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UNIVERSITÀ DEGLI STUDI
DI MILANO

Centre for Metrological
Traceability in
Laboratory Medicine
(CIRME)

site: <http://users.unimi.it/cirme>

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CELEBRATING
Years

The long and winding road to the standardization of HbA₂

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CIRME

Dept. Physiopathology and Transplantation

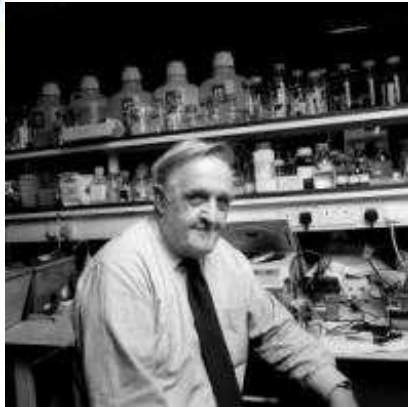
Università degli Studi di Milano

Contents

- **Why HbA₂ is important**
- **State of the art**
- **Activities of the IFCC WG-HbA2**

Contents

- **Why HbA₂ is important**
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- Activities of the IFCC WG-HbA₂



World Distribution, Population Genetics, and Health Burden of the Hemoglobinopathies

Thomas N. Williams¹ and David J. Weatherall²

¹Kenya Medical Research Institute/Wellcome Trust Programme, Centre for Geographical Research, Kilifi District Hospital, PO Box 230, Kilifi, Kenya

²Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX39DU, United Kingdom

Correspondence: twilliams@kilifi.kemri-wellcome.org

Table 1. A breakdown of the annual number of births with the different hemoglobin disorders

Annual births with major hemoglobin disorders	
β -thalassemia major	22,989
HbE β thalassemia	19,128
HbH disease	9568
Hb Bart's hydrops (α^0/α^0)	5183
SS disease	217,331
S β thalassemia	11,074
SC disease	54,736

From available data (Modell and Darlison 2008; Weatherall 2010).

