

# MALDI-TOF analysis of whole blood: its usefulness and potential in the assessment of HbA1c levels

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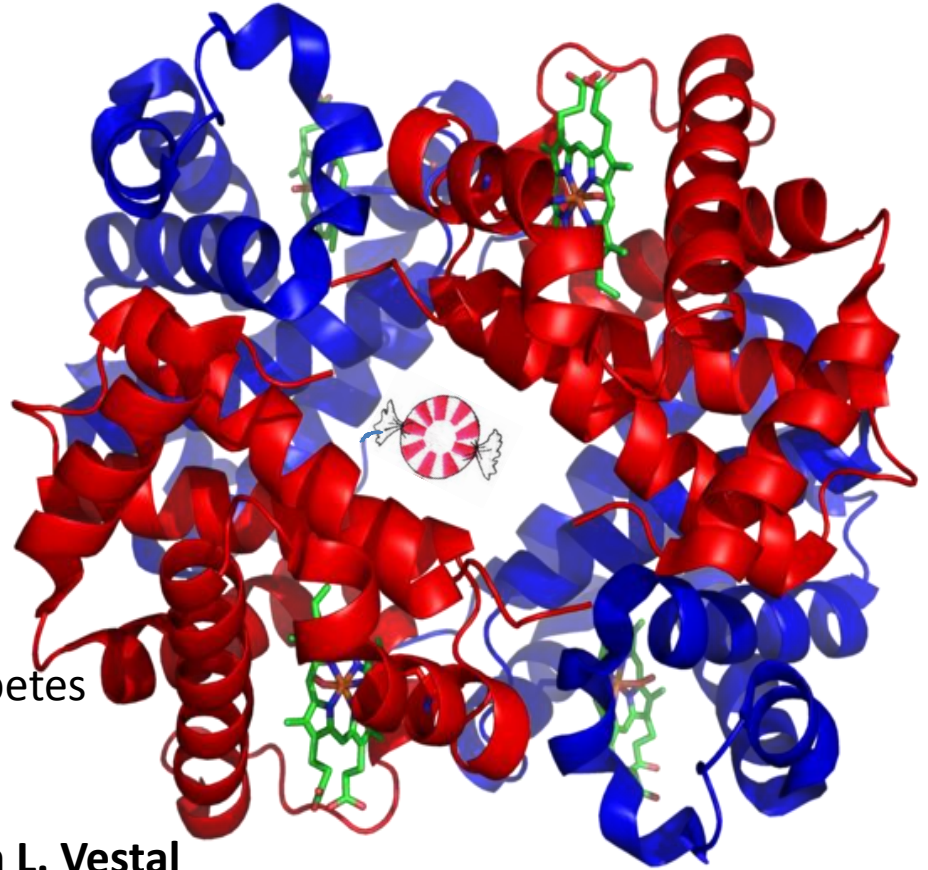
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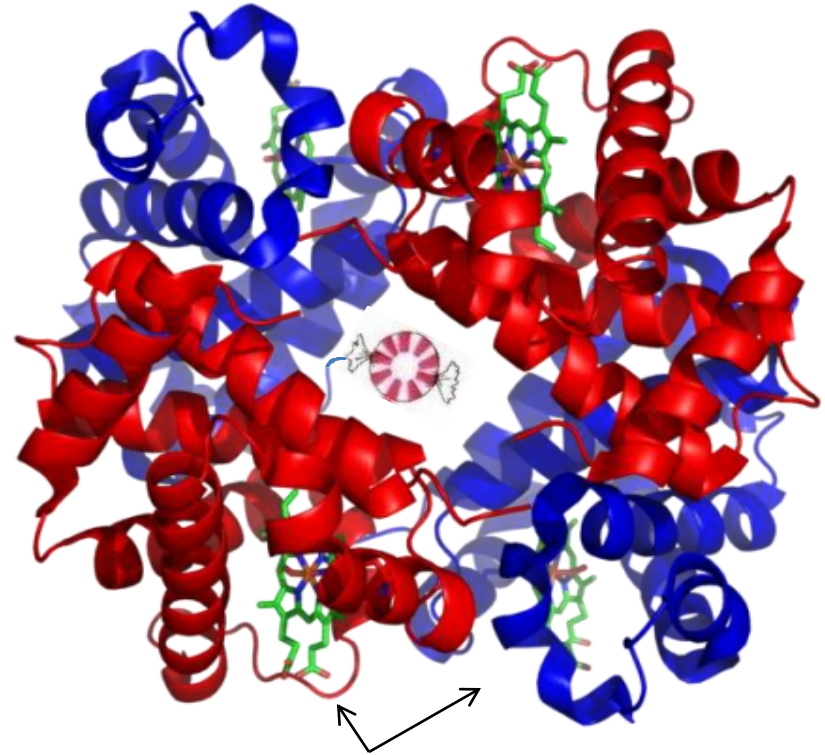


# Hemoglobin (Hb) and HbA1c

## Hemoglobin

Protein in red blood cells (erythrocytes)

- Primary function is respiratory
  - Transports oxygen from lungs to tissue
- Four globulin chains
  - Normal adult
    - 2 alpha-chains ( $\alpha$ Hb)
    - 2 beta-chains ( $\beta$ Hb)
  - All chains contain an embedded heme-group



## Hemoglobin A1c

- Glycation of N-terminal valine of the  $\beta$ Hb
  - Non-enzymatic, [glucose]-dependent
- Serves as an average measure of blood glucose over the past 2 to 3 months
- $\beta$ Hb contains 11 lysine residues that can also undergo glycation

# Diabetes Mellitus / diagnosis and monitoring

## Diabetes

- **Group of metabolic diseases that result in high blood sugar levels**
- 9% of the US population ( 30 million people) have diabetes
- 8 million are undiagnosed
- 80 million people are pre-diabetic; 90% of them are undiagnosed



## Diagnosis and monitoring

-**Quantitation blood glucose:** (fluctuates with diet, fasting, multiple measurements)

Fasting levels:	normal	70 and 99 mg/dL
	pre-dia.	100-125 mg/dL
	diabetic	126 mg/dL

-**Quantitation of HbA1c** (% N-terminal beta chain glycation)

-HPLC, Capillary EP, ELISA (fasting not required, single measurement)

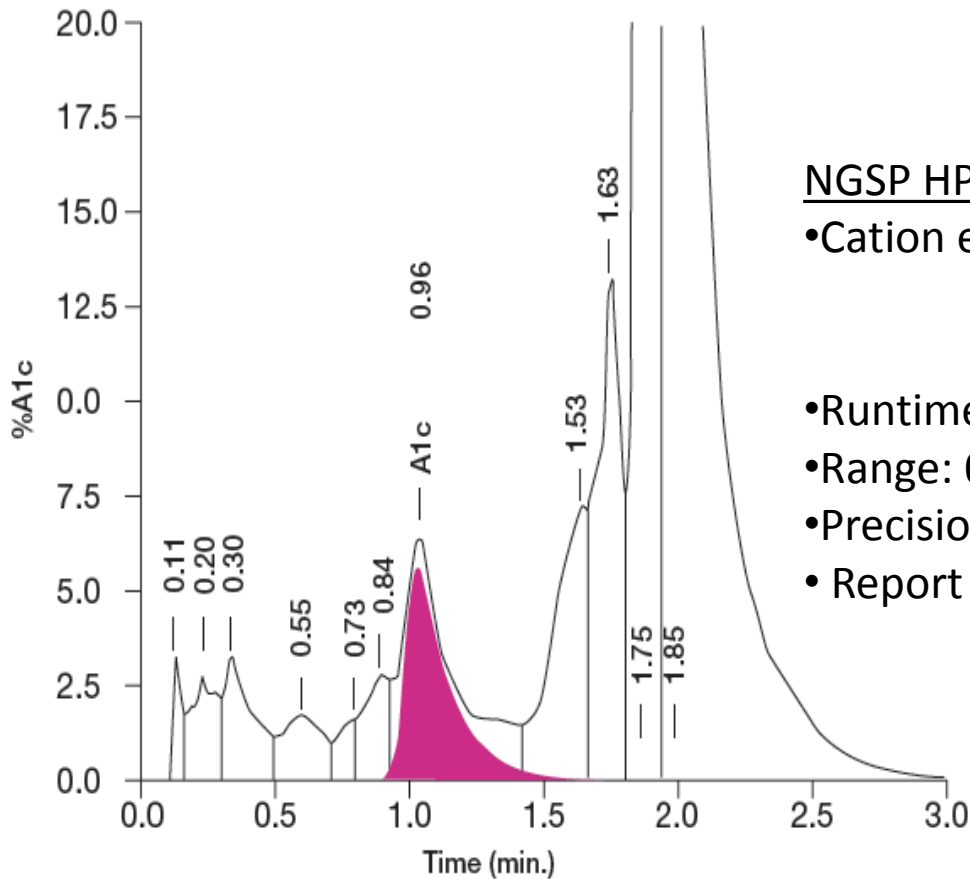
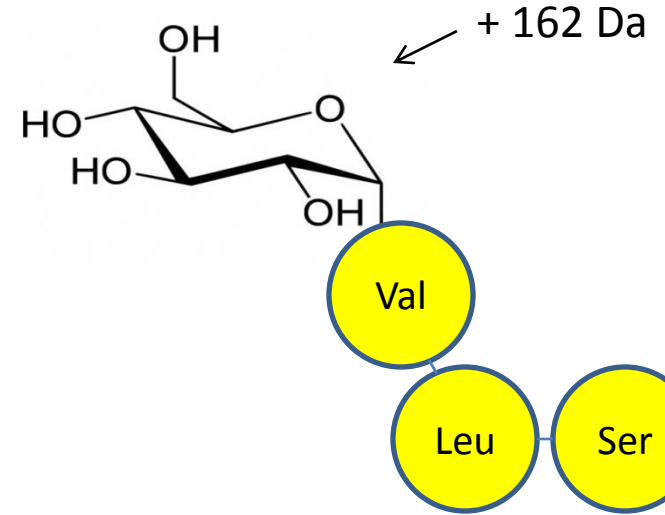
normal	< 5.7%
pre-diabetic	5.7-6.4%
diabetic	> 6.5%



