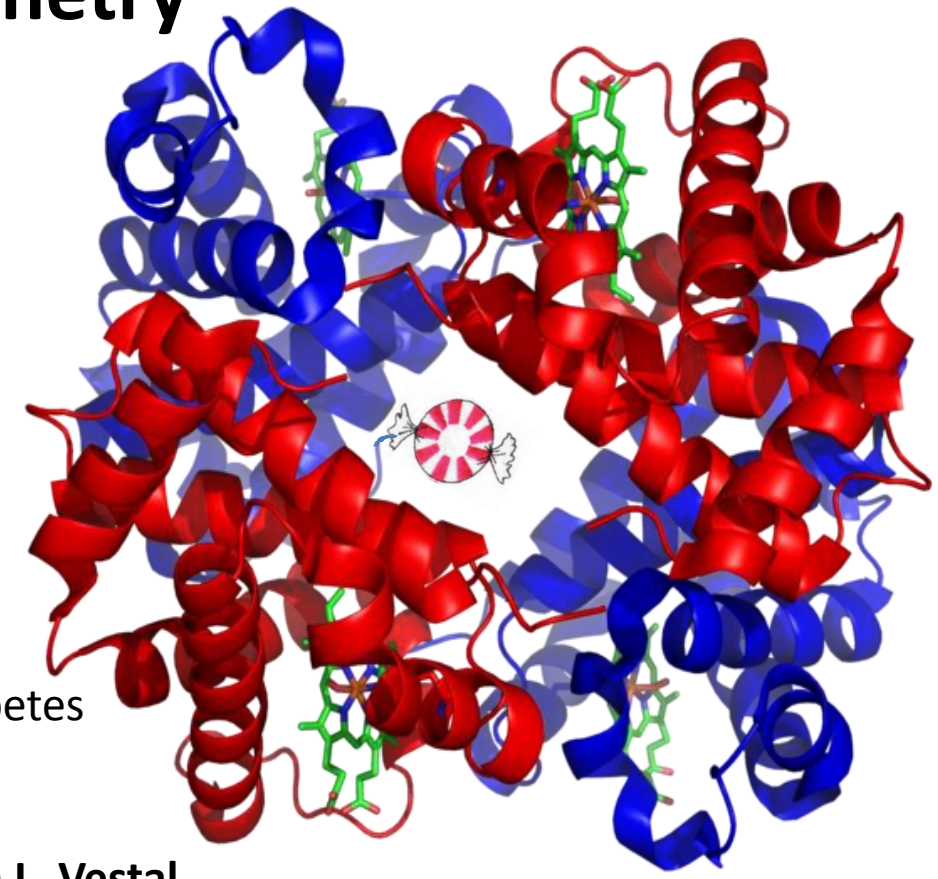
Quantification of Glycated Hemoglobin by MALDI-TOF Mass Spectrometry



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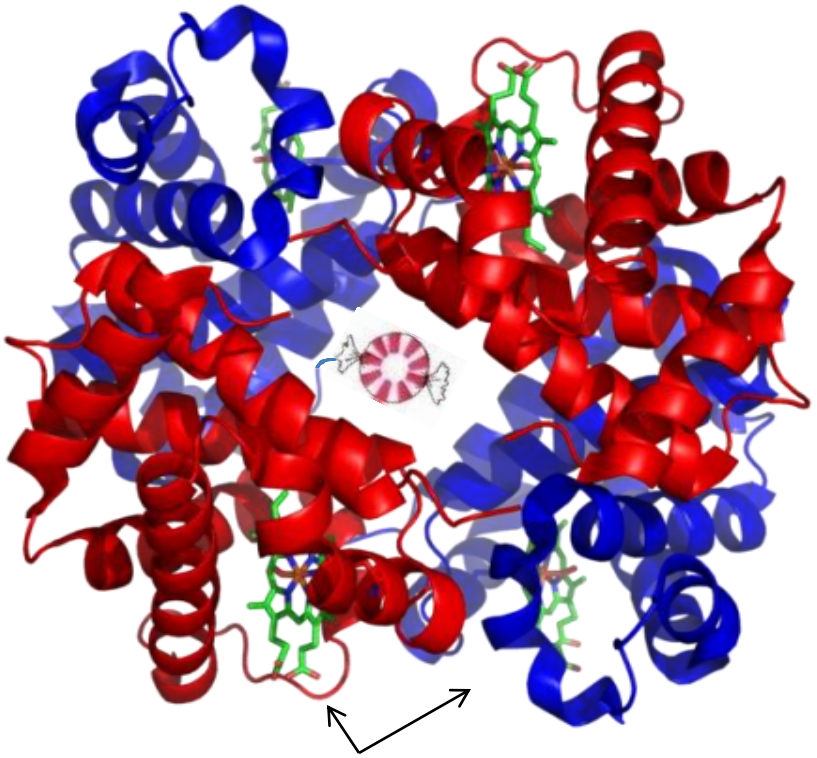
Hemoglobin (Hb) / Hb-A1c

Hemoglobin

Protein in red blood cells (erythrocytes)

- Primary function is respiratory
  - Transports oxygen / carbon dioxide

- Four globulin chains
  - Normal adult
    - 2 alpha-chains (α-Hb)
    - 2 beta-chains (β-Hb)
  - All chains contain an embedded heme-group



Heme (green)

Hemoglobin A1c

- Glycation of N-terminal valine of the Hb beta-chain
  - Non-enzymatic, [glucose]-dependent

-Serves as an average measure of blood glucose over the past 2 to 3 months

- β-Hb contains 11 lysine residues that can also undergo glycation

Diabetes Mellitus / diagnosis and monitoring

<u>Diabetes</u>

- ***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

<u>Diagnosis and monitoring</u>

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

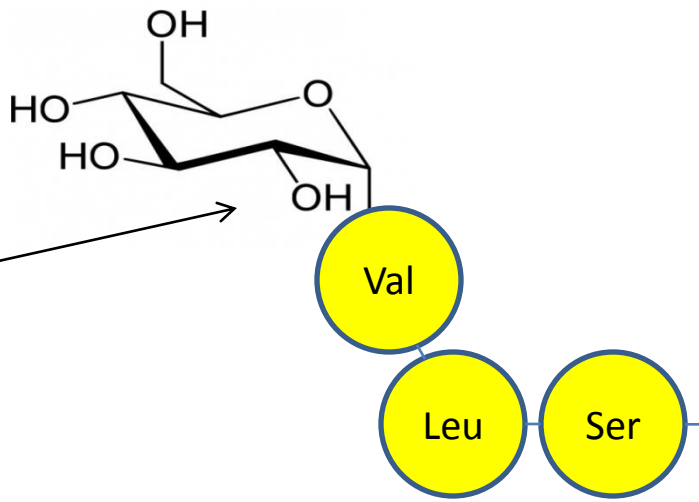
Fasting levels:	normal	70 and 99 mg/dL
	pre-dia.	100-125 mg/dL
	diabetic	126 mg/dL

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

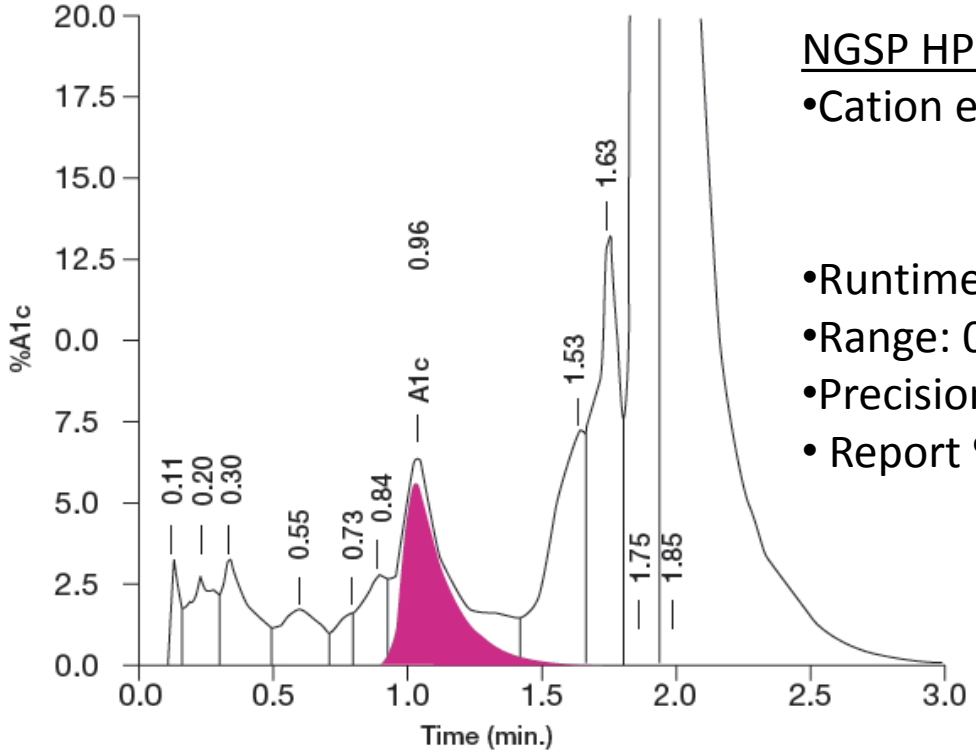
-HPLC, ELISA (fasting not required, single measurement)

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

NGSP techniques and MALDI-TOF



- Glycation causes change in molecular charge and mass
  - Glucose addition
    - reduction in molecular charge (-1)
    - addition (+ 162 Da) in molecular mass



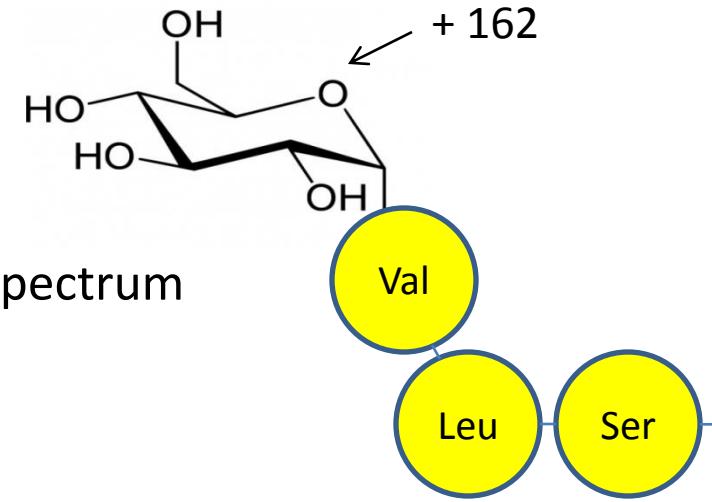
Example HPLC Chromatogram

<u>NGSP HPLC Method</u>

- Cation exchange chromatography
  - difference in charge state
  - Heme abs. (415nm)
- Runtime: 3 min
- Range: 0 – 20% HbA1c
- Precision ≤ 2%
- Report % A1c as a ratio

  [A1c / (Hβ + A1c)] * 100 = % A1c

Why MALDI-TOF ?