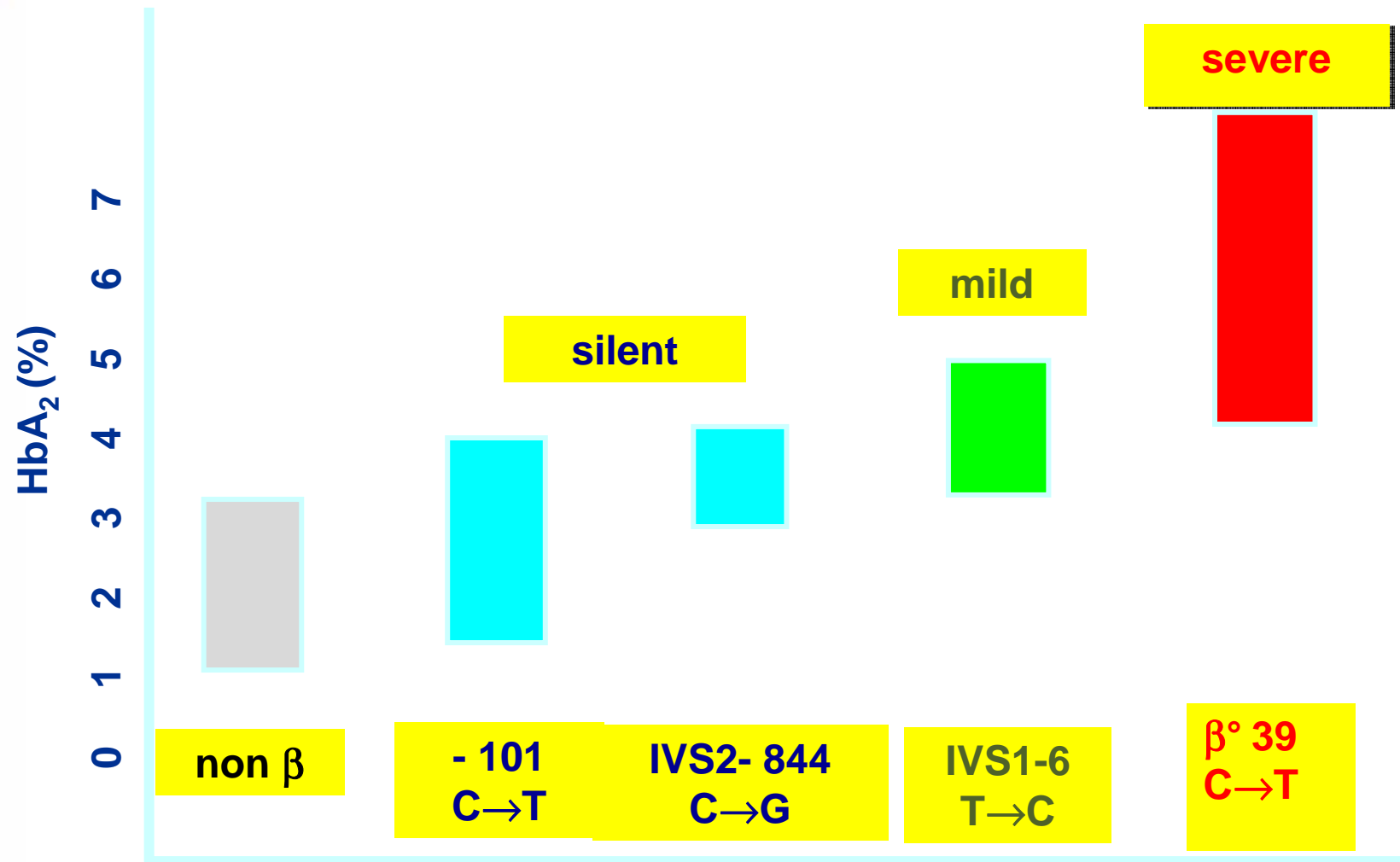


Figure 2. The world distribution of the origins of the α and β thalassemias. (From Weatherall and Clegg 2001; reprinted, with permission, from the author.)

The importance of Hb A₂ measurement

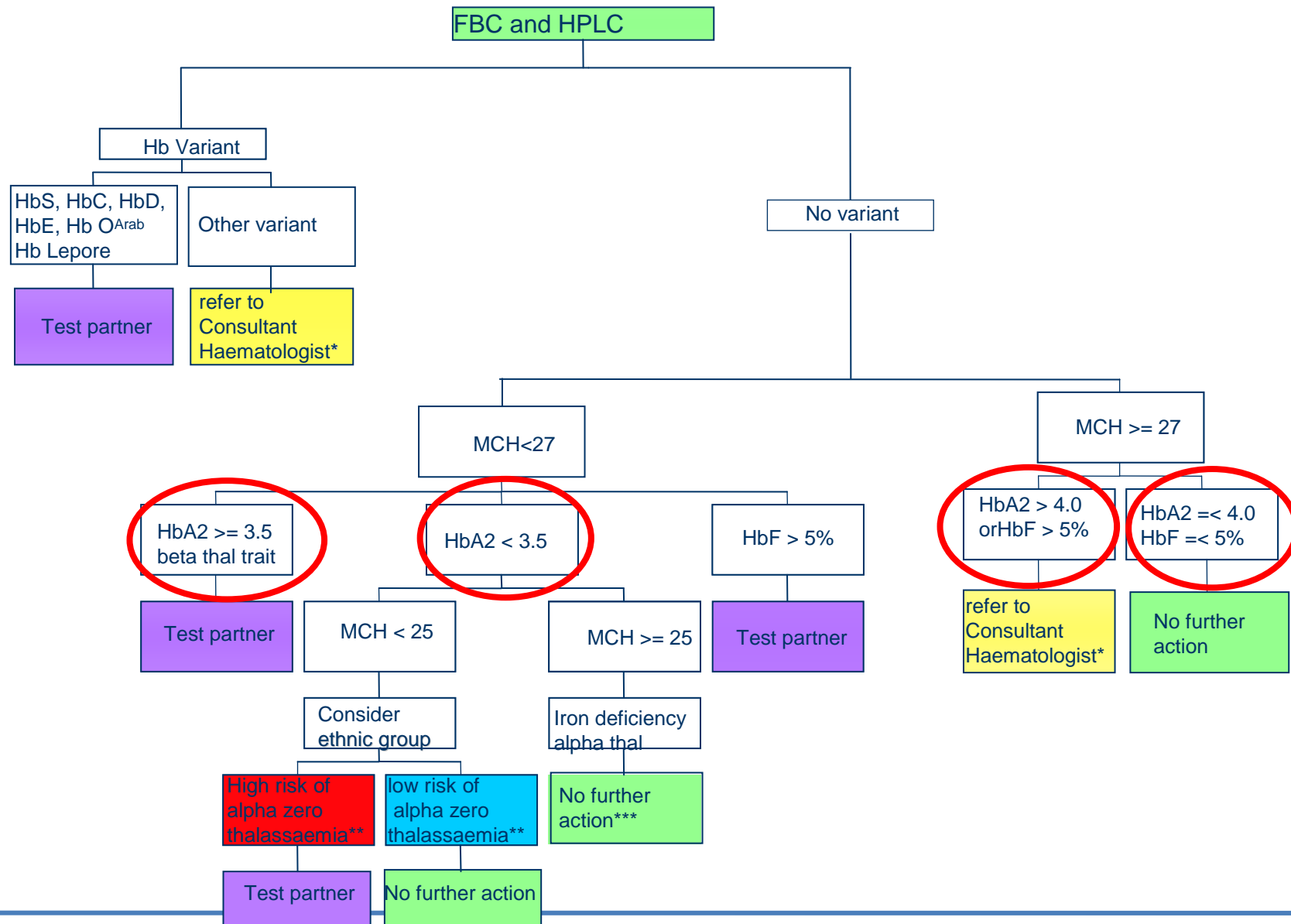
- **Hb A₂ measurement is used as a marker for beta thalassemia trait**
- **Carrier detection is important because:**
 - β -thalassemia carriers are asymptomatic but homozygous β -thalassemia is a life-threatening disorder
 - Women should be screened for β -thal trait (high risk areas)
 - Carriers: recommend partner testing prediction of genetic risk
- **Failure to detect condition may result in newborn with a medically significant condition**