# NGSP techniques

(National Glycohemoglobin Standardization Program)

- Glucose addition  
- reduction in molecular charge (-1)



Example HPLC Chromatogram

## NGSP HPLC Method

- Cation exchange chromatography
  - difference in charge state
  - Heme abs. (415nm)

• Runtime: 3 min

• Range: 0 – 20% HbA1c

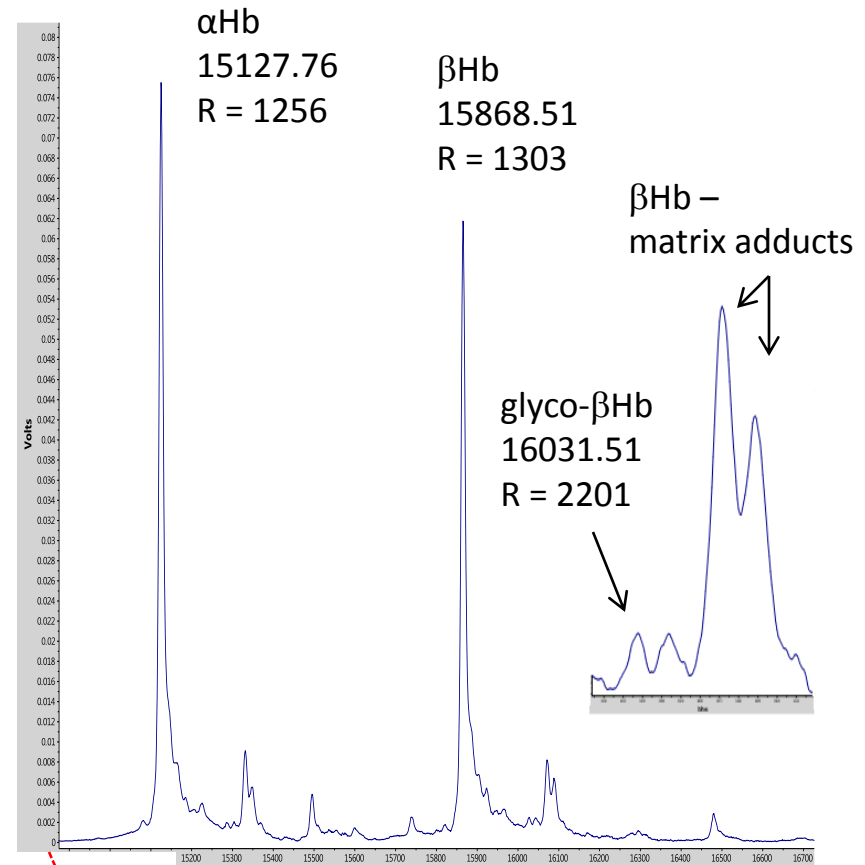
• Precision  $\leq 2\%$

• Report % HbA1c as a ratio

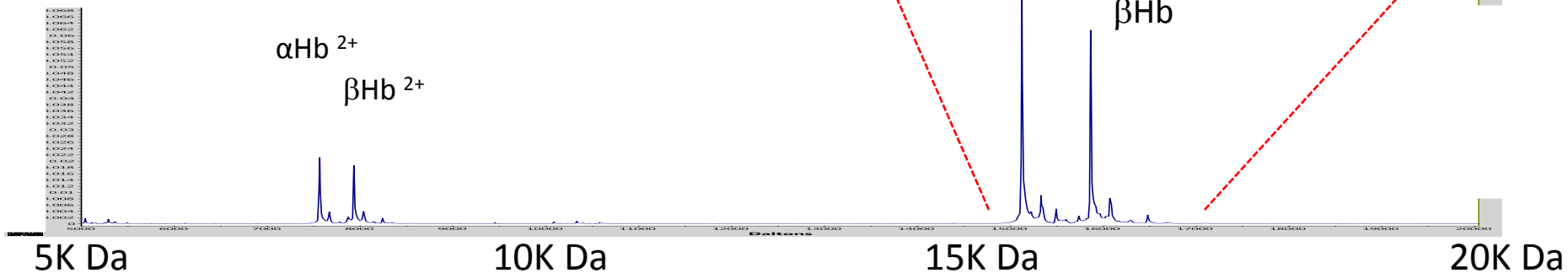
$$[\text{HbA1c} / (\text{H}\beta + \text{HbA1c})] * 100 = \% \text{ A1c}$$

# Why MALDI-TOF ?

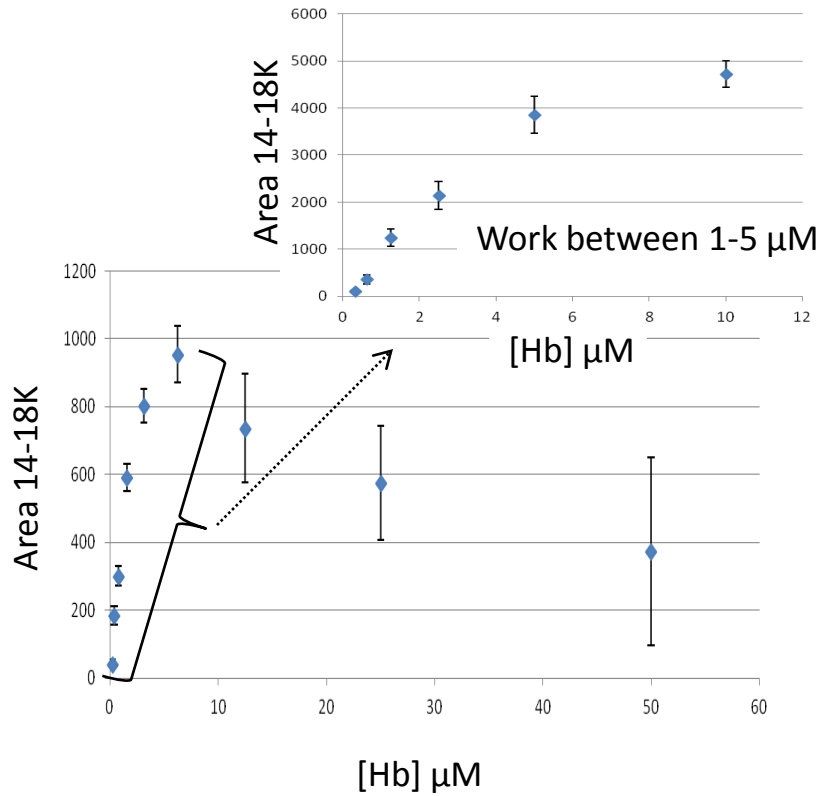
- glucose addition adds 162 Da to molecular mass
- accurate across a wide mass range  
linear mode 500 – 100000 and beyond  
in single spectrum
- precise, quantitative
- requires  $\ll 1 \mu\text{L}$  of sample
- fast, high throughput
  - single analyses time scale  $\sim 20$  seconds
  - multiple replicates analyses,  $5x \sim$  minutes
  - reanalysis may be done if necessary
- provides additional information



mass spectrum of whole blood (5 -20 kDa)



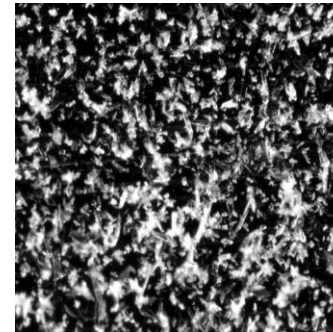
# [Where] to work



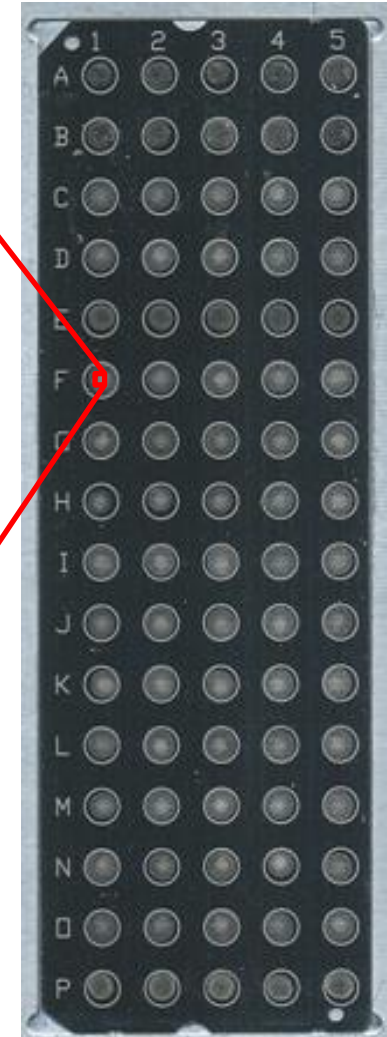
- Serial dilution 50.0 – 0.2 μM Hb
- quantitative response ~ 0.2- 5.0 μM
- **1:2000 dilution of whole blood** ~ 2.0 μM Hb
- measurements; 5x each (error ± 2x Std. Dev.)