- accurate across a wide mass range
  linear mode 500 – 100000 and beyond in single spectrum

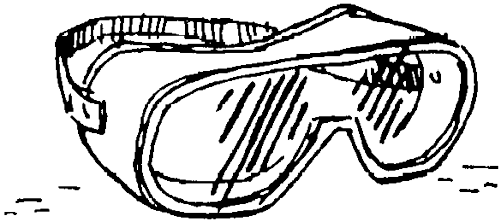
- precise, quantitative

- requires << 1 µL of sample

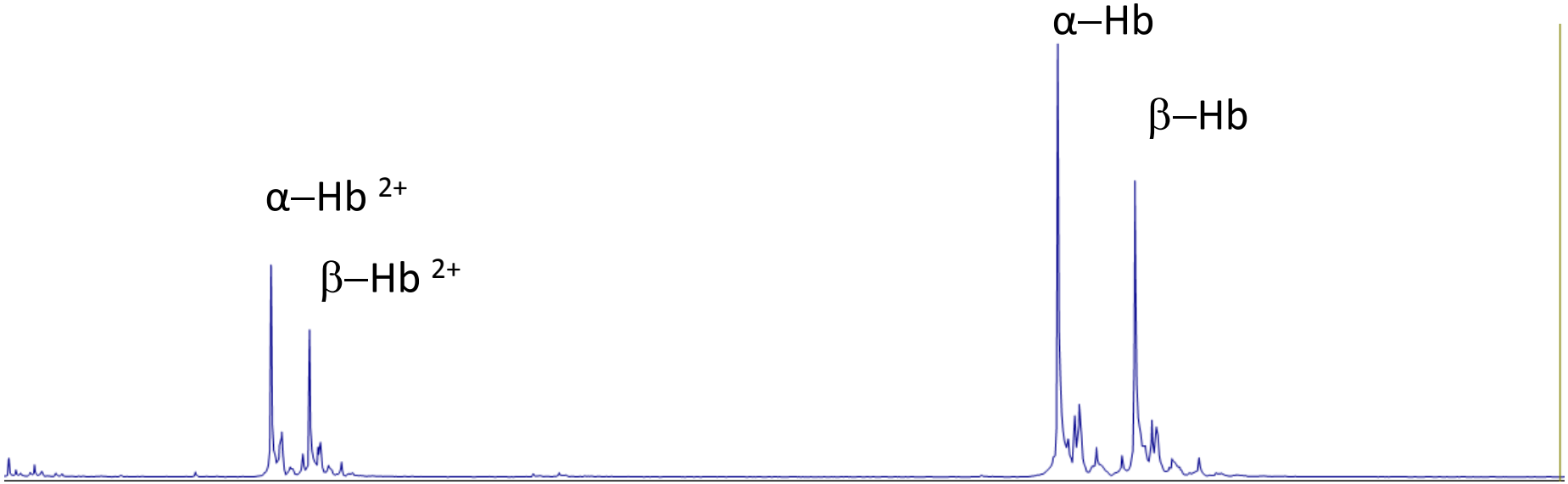
- fast, high throughput
  - single analyses time scale ~ 20 seconds
  - multiple replicates analyses, 5x ~ minutes
  - reanalysis may be done if necessary

- Potential for the provision of addition information

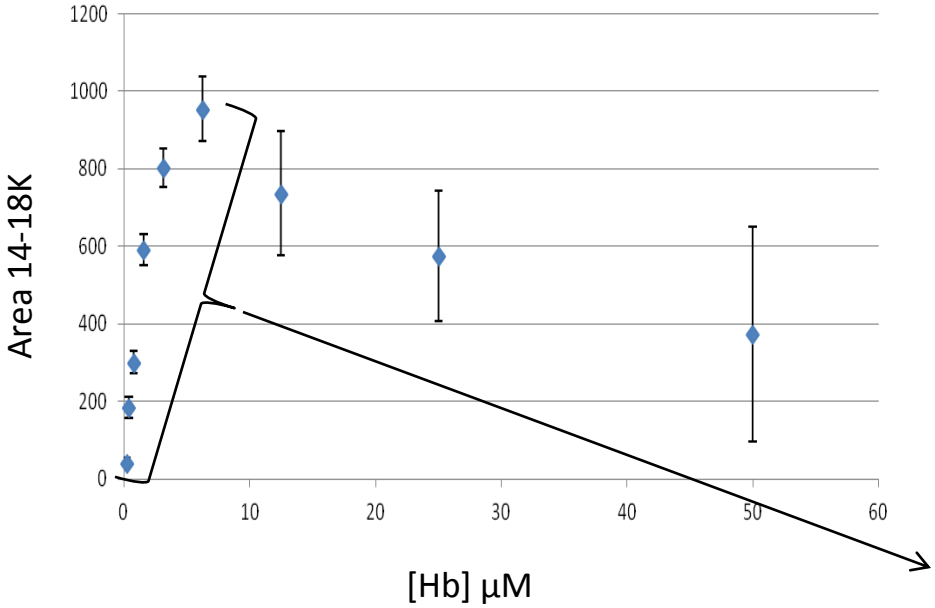
Investigations



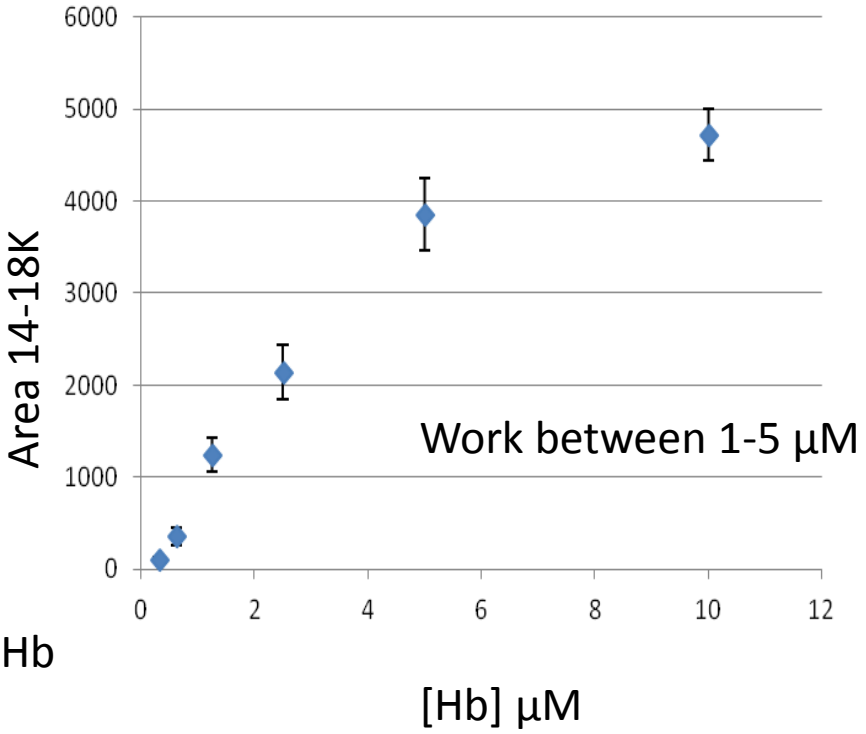
- ***Practicality*** – method development
- ***Reproducibility*** - precision of procedure / measurements
- ***Stability*** - of target analyte
- ***Range*** - measurement across clinically relevant domain
- ***Accuracy*** - comparison with NGSP validated technique (HPLC)



[Where] to work ?



Range: 0.2 – 10 µM
Signal: 14-18 kDa
Measurements: 5x each



- Serial dilution 50.0 – 0.2 µM Hb

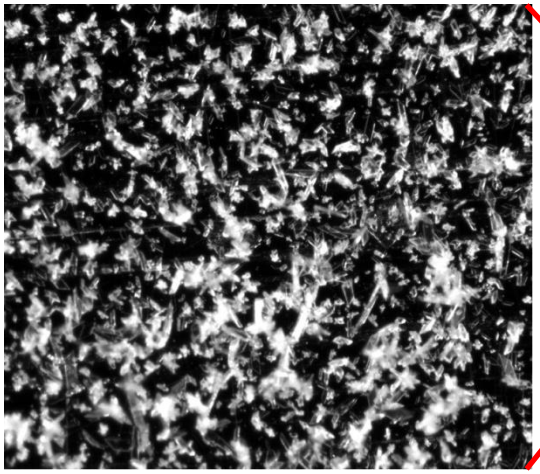
- quantitative response ~ 0.2- 5.0 µM

- ***1:2000 dilution of whole blood*** ~ 2.0 µM Hb

Sample Preparation

- Sinapinic acid (30% CH_3CN, 0.1% TFA)
- µFocus MALDI Plate 2600 µm (Hudson Surface Technology)
- Samples spotted as 5x replicates

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



Crystals ~ 10-20 µM

Sample Acquisition

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

Capabilities:

- Max accelerating voltage 20 kV

- Max laser pulse frequency 5000 Hz

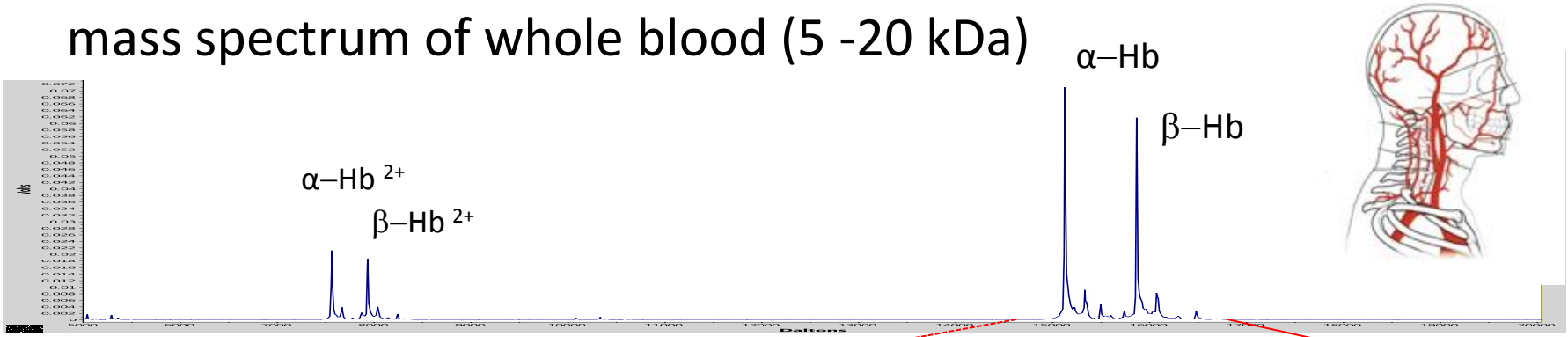
- Max scan speed 10 mm/s

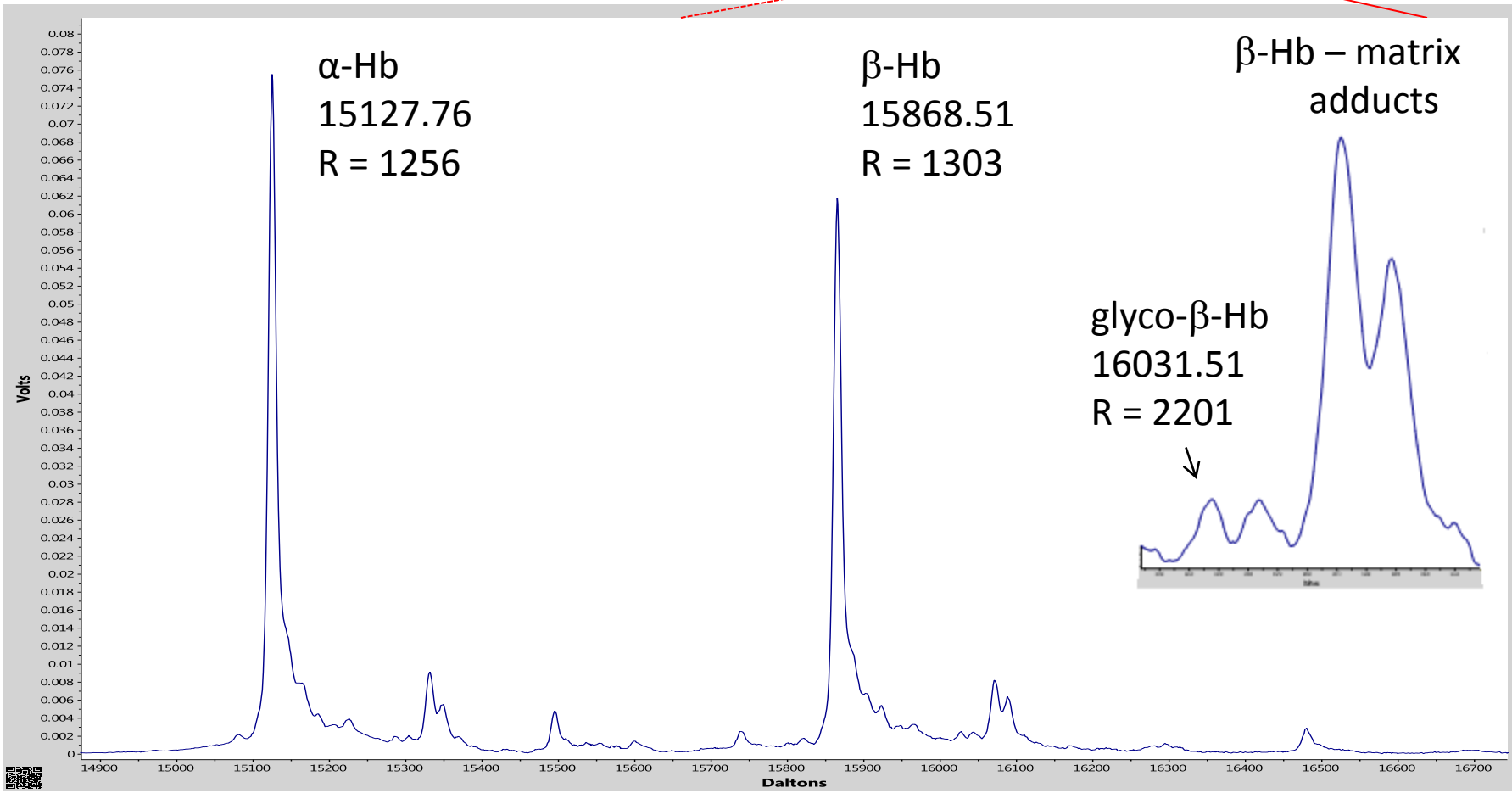
<u>Acquisition parameters</u>

- 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 µJ
- Scan rate 1 mm/s
- Spot size 2.6 µm
- 100 µm raster to cover each sample position

Red = adjustable parameters that determine acquisition speed

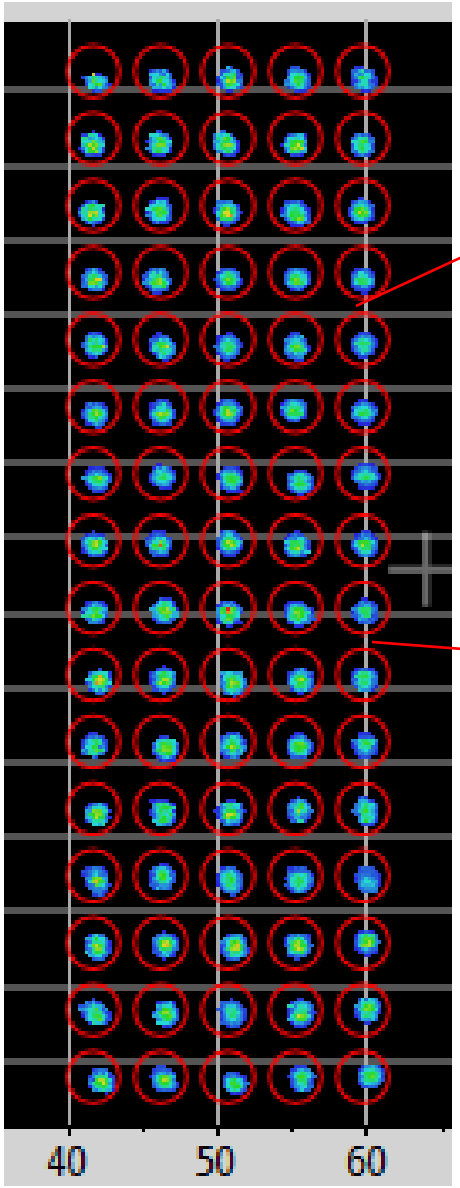
mass spectrum of whole blood (5 -20 kDa)



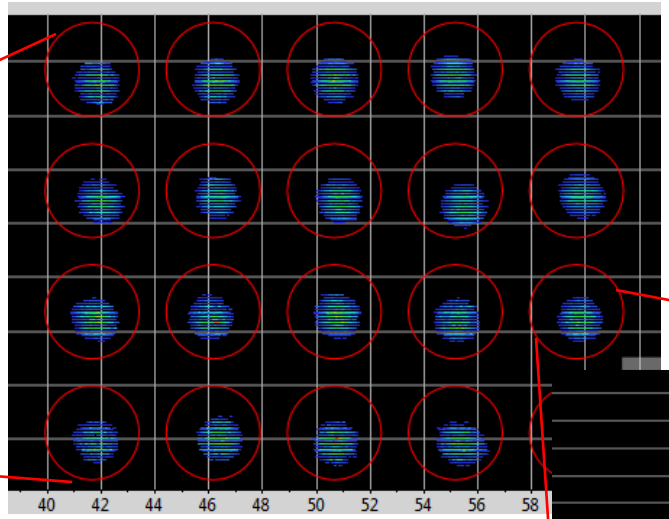


Sample Acquisition cont.

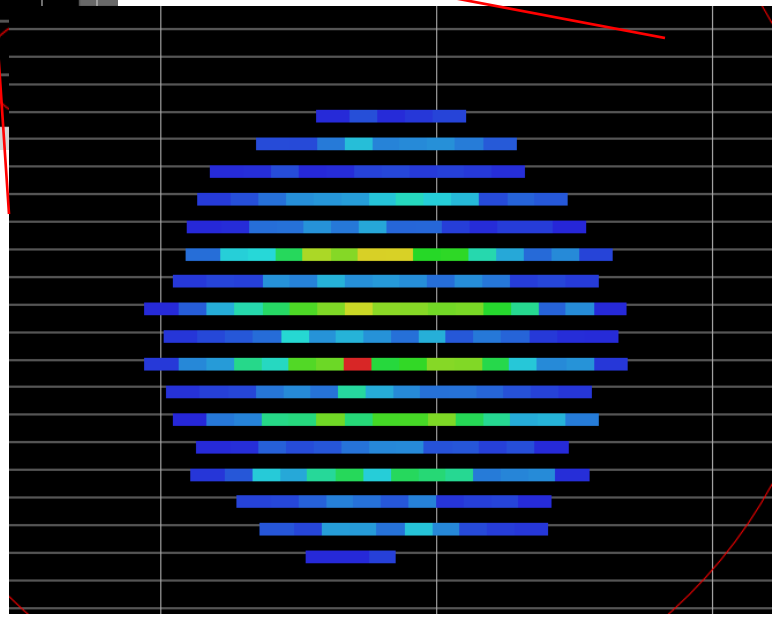
Microscope slide-sized plate



16 samples / 5x replication.

