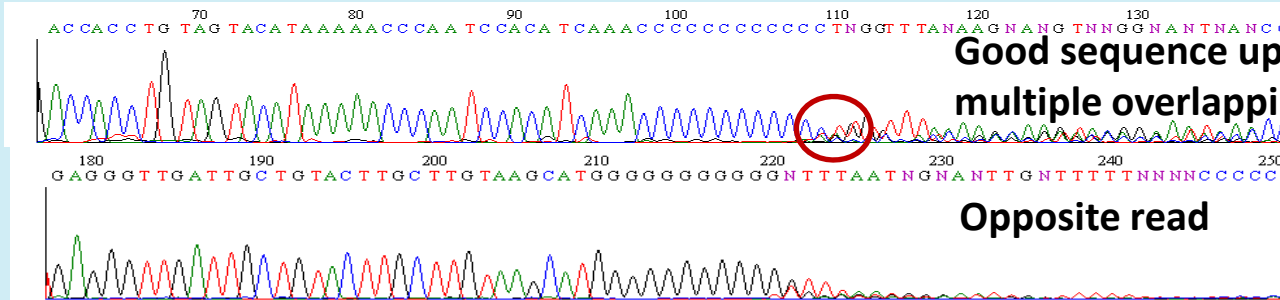
Minimum HVI +HVII  
sequences 16024-  
16365 and 73-340