# Sample Preparation

- Sinapinic acid (30% CH<sub>3</sub>CN, 0.1% TFA)
- μFocus MALDI Plate 2600 μm  
-(Hudson Surface Technology)
- Samples 5x replicates



Crystals ~ 10-20 μM



## Other matrices examined

- Alpha-cyano
- DHB
- Super-DHB
- HABA
- 3-HPA
- Ferrulic acid
- 3,4,5 Trimethoxycinnamic acid
- Trans 3,5-*bis* (trifluoromethyl) cinnamic acid

# Sample Acquisition

- SimulTOF 100 MALDI-TOF mass spectrometer (SimulTof Systems, Sudbury, MA)

## *Capabilities:*

- *Max accelerating voltage 20 kV*
- *Max laser pulse frequency 5000 Hz*
- *Max scan speed 10 mm/s*

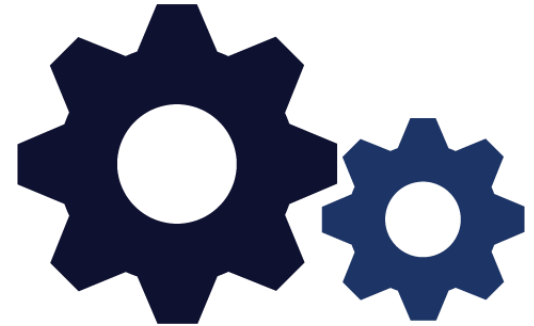
## Acquisition parameters

- Linear mode using positive-ion polarization
- Acceleration voltage 20 kV
- Mass range 5000 – 20,000 Dalton
- Focus mass 15,000
- Laser pulse frequency 1000 Hz
- Laser pulse energy 12  $\mu$ J
- *Scan rate 1 mm/s*
- *Spot size 2.6  $\mu$ m*
- *100  $\mu$ m raster to cover each sample position*

*Red = adjustable parameters that determine acquisition speed*



# Post Acquisition Data Processing



- **Individual Spectra** collected in 100 shot bundles
- **Average all spectra** > 20 mV signal intensity / spot
- **Baseline corrected** spot-averaged spectra
- **Calibrate** spot-averaged spectra
  - M<sup>+1</sup> and M<sup>+2</sup> ions of hemoglobin  $\alpha$  and  $\beta$  subunits  
( $\alpha$  = 7,564.37, 15,127.74,  $\beta$  = 7,934.75, 15,868.51 Da)
- **Quantify** by integration of signals from  $\beta$ Hb and glyco- $\beta$ Hb = ( $\beta$ Hb + 162 (glucose))
- **Report** as a ratio of the percentage of total glycation on the  $\beta$  chain

$$\left[ \frac{\text{glyco-}\beta\text{Hb}}{\beta\text{Hb} + \text{glyco-}\beta\text{Hb}} \right] * 100 = \% \text{ glyco-}\beta\text{Hb}$$

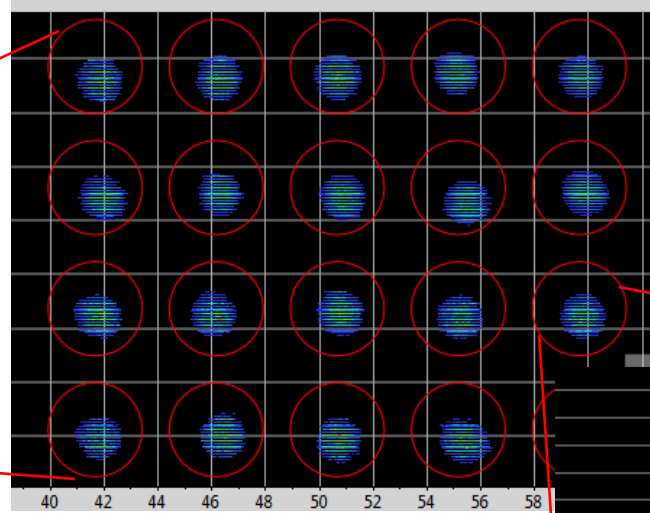
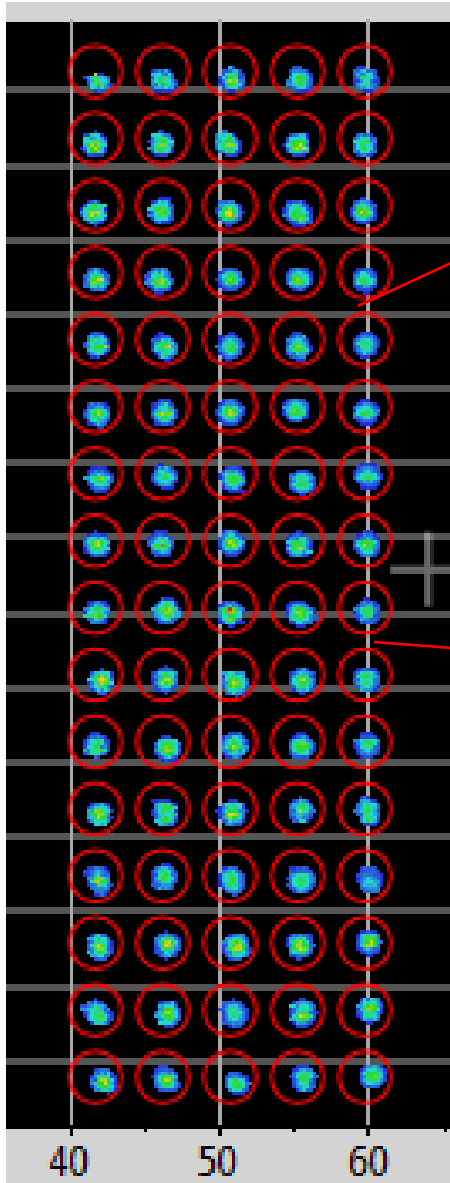
(can also be done for glyco- $\alpha$ Hb)



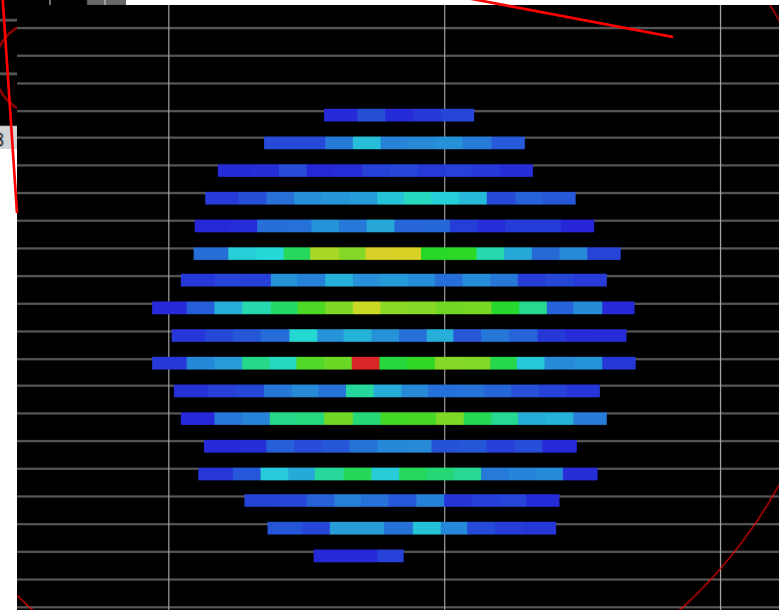
Microscope slide-sized plate

# Acquisition / processing cont.

16 samples / 5x replication.



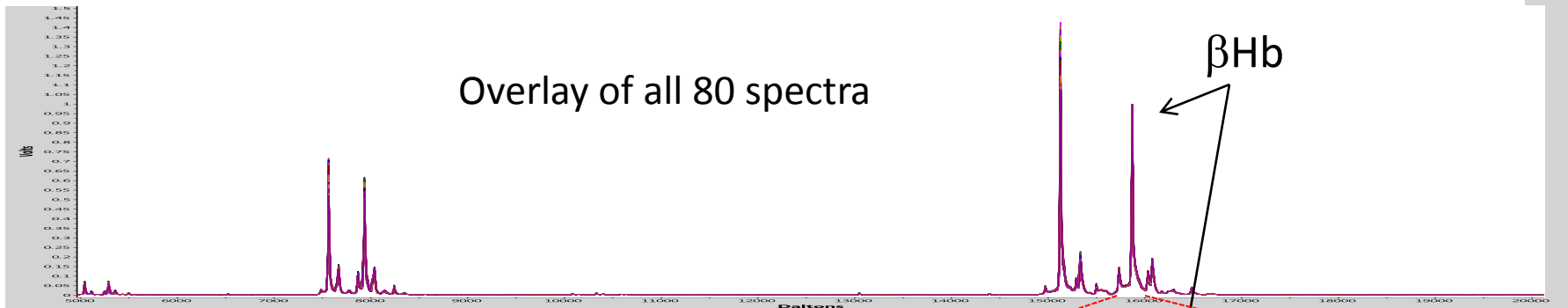
100 shots / spectrum  
~ 200 spectra / spot