HbA₂ in various β -thalassemia genotypes



(R. Galanello, 2002)

NSC&TSP: High Prevalence Screening



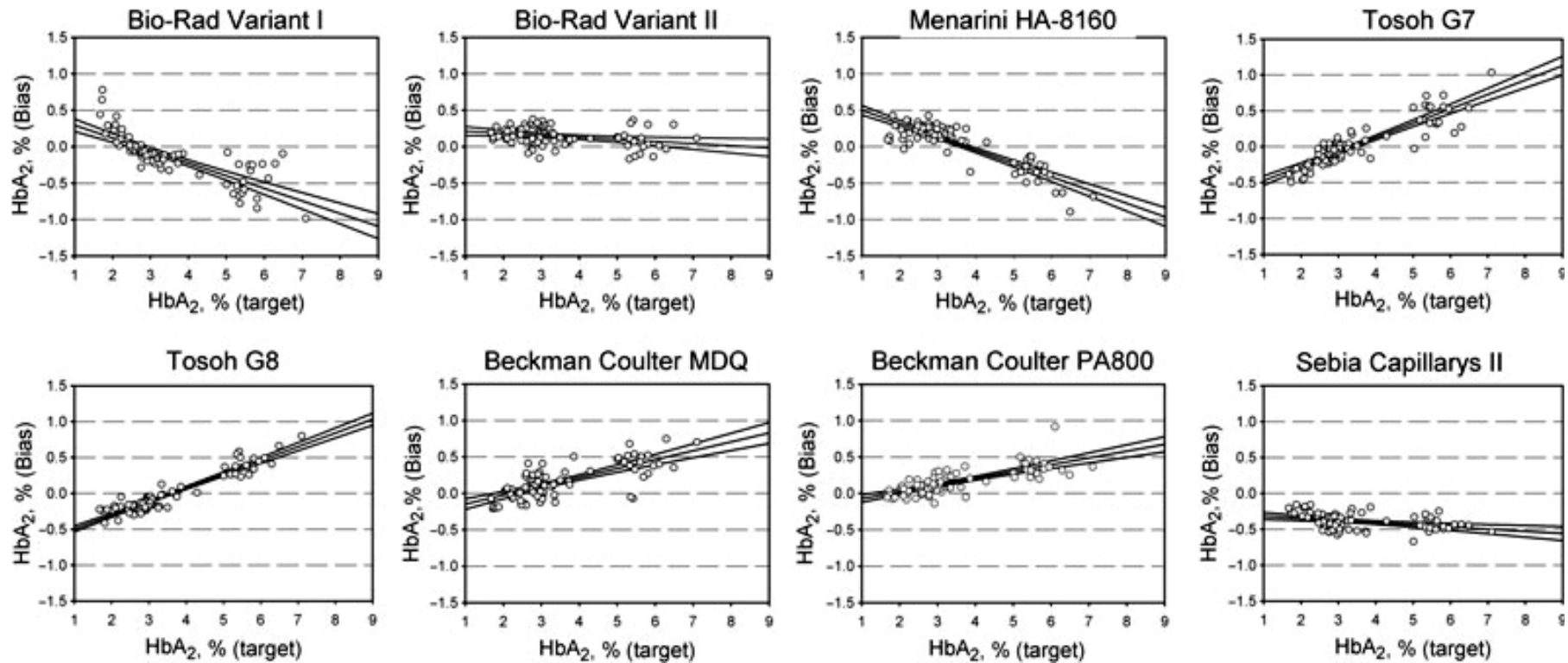
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Interlaboratory comparison of current high-performance methods for HbA₂