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

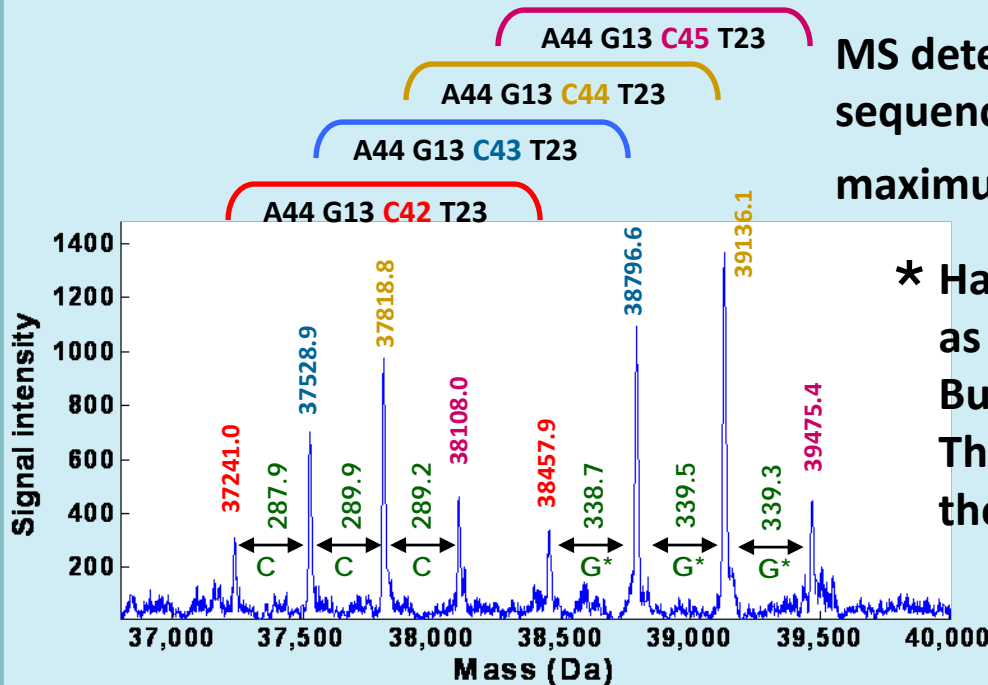
# Length Heteroplasmy Detection

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



Good sequence up to C stretch, then multiple overlapping reads

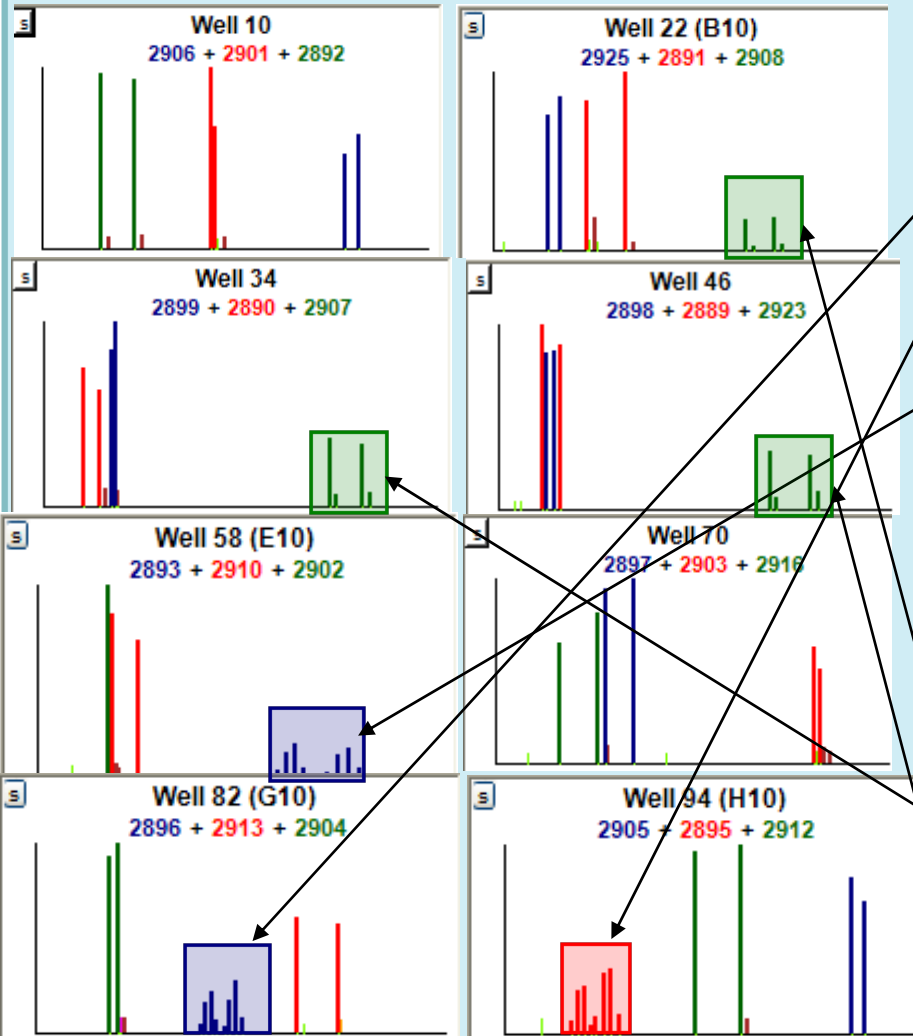
Opposite read



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.

# Length Heteroplasmy Example



2901: 15893..16012: A47 G18 C25 T30  
 2925: 15937..16041: A35 G14 C24 T32  
 2899: 15985..16073: A25 G16 C21 T27  
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 2897: 16055..16155: A31 G12 C30 T28  
 2896: 16102..16224: A44 G12 C42 T24  
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 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

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# SNP Heteroplasmy Detection