Each pixel = 1 spectrum

# Reproducibility

Overlay of all 80 spectra



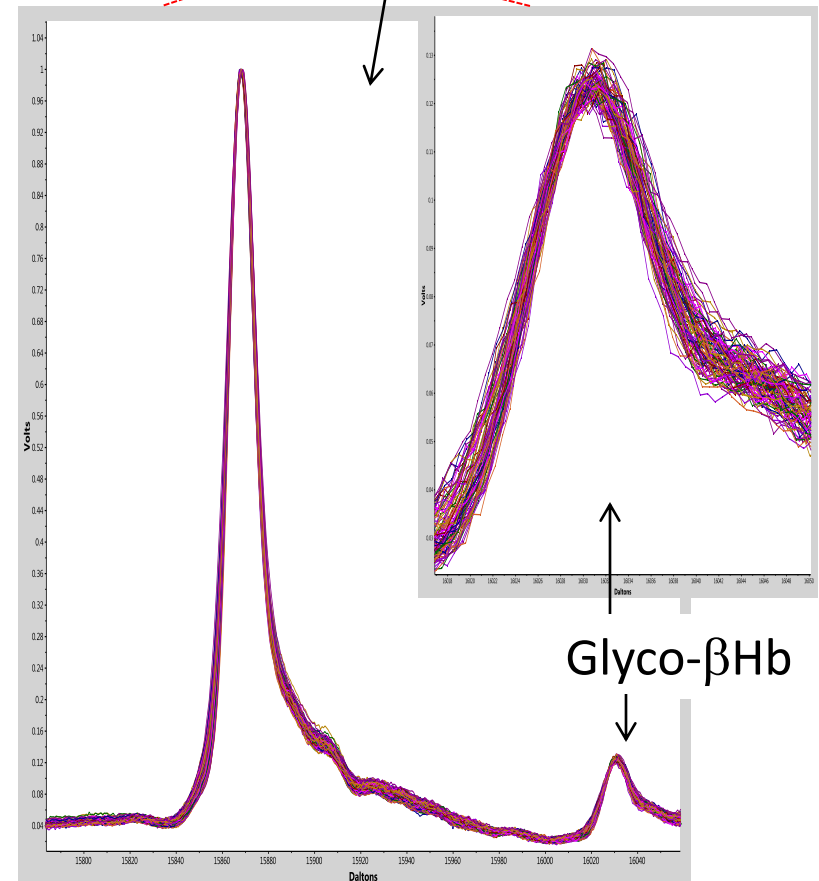
## Reproducibility experiment

- 1 sample across plate
- Processed 16 samples  
5x replicates
- Ave CV for 16 < 1.00%
- CV for 16 glyco ratios 1.22 %

80 spots

~ 16,000 spectra

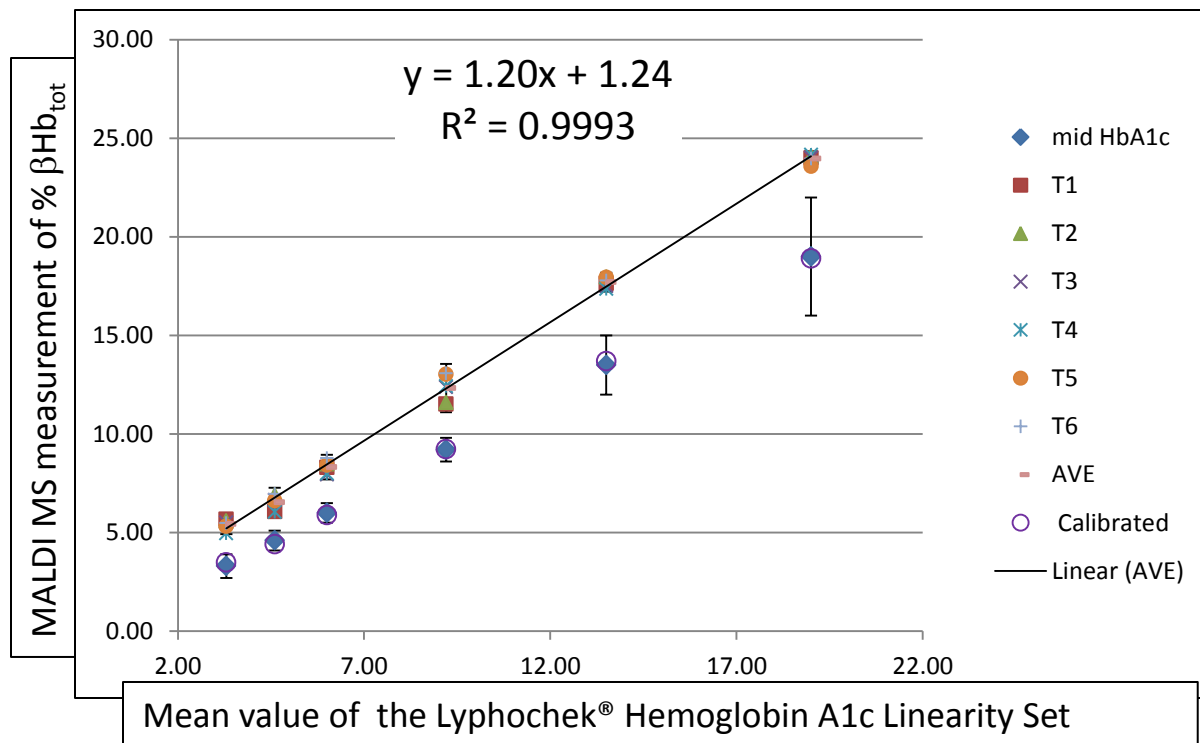
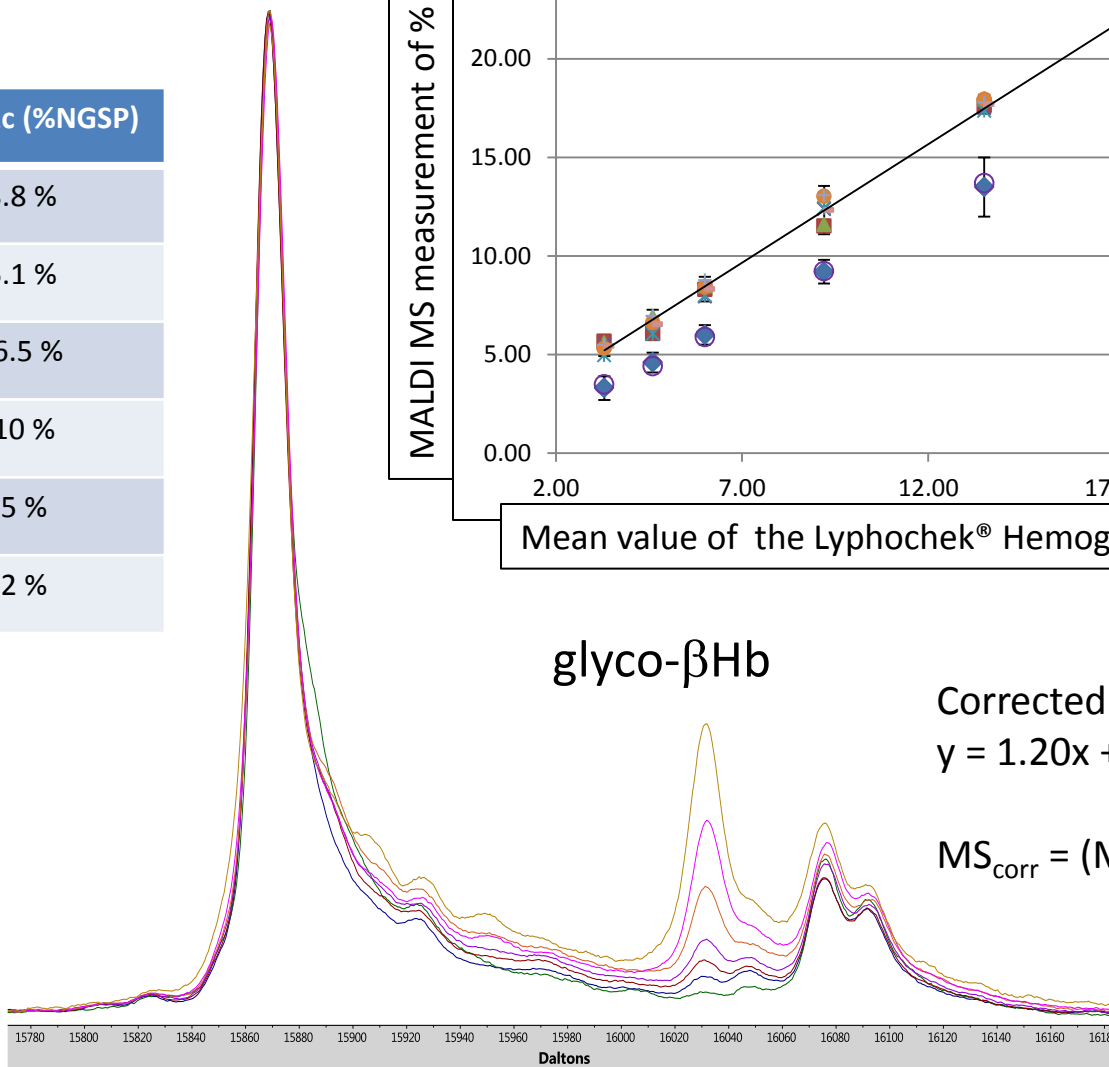
	GlyHb/Hb%	CV %
T 1	13.52	1.39
T 2	13.48	0.71
T 3	13.20	0.62
T 4	13.07	0.99
T 5	13.07	0.96
T 6	13.11	0.90
T 7	12.98	0.67
T 8	12.99	1.19
T 9	13.05	0.96
T 10	12.99	0.99
T 11	13.08	0.92
T 12	13.01	0.66
T 13	13.16	1.00
T 14	13.15	0.84
T 15	13.22	0.70
T 16	13.17	0.85
<b>Average</b>	<b>13.14</b>	<b>0.90</b>
<b>Std Dev</b>	<b>0.16</b>	
<b>Rel Std Dev</b>	<b>1.22</b>	



# Analysis of Lyphochek® Hemoglobin A1c Linearity Set

	HbA1c (%NGSP)
Level 1	2.7 - 3.8 %
Level 2	4.1 - 5.1 %
Level 3	5.5 - 6.5 %
Level 4	8.4 - 10 %
Level 5	12 - 15 %
Level 6	16 - 22 %