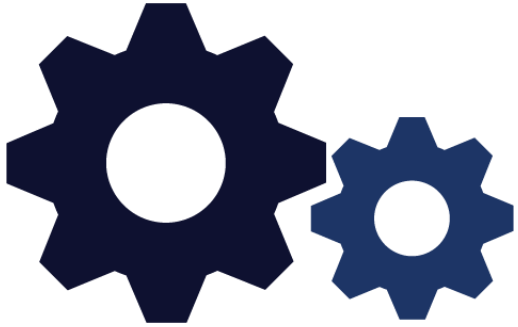


100 shots / spectrum
~ 200 spectra / spot



Each pixel = 1 spectrum

Post Acquisition Data Processing

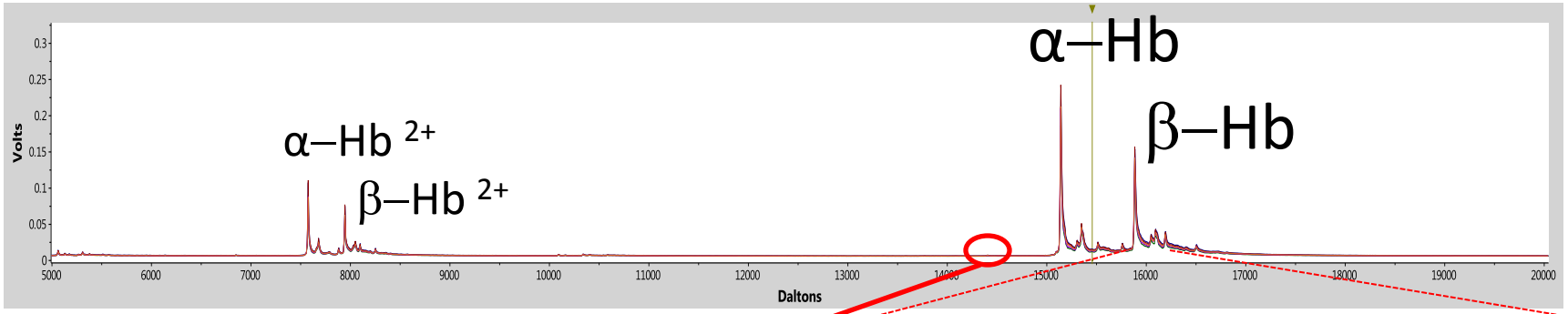


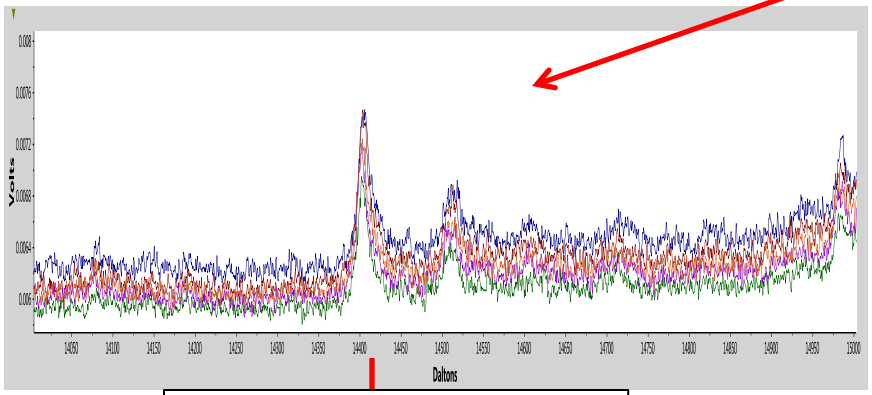
- ***Average all spectra > 20 mV*** signal intensity / spot
- ***Baseline corrected*** spot-averaged spectra
- ***Calibrate*** spot-averaged spectra
  - M^{+1} and M^{+2} ions of hemoglobin α and β subunits
    (α = 7,564.37, 15,127.74, β = 7,934.75, 15,868.51 Da)
- ***Quantify*** by integration of signals from β–Hb and (β–Hb + 162 (glucose))
- ***Report*** as a ratio of the percentage of total glycation on the β chain

$$[\ (H\beta + 162) / (H\beta + (H\beta+162))\ ]\ * 100 = \%\ \text{Glyco-}\beta\text{–Hb}$$

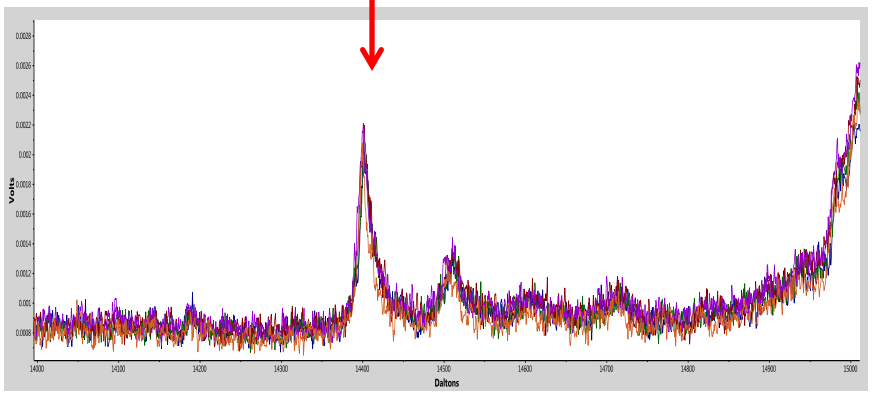
(can also be done for glyco-α–Hb)

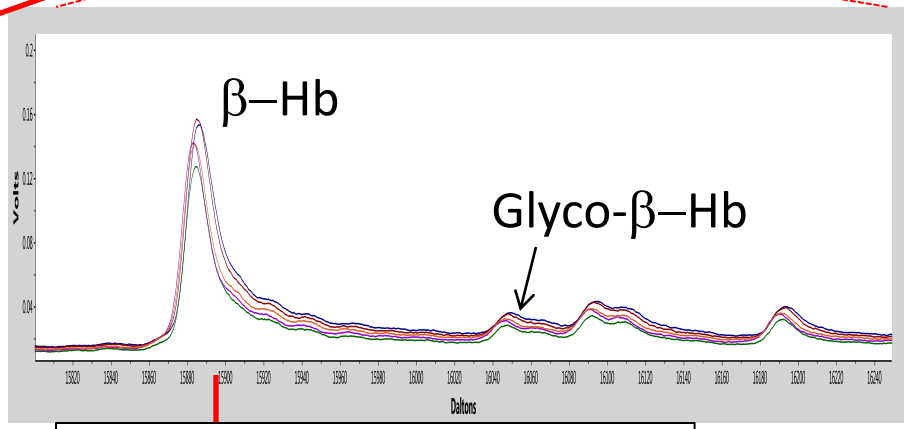
Data Processing



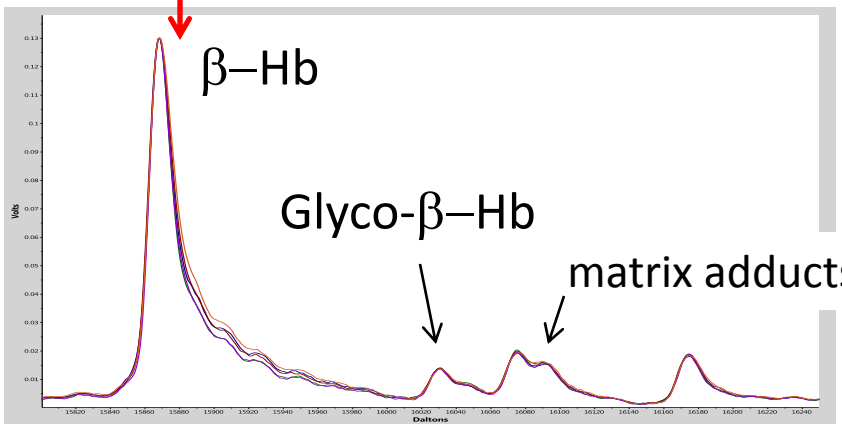


1. Baseline correction

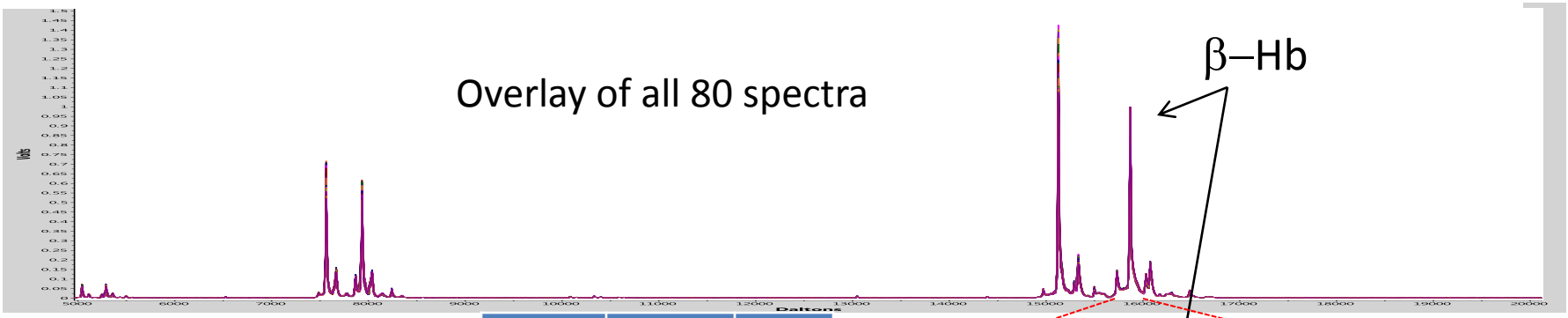




2. Calibration & normalization



Reproducibility

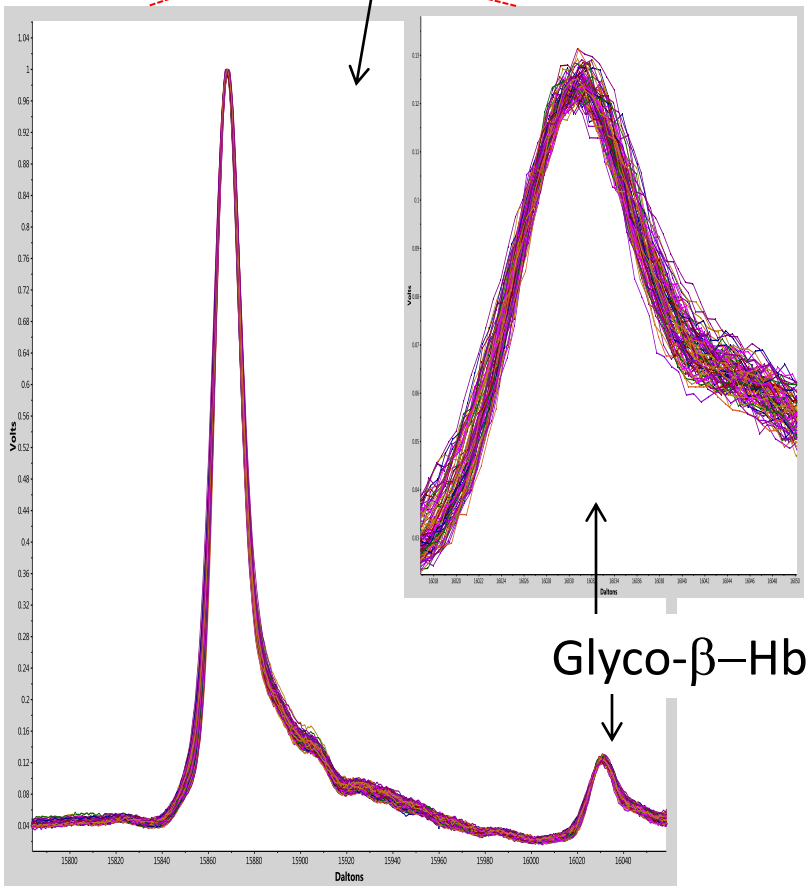


<u>Reproducibility experiment</u>

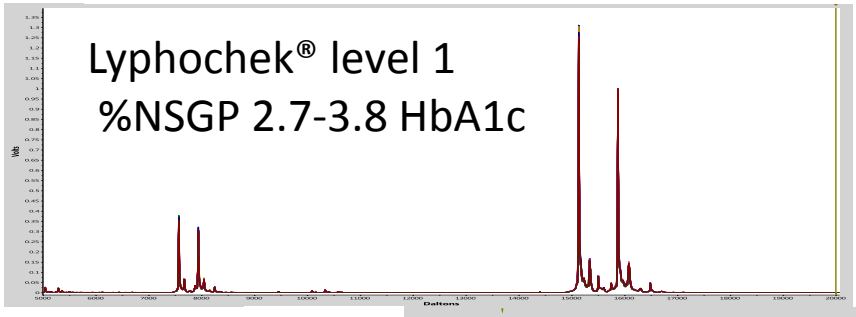
- 1 sample across plate
- Processed 16 samples
  5x replicates
- Ave CV for 16 < 1.00%
- CV for 16 Glyco ratios 1.22 %

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
Average	13.14	0.90
Std Dev	0.16	
Rel Std Dev	1.22	