From sequence profile

AF-4: 16024-16365

C 16176 N

T 16362 C

C/T heteroplasmy

C/T heteroplasmy

Observed profile

16102..16224: A45 G13 C41 T24

16102..16224: A45 G13 C40 T25

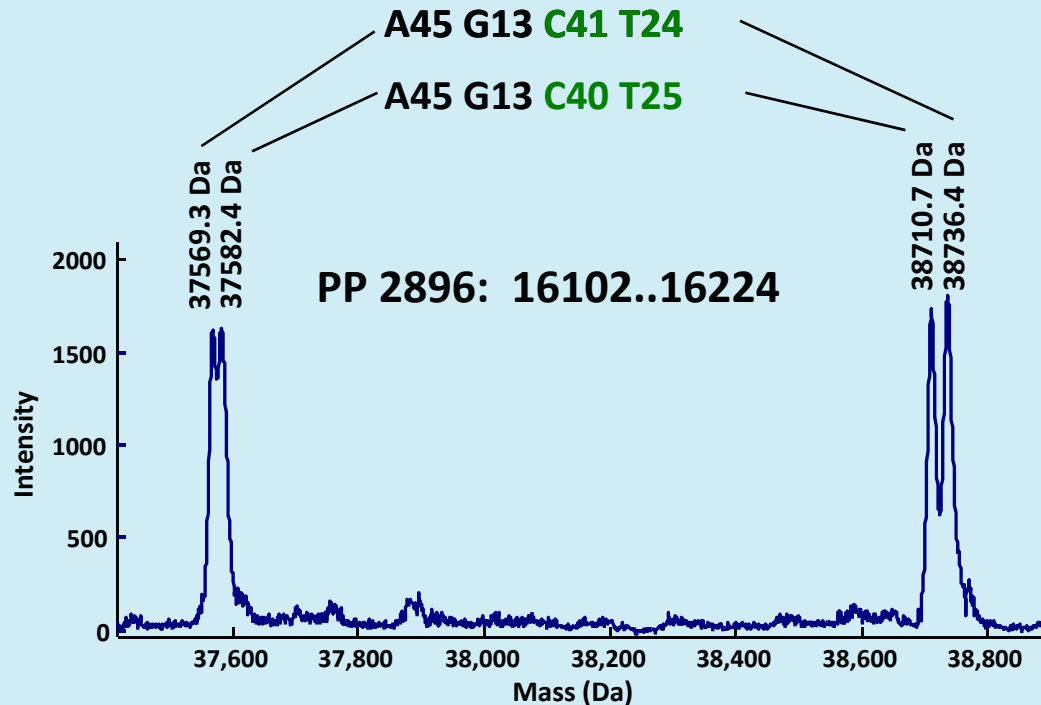
16130..16224: A36 G7 C33 T19

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

# SNP Heteroplasmy Detection

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

Contributor	Sample	Hetero- plasmies	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 five 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



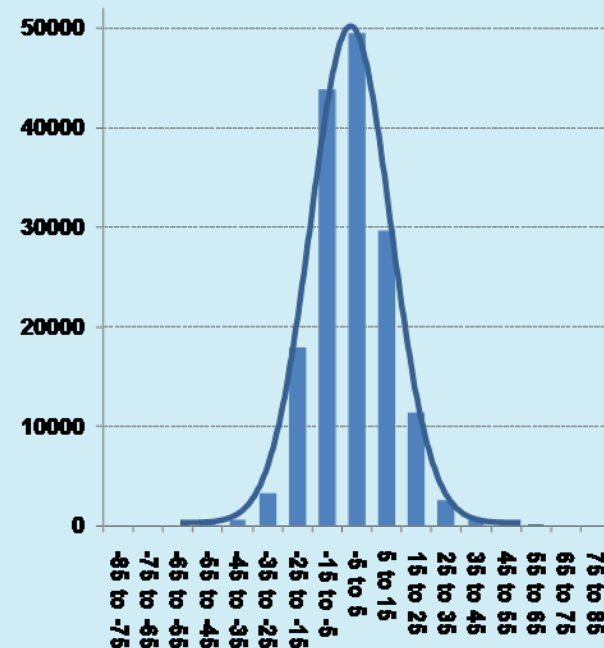


# Reproducibility

- 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 mis-assignments (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  $10.12 \pm 8.04$  ppm



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# ***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 about 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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***Questions?***

# ***Contact Information***

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***Note:*** All images and charts courtesy of Tom Hall, Ph.D.