$\beta$  Hb



glyco- $\beta$ Hb

Corrected data

$$y = 1.20x + 1.24 \therefore x = (y - 1.24) / 1.20$$

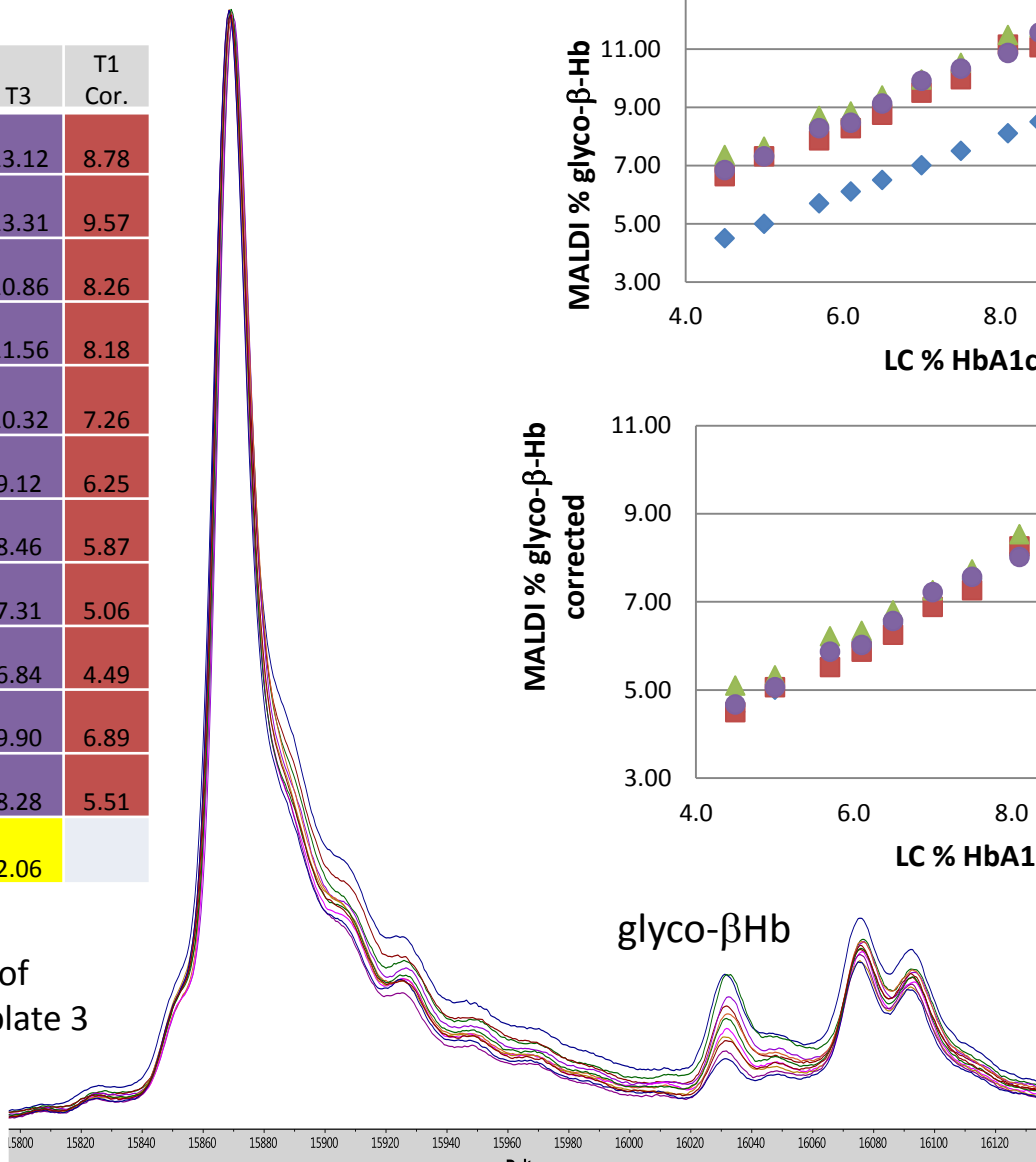
$$MS_{\text{corr}} = (MS - y \text{ intercept}) / \text{slope}$$

# Blood Samples Jan. 2015, 3 plates, 11 samples

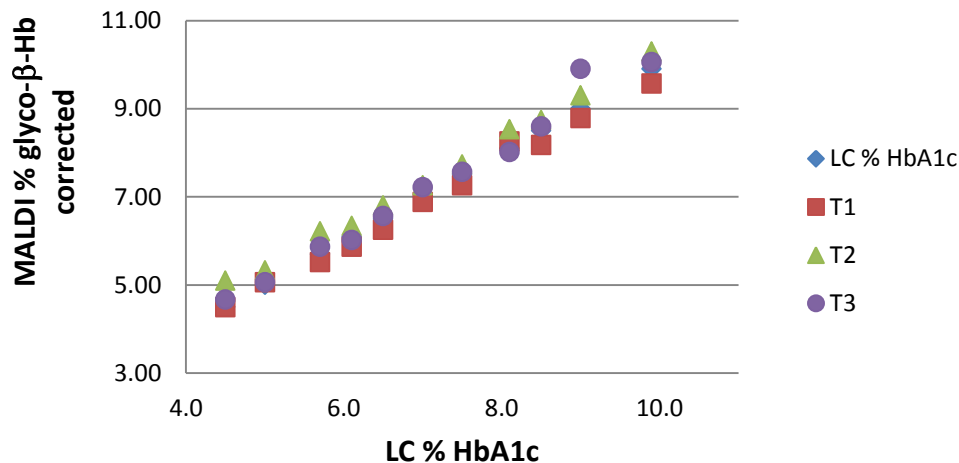
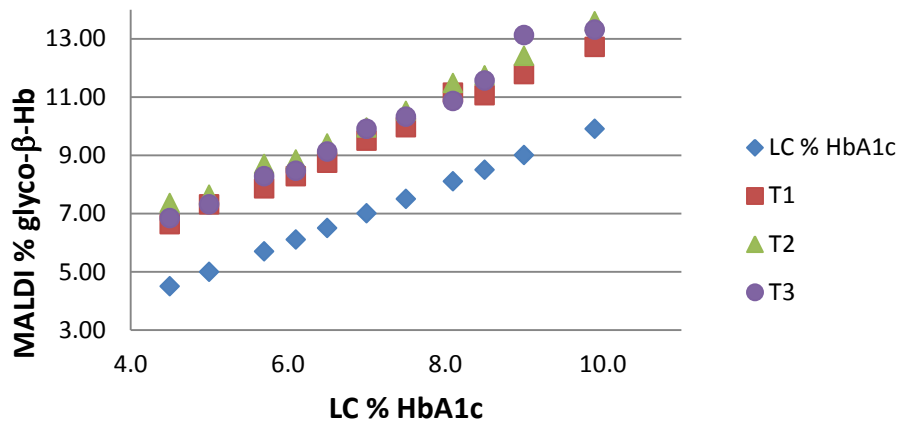
$\beta$ Hb

LC % A1c	T1	T2	T3	T1 Cor.
9.00	11.78	12.40	13.12	8.78
9.90	12.72	13.59	13.31	9.57
8.10	11.15	11.47	10.86	8.26
8.50	11.05	11.73	11.56	8.18
7.50	9.95	10.52	10.32	7.26
6.50	8.74	9.40	9.12	6.25
6.10	8.28	8.85	8.46	5.87
5.00	7.31	7.64	7.31	5.06
4.50	6.63	7.35	6.84	4.49
7.00	9.50	9.95	9.90	6.89
5.70	7.86	8.70	8.28	5.51
Ave				
% CV	2.09	2.43	2.06	