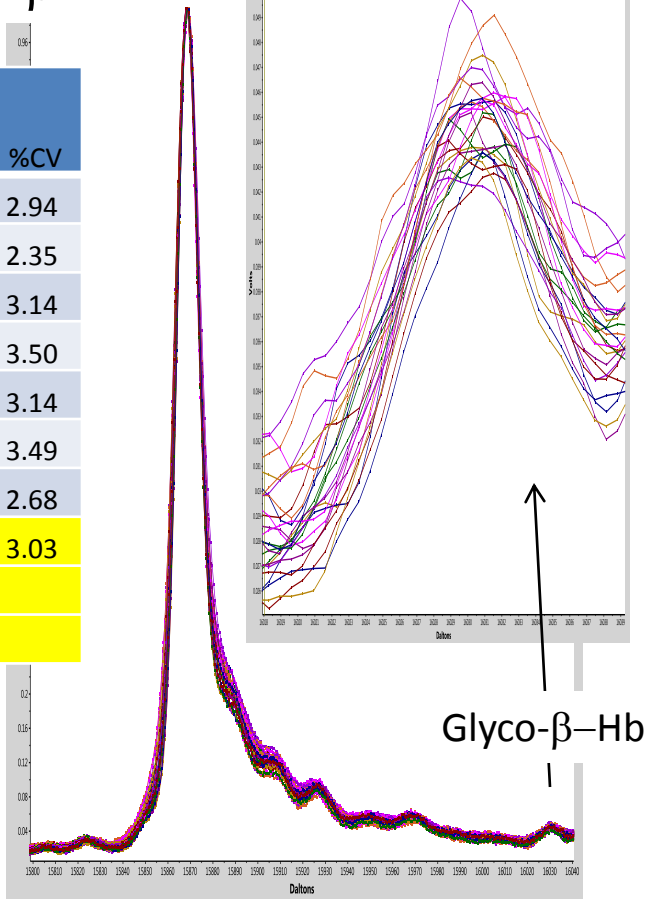


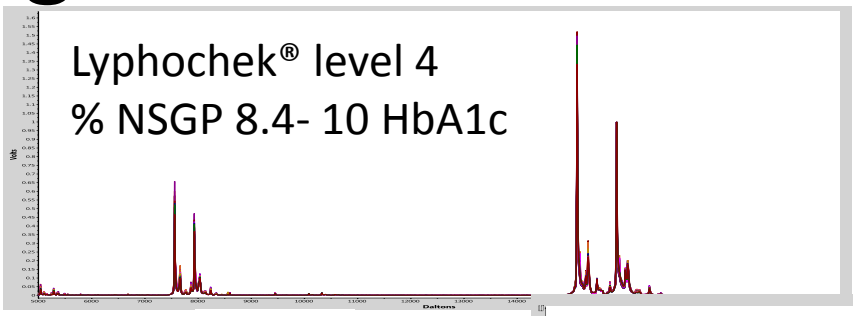
Low and mid range HbA1c Stds



β–Hb

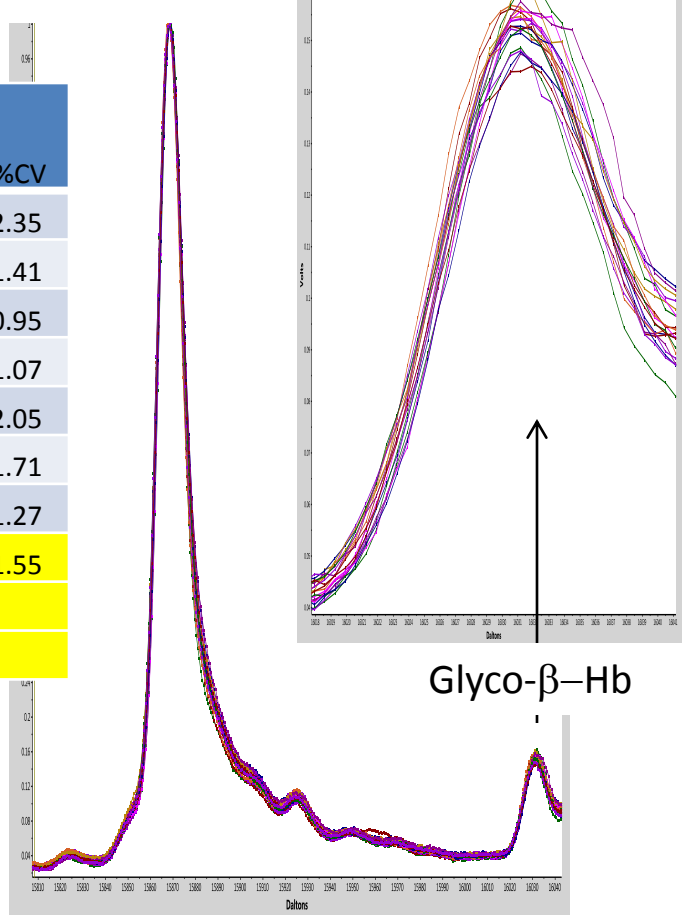
Level 1 Std	GlyHb/ Hb%	%CV
T1	5.19	2.94
T2	4.96	2.35
T3	4.99	3.14
T4	4.94	3.50
T5	5.07	3.14
T6	4.75	3.49
T7	4.91	2.68
AVE	4.97	3.03
Std Dev	0.14	
% CV	2.74	



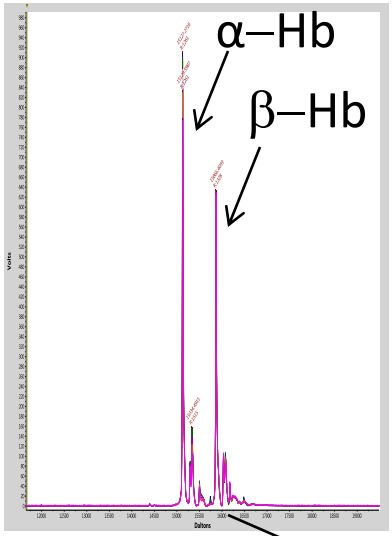


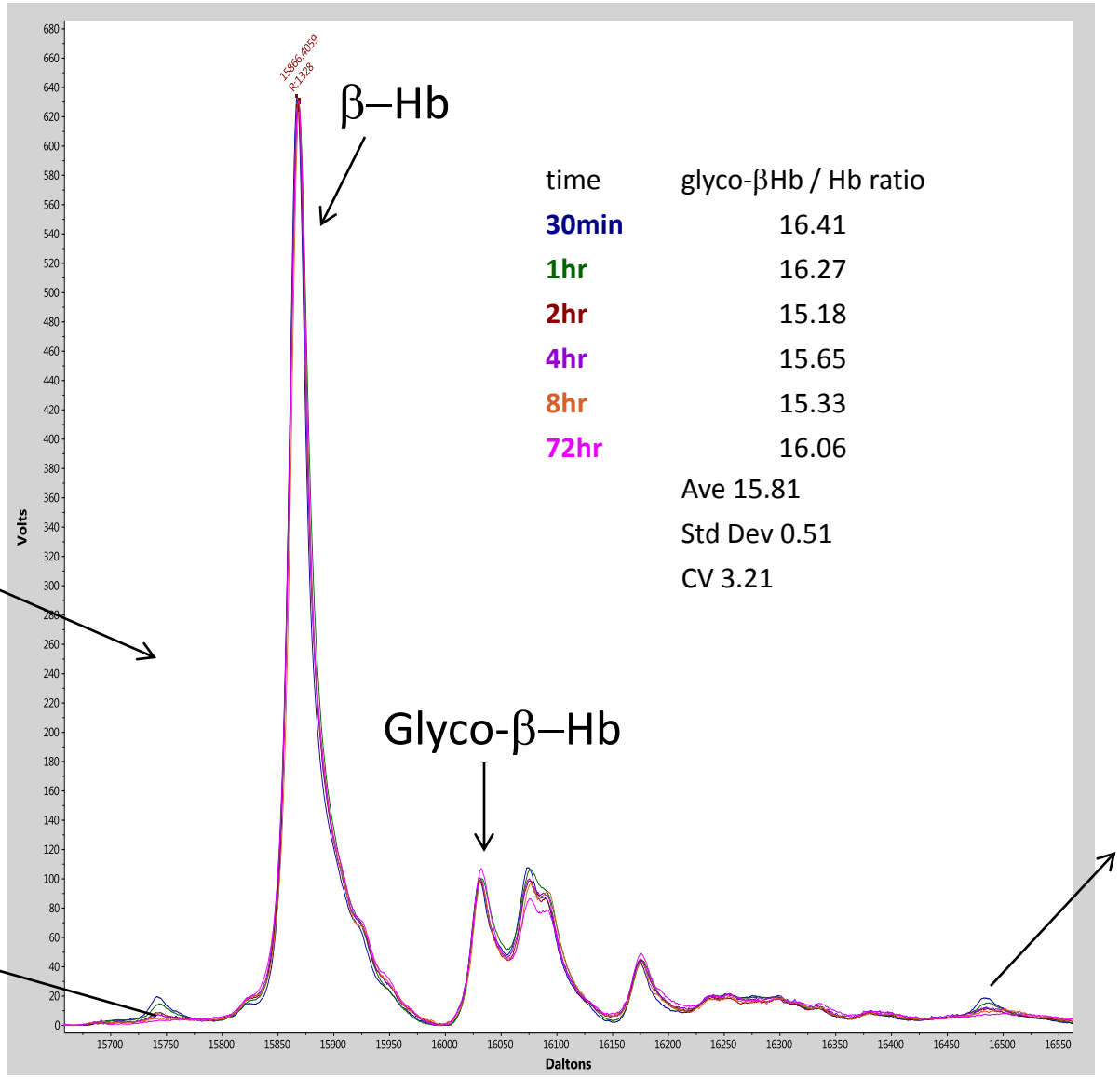
β–Hb

Level 4 Std	GlyHb/ Hb%	%CV
T1	13.97	2.35
T2	13.81	1.41
T3	14.12	0.95
T4	13.48	1.07
T5	13.58	2.05
T6	13.62	1.71
T7	13.36	1.27
Ave	13.70	1.55
Std Dev	0.21	
% CV	1.55	

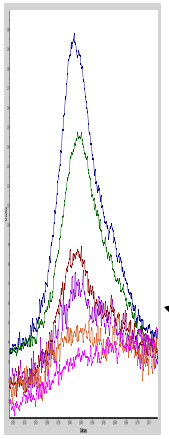


Stability / Time Course of Glyco-β-Hb / Hb Ratio





time	glyco-βHb / Hb ratio
30min	16.41
1hr	16.27
2hr	15.18
4hr	15.65
8hr	15.33
72hr	16.06
	Ave 15.81
	Std Dev 0.51
	CV 3.21



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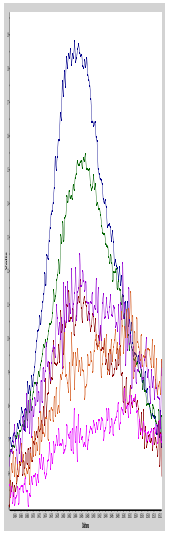
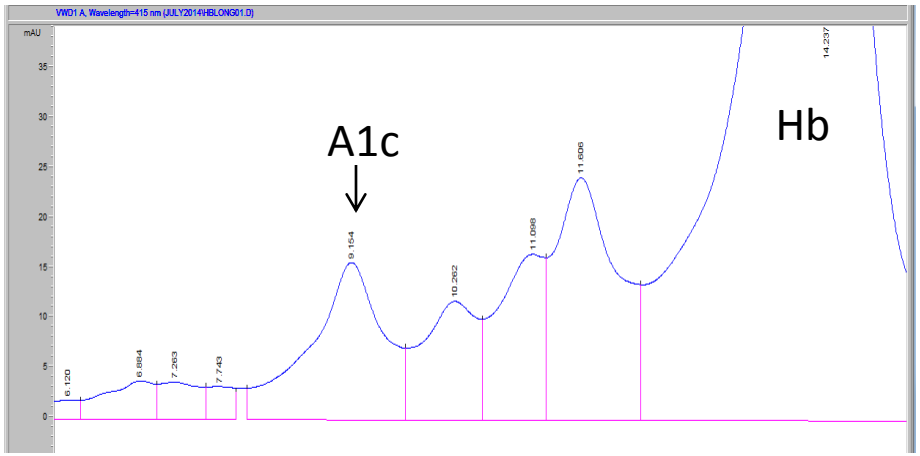


Some things are happening ?