Overlay of spectra plate 3



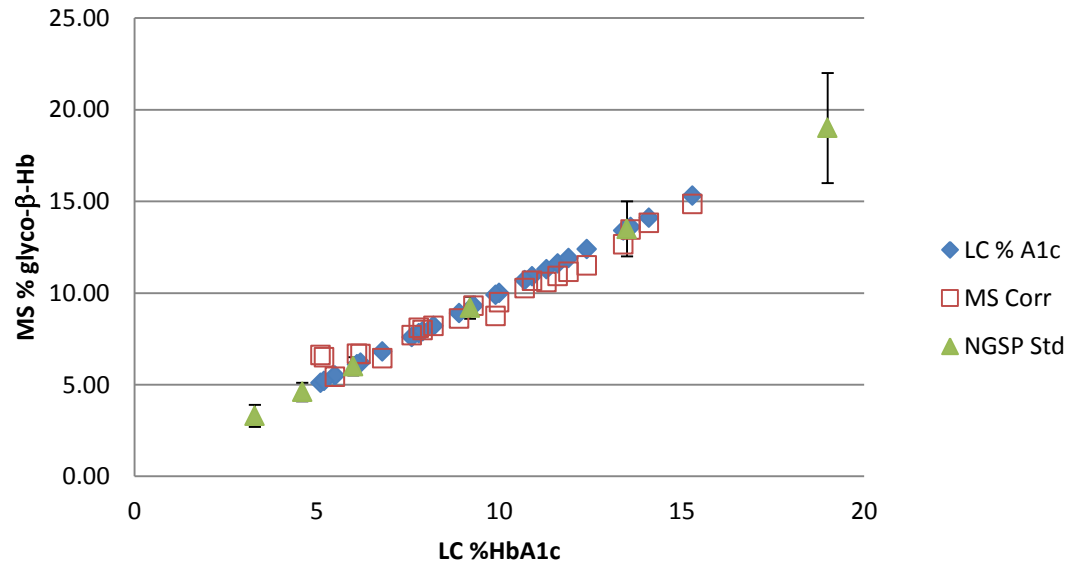
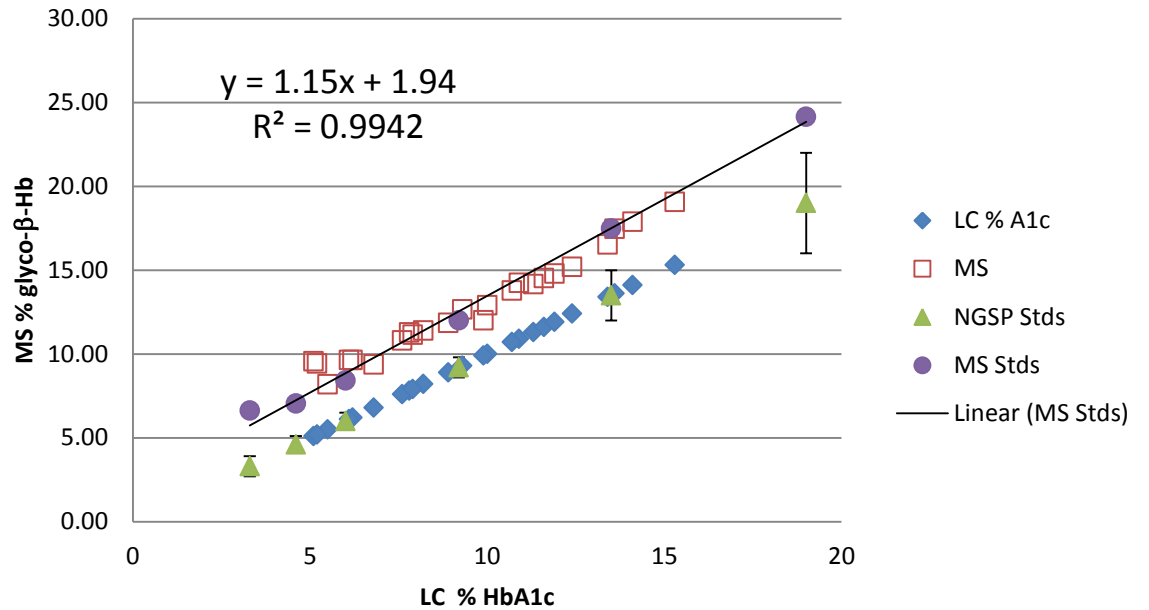
3 plates, 11 samples, 5x replicates



Corrected data  
 $MS_{\text{corr}} = (MS - 1.24) / 1.20$

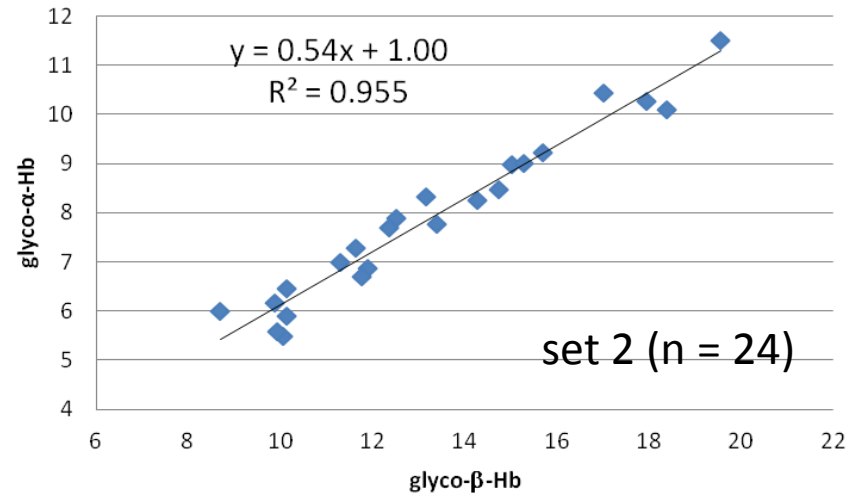
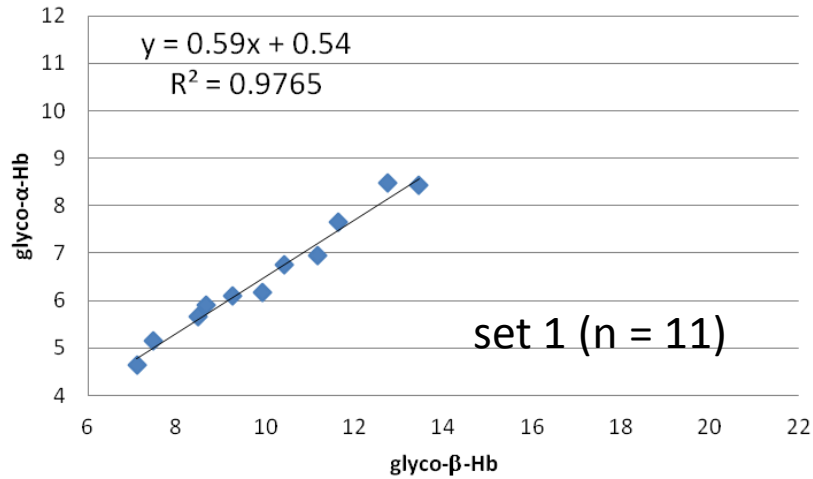
# Data Set 2

LC % HbA1c	MS	MS Corr
14.10	17.88	13.83
10.90	14.24	10.67
8.20	11.39	8.20
10.00	12.89	9.50
10.70	13.77	10.26
7.80	11.28	8.10
12.40	15.20	11.50
11.30	14.15	10.60
13.60	17.46	13.47
11.60	14.53	10.92
11.90	14.79	11.15
9.90	12.01	8.74
7.60	10.80	7.69
6.80	9.36	6.44
15.30	19.07	14.86
6.20	9.63	6.67
5.50	8.19	5.42
6.10	9.65	6.69
5.10	9.55	6.61
5.20	9.43	6.50
7.90	11.14	7.98
8.90	11.86	8.61
13.40	16.53	12.66
9.30	12.66	9.30

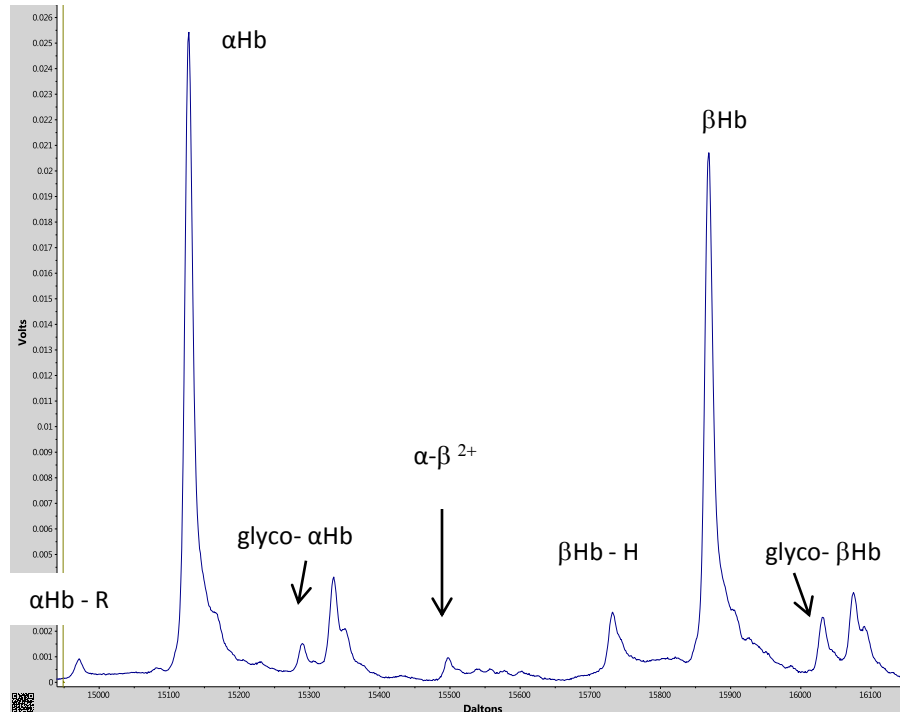


$$\text{MS correction} = (\text{MS} - 1.94) / 1.15$$

# Relationship between $\alpha$ -chain and $\beta$ -chain glycation



Glyco- $\beta$	Glyco- $\alpha$	Glyco- $\alpha$ / Glyco- $\beta$
12.43	8.38	0.67
13.21	8.07	0.61
11.16	6.77	0.61
11.45	7.15	0.62
10.27	6.36	0.62
9.09	5.82	0.64
8.53	5.96	0.70
7.42	4.91	0.66
6.94	4.43	0.64
9.78	5.88	0.60
8.28	5.41	0.65
	<b>Ave</b>	<b>0.64</b>
	<b>Std Dev</b>	<b>0.03</b>
	<b>CV (n=11)</b>	<b>4.80</b>

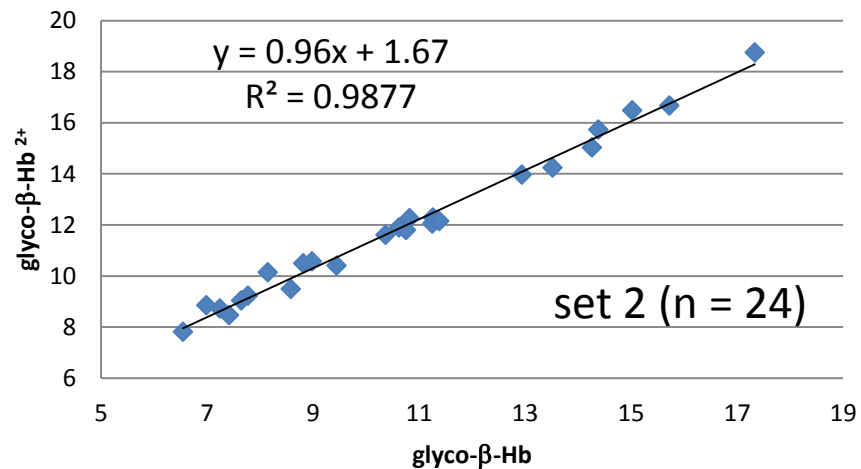
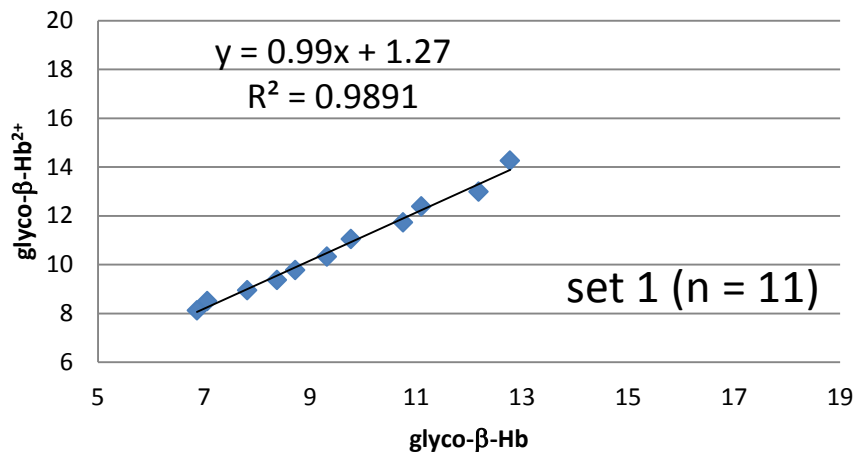


Data set 2  
glyco- $\alpha$ Hb / glyco- $\beta$ Hb

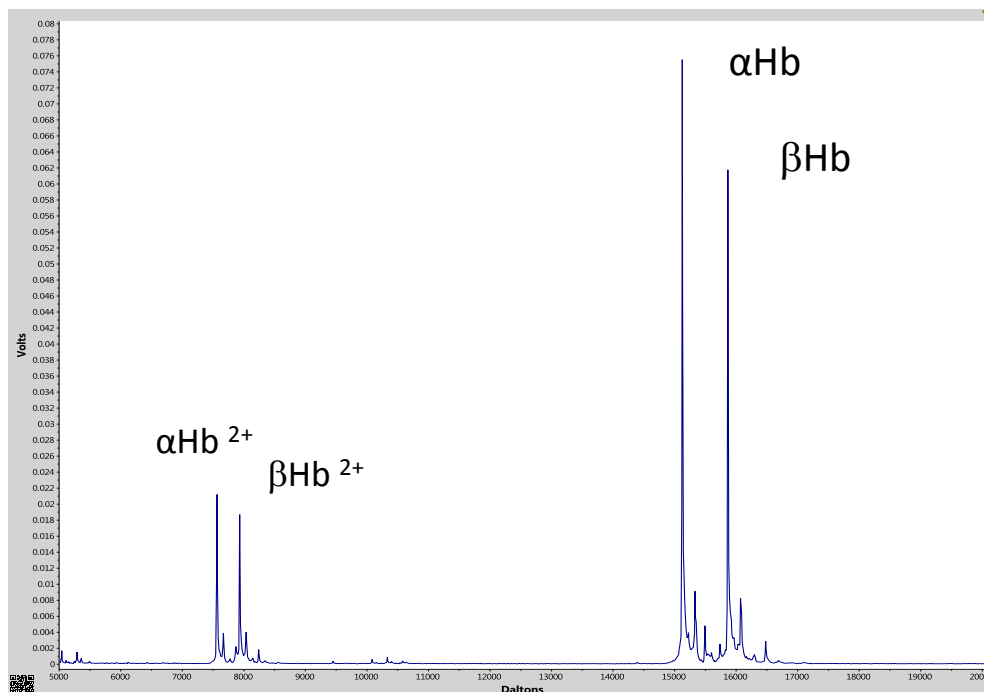
<b>Ave</b>	<b>0.63</b>
<b>Std Dev</b>	<b>0.04</b>
<b>CV (n=24)</b>	<b>6.51</b>

**High = 0.73**  
**Low = 0.57**

# Relationship between singly and doubly charged $\beta$ -chain glycation



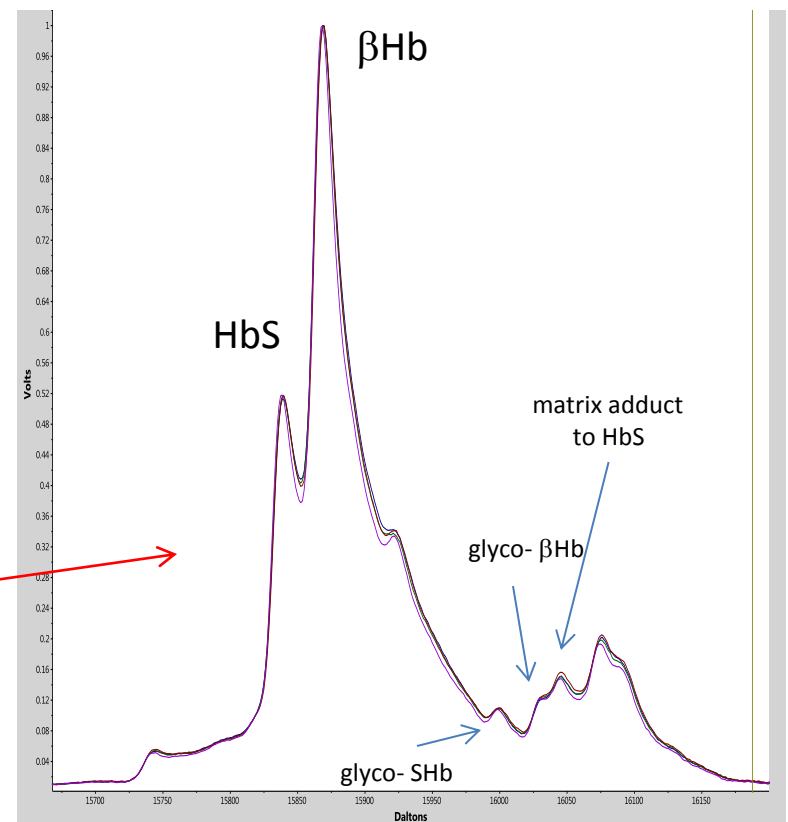
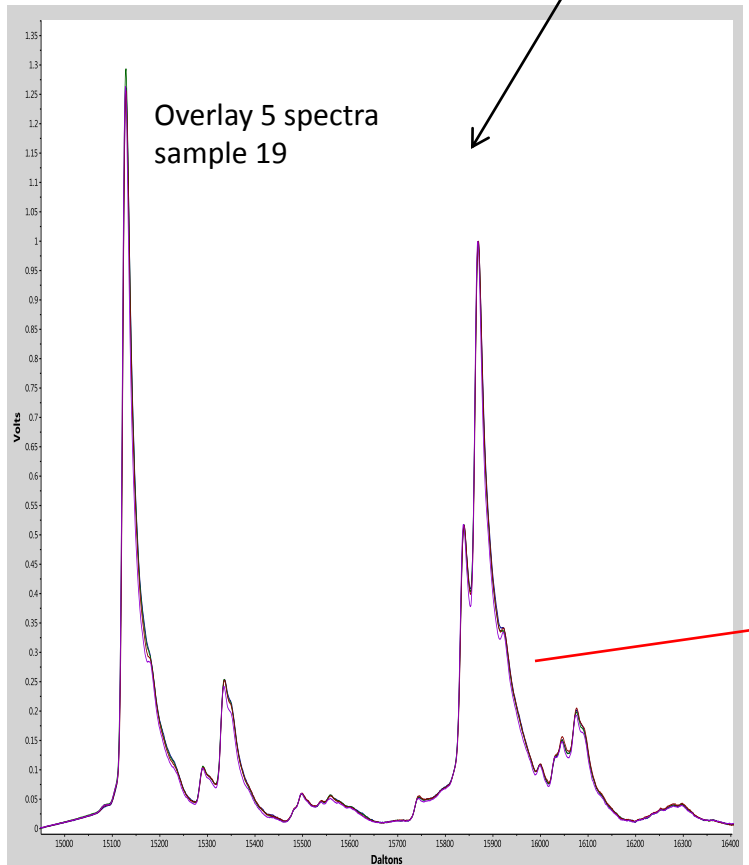
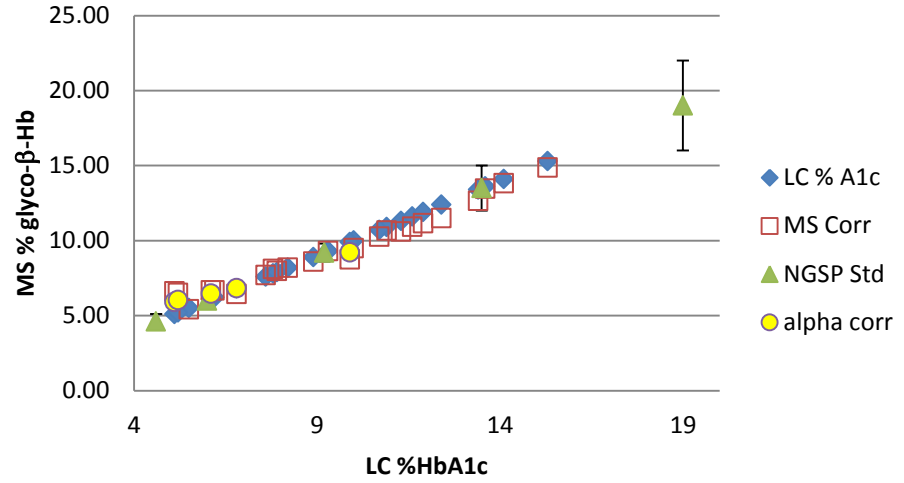
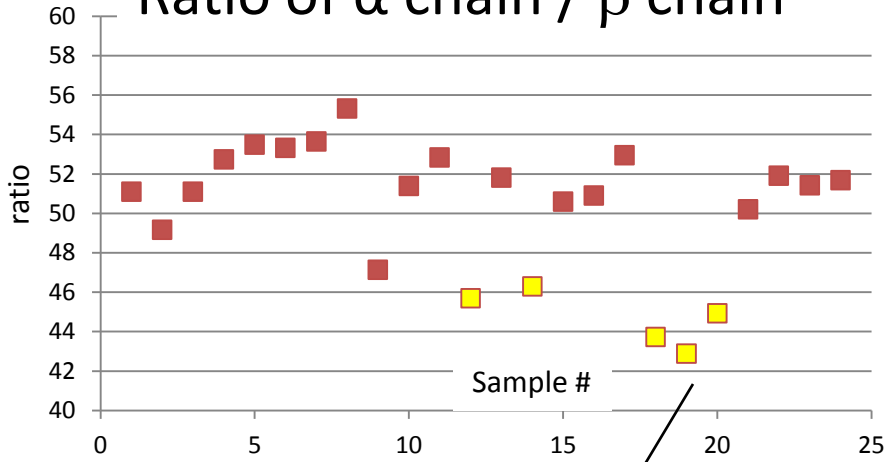
Glyco- $\beta$	Glyco- $\beta^{2+}$	Glyco- $\beta^{2+}$ / Glyco- $\beta$
12.43	12.99	1.04
13.21	14.25	1.08
11.16	11.73	1.05
11.45	12.38	1.08
10.27	11.04	1.08
9.09	9.77	1.08
8.53	9.37	1.10
7.42	8.50	1.15
6.94	8.12	1.17
9.78	10.33	1.06
8.28	8.95	1.08
	Ave	1.09
	Std Dev	0.04
	CV (n=11)	3.55