Isolation of A1c for Calibration Curve Construction



- Agilent 1100 series LC
- Mono S 5/50 GL column

Buffers

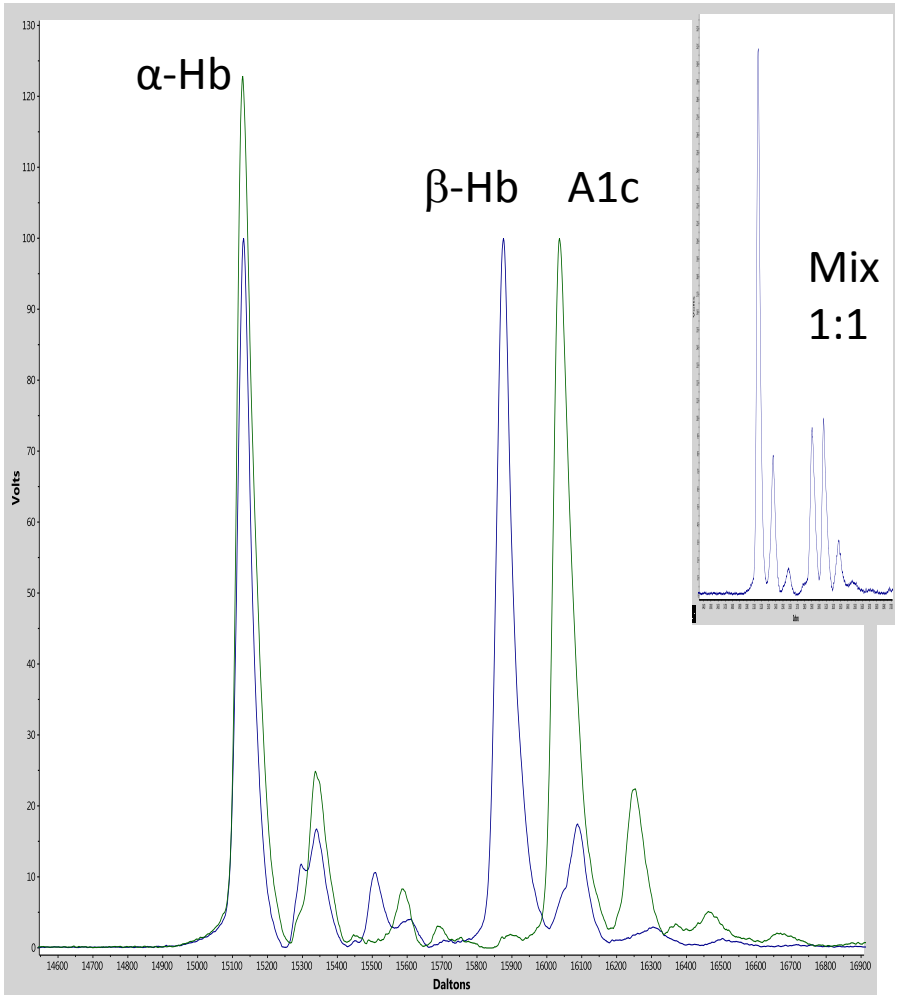
A = 10 mM Na Malonate pH 5.7

B = 10 mM Na Malonate pH 5.7, 0.3 M LiCl

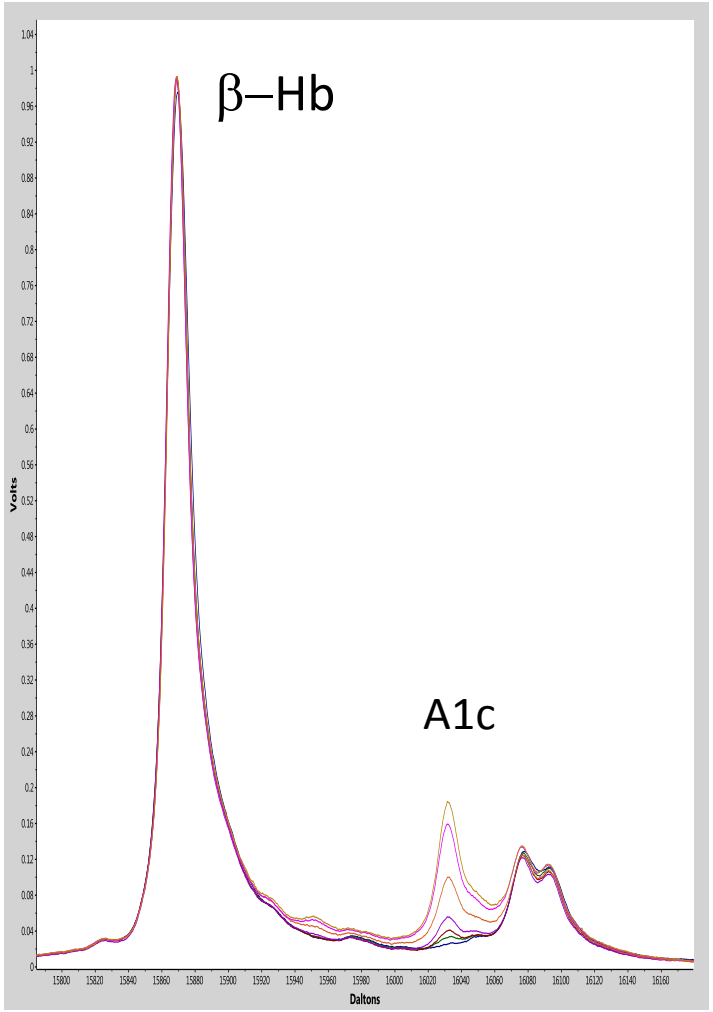
Detection 415 nm

***CLIN. CHEM*. 32/10, 1867-1 872 (1986)**

Measurement of Hemoglobin A1C by a New Liquid-Chromatographic Assay: Methodology, Clinical Utility, and Relation to Glucose Tolerance Evaluated. Jan-Olof Jeppsson,' Per Jemtorp, Sundkvıst, KanEnglund, and Virve Nylunde

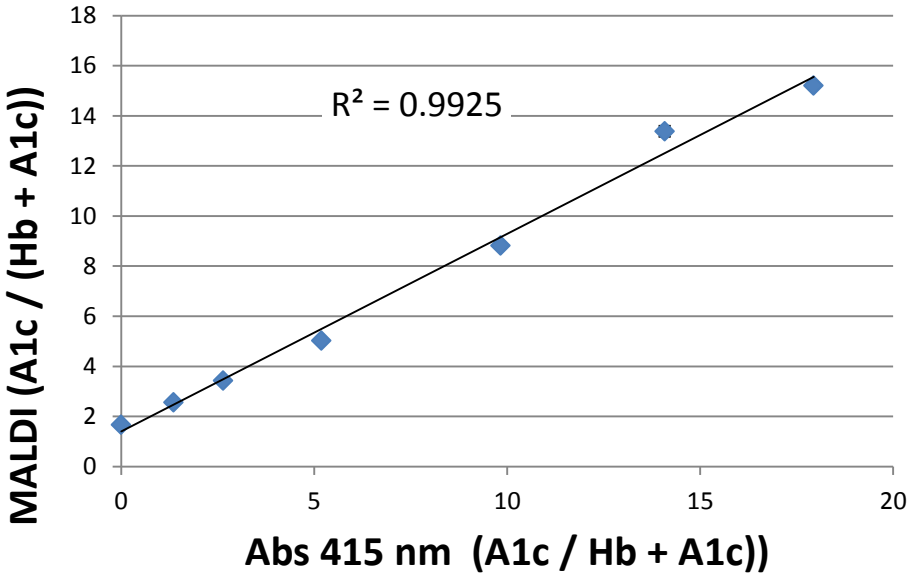


Spectra from purified materials



Calibration Curve Cont.

Avg	Std	CV
1.67	0.07	4.03
2.56	0.02	0.74
3.42	0.05	1.35
5.02	0.04	0.81
8.82	0.08	0.95
13.38	0.22	1.62
15.20	0.07	0.48



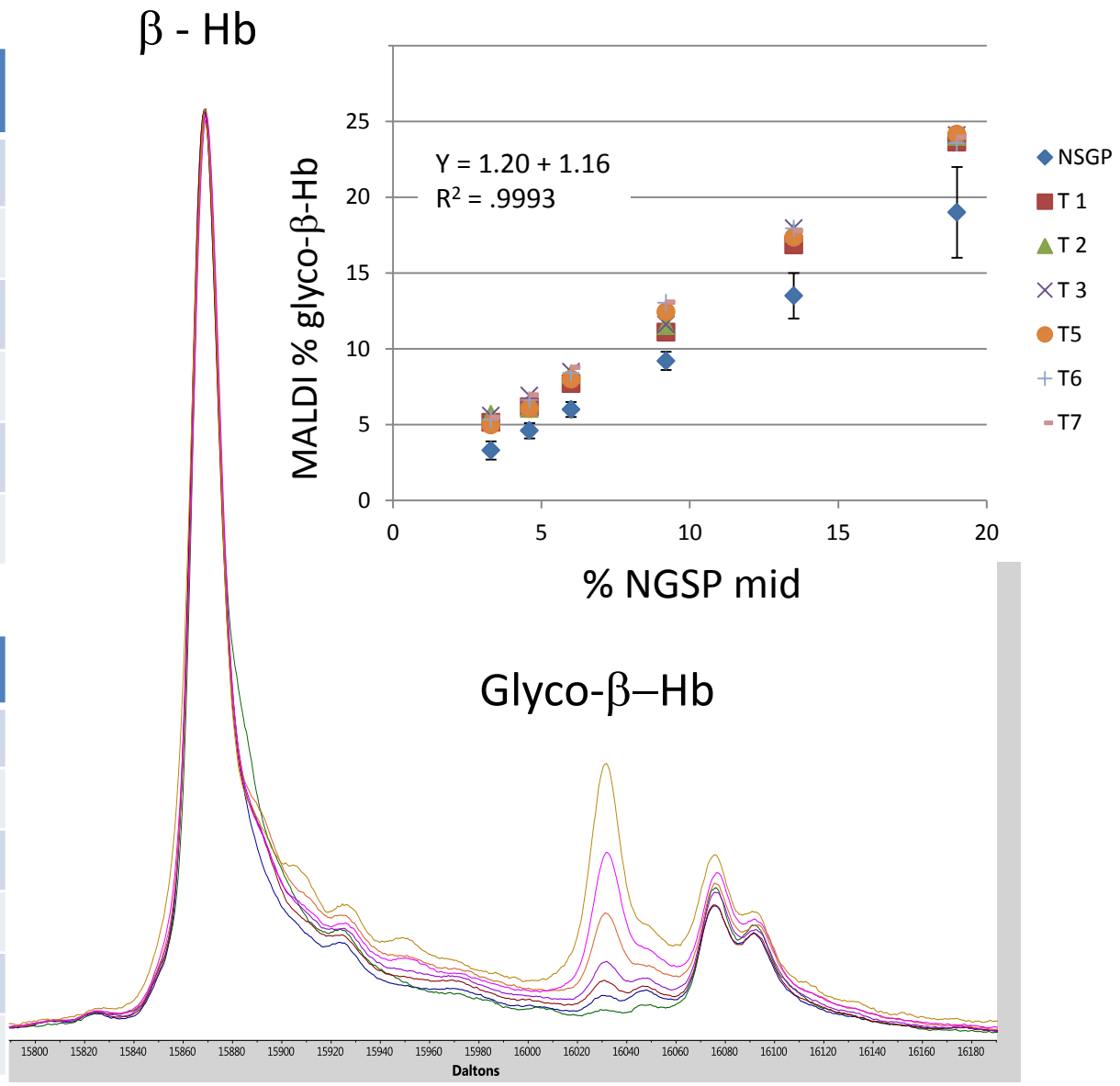
H_2O µL	A1c µL	Hb µL	Matirx µL	[Hb] µM	[A1c] µM	% A1c	% A1c/Hb +A1c
20	0	20	60	1.88	0		
19	1	20	60	1.88	.026	1.37	1.36
18	2	20	60	1.88	.051	2.73	2.64
16	4	20	60	1.88	.104	5.47	5.19
12	8	20	60	1.88	.208	10.93	9.83
8	12	20	60	1.88	.312	16.44	14.08
4	16	20	60	1.88	.416	21.92	17.94

Linear response across clinically relevant range

Analysis of Lyphochek® Hemoglobin A1c Linearity Set

	Expected 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 %

mid Std	Ave	Std Dev	CV
3.3	5.39	0.26	4.75
4.6	6.49	0.39	5.99
6	8.23	0.37	4.46
9.2	12.16	0.77	6.34
13.5	17.58	0.39	2.20
19	23.92	0.23	0.95



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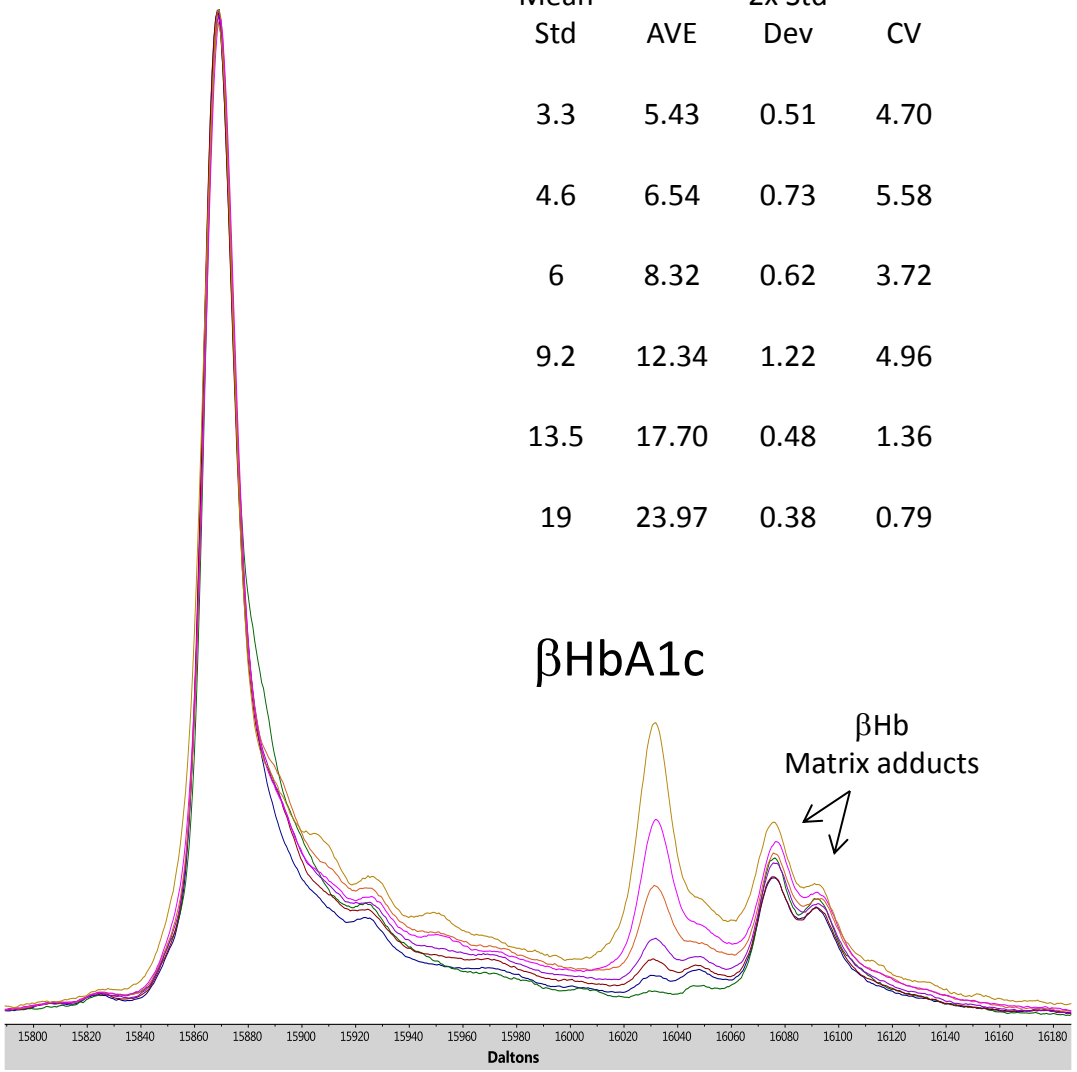
Level 4	8.4 – 10 %
Level 5	12 – 15 %
Level 6	16 – 22 %

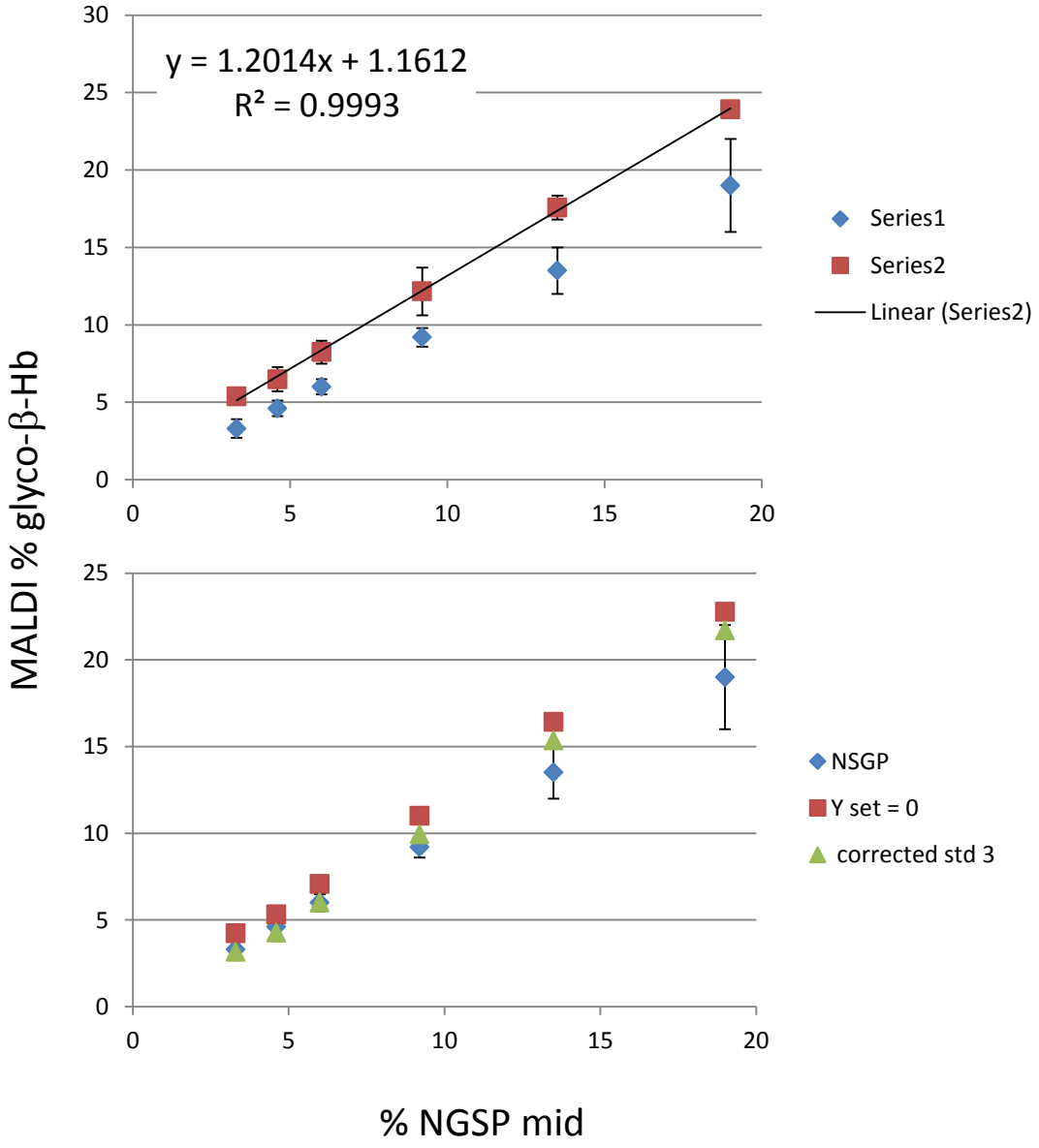
βHb

Mean Std	AVE	2x Std Dev	CV
3.3	5.43	0.51	4.70
4.6	6.54	0.73	5.58
6	8.32	0.62	3.72
9.2	12.34	1.22	4.96
13.5	17.70	0.48	1.36
19	23.97	0.38	0.79