Ave	1.13
Std Dev	0.06
CV (n=24)	5.40

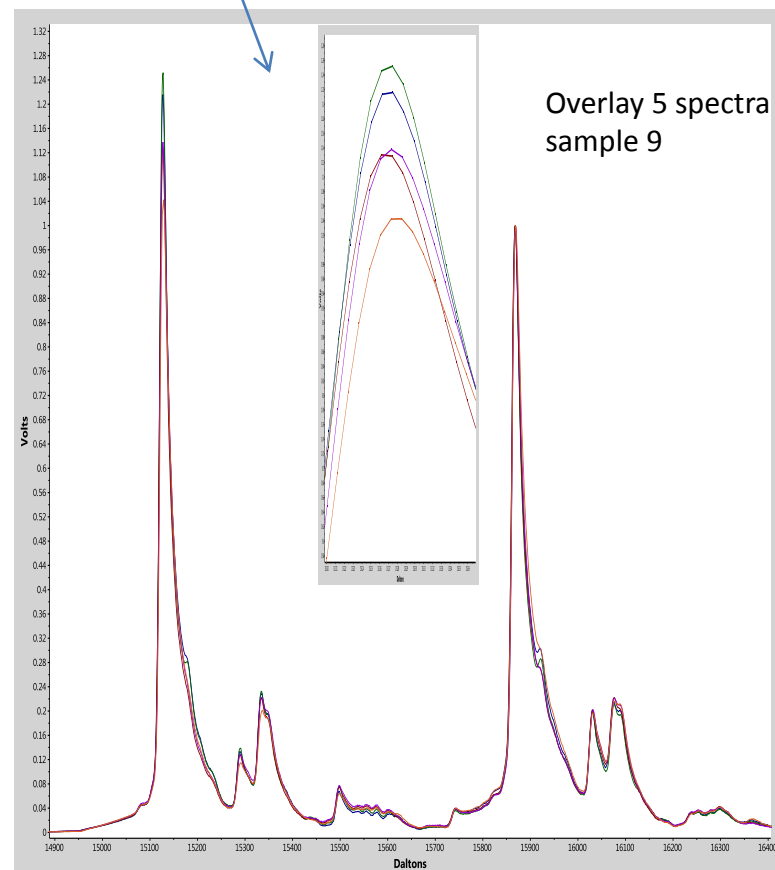
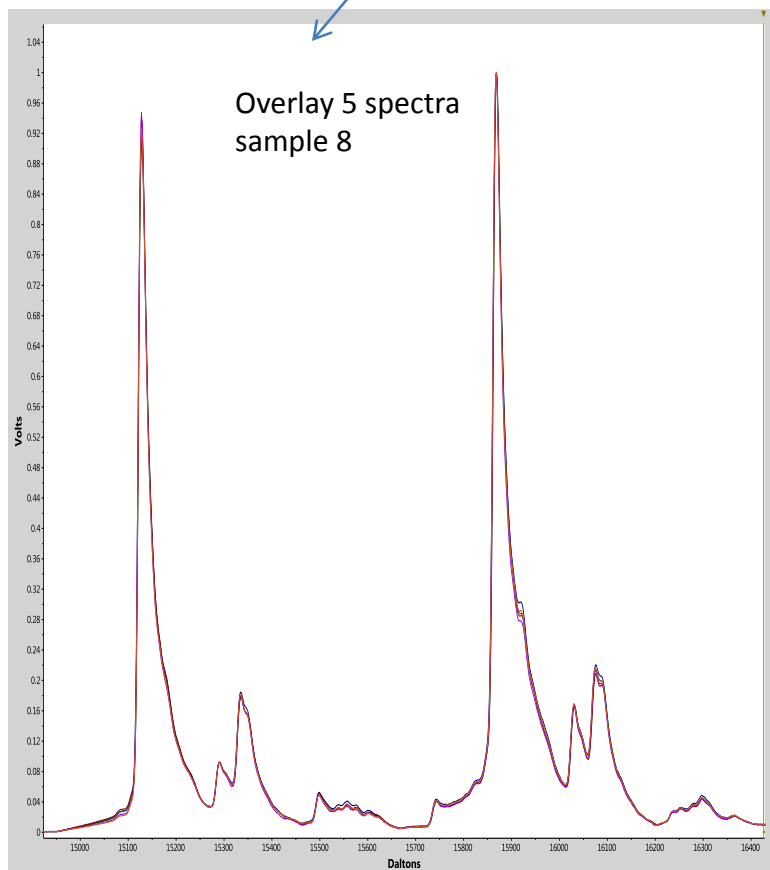
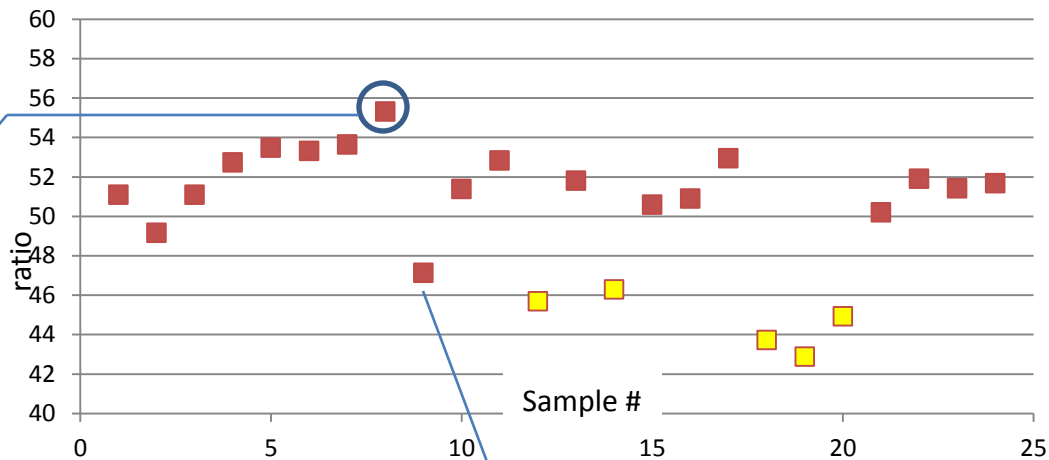
High = 1.27  
Low = 1.05

# Ratio of $\alpha$ chain / $\beta$ chain

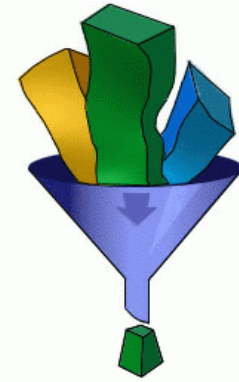




Ratio of  $\alpha$  chain /  $\beta$  chain



# Summary



## MALDI-TOF mass spectrometry

- Promising analytical platform for measuring glycated hemoglobin
- Diluted whole blood sample, no fasting required
- Reproducible / quantitative
- Accurate across clinically relevant range
- Fast, potentially high-throughput, reduced cost of analysis

# Future



- Develop methods that use additional MS information to verify % glyco- $\beta$ Hb measurements
  - glyco- $\alpha$ Hb ratio
  - glyco- $\beta$ Hb <sup>2+</sup> ratio
- Continue to explore the relationship between  $\alpha$  and  $\beta$  chain glycation and parent  $\alpha$  and  $\beta$  chain intensities for potential variant detection / identification
- Characterize calibration standards better
- Automate sample handling
- Run more samples