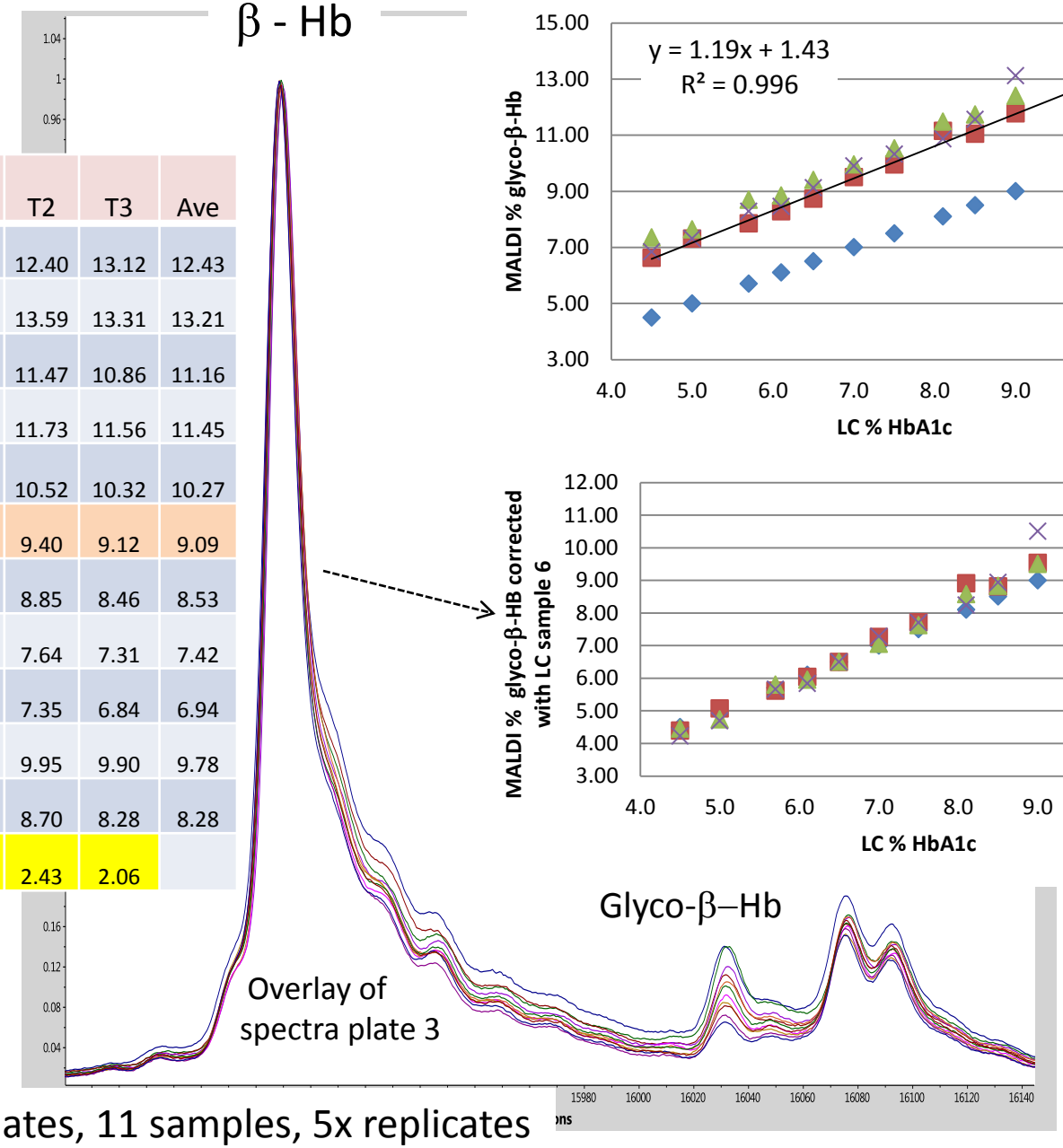
Lyphochek® Hemoglobin A1c Linearity Set cont.

	HbA1c %NGSP	Ave	Y set = 0	Corr.std 3
Level 1	2.7 - 3.8 %	5.39	4.23	3.16
Level 2	4.1 - 5.1 %	6.49	5.33	4.26
Level 3	5.5 – 6.5 %	8.23	7.07	6.00
Level 4	8.4 – 10 %	12.16	11.00	9.93
Level 5	12 – 15 %	17.58	16.42	15.35
Level 6	16 – 22 %	23.92	22.76	21.69



Blood Samples Jan. 2015, 3 plates, 11 samples

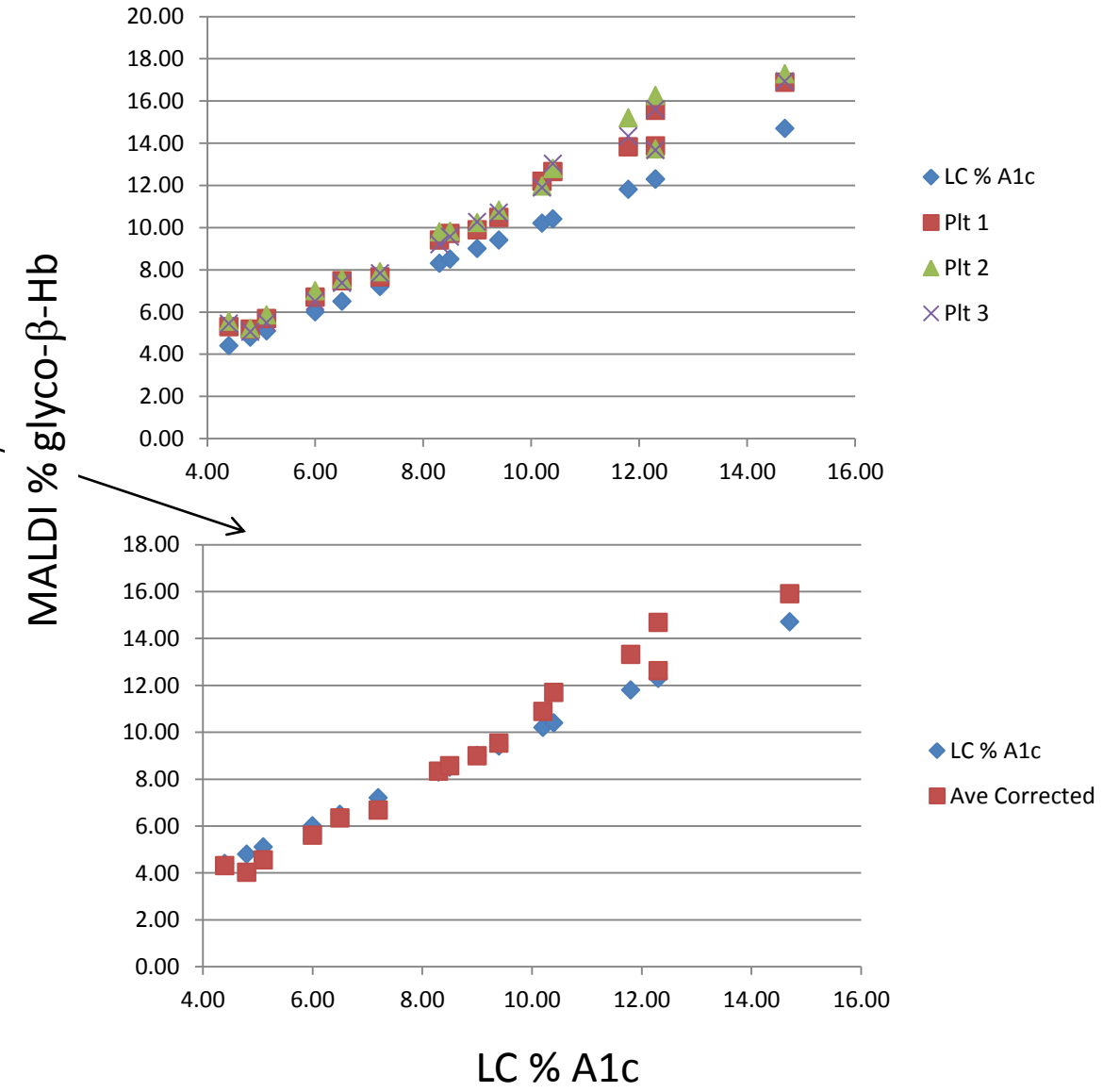
LC % A1c	T1	T2	T3	Ave
9.00	11.78	12.40	13.12	12.43
9.90	12.72	13.59	13.31	13.21
8.10	11.15	11.47	10.86	11.16
8.50	11.05	11.73	11.56	11.45
7.50	9.95	10.52	10.32	10.27
6.50	8.74	9.40	9.12	9.09
6.10	8.28	8.85	8.46	8.53
5.00	7.31	7.64	7.31	7.42
4.50	6.63	7.35	6.84	6.94
7.00	9.50	9.95	9.90	9.78
5.70	7.86	8.70	8.28	8.28
Ave % CV	2.09	2.43	2.06	



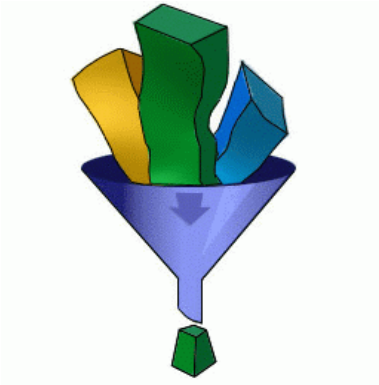
3 plates, 11 samples, 5x replicates

Samples Nov 2014, 3 plates, 16 samples

LC % A1c	Plt 1 % glyco-β-Hb	Plt 2 % glyco-β-Hb	Plt 3 % glyco-β-Hb	AVE
11.80	13.82	15.19	14.33	14.45
5.10	5.69	5.86	5.48	5.68
7.20	7.66	7.91	7.84	7.80
6.50	7.47	7.56	7.37	7.47
8.30	9.41	9.80	9.20	9.47
9.40	10.48	10.81	10.70	10.67
6.00	6.71	7.00	6.50	6.73
9.00	9.88	10.24	10.26	10.13
4.80	5.18	5.22	5.06	5.15
12.30	13.86	13.72	13.67	13.75
12.30	15.56	16.26	15.61	15.81
4.40	5.29	5.56	5.45	5.43
14.70	16.89	17.29	16.92	17.03
10.20	12.20	11.96	11.89	12.02
10.40	12.66	12.79	13.02	12.82
8.50	9.72	9.82	9.56	9.70
Ave CV	% 1.73	% 2.55	% 2.56	



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 estimates of glyco-β-Hb

  glyco-α-Hb ratio

  glyco-β-Hb $^{2+}$ ratio
- Investigate relationship between α and β chain glycation
- Automate sample handling
- Implement calibration standard into analysis
- Run more samples