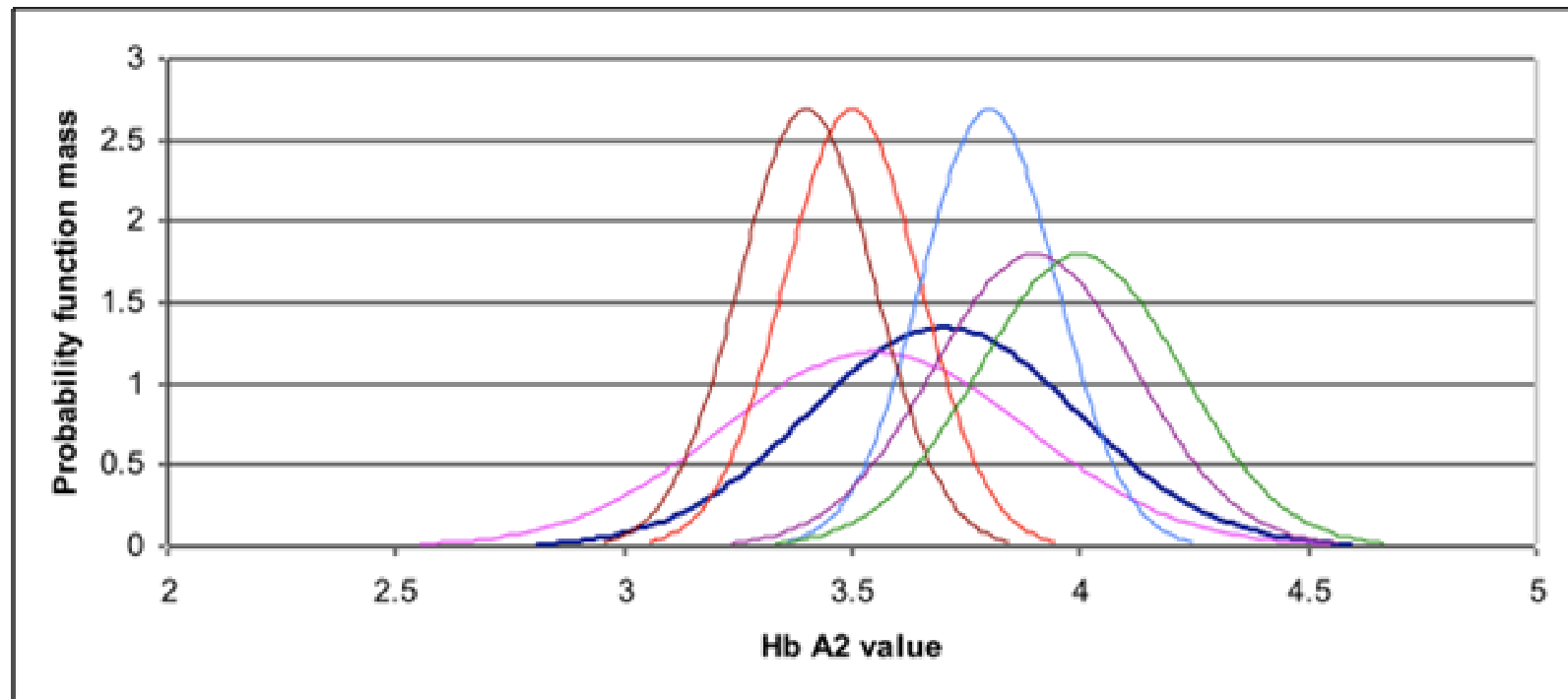
R. PALEARI*, B. GULBIS†, F. COTTON†, A. MOSCA*



UKNEQAS

UK National External Quality Assessment Scheme

Borderline sample: Hb A₂ 3.7%





Centro di Riferimento Sicurezza di Qualità
Valutazione esterna di qualità
SCREENING Hb-Ciclo 2015

Analita: **HbA2** %
Risultato atteso: 3.0

	N.	Out	Media	C.V.	S.D.	Med.na
Tutti	101	3	3.33	13.26	0.44	3.30
Tuo Metodo	25	1	3.03	4.41	0.13	3.00

Campione	5 (Scad. 25/08/2015)
Tuo risultato	2.9

	Diff. S	Diff. %
Tutti	-0.97	-12.91
Tuo Metodo	-1.00	-4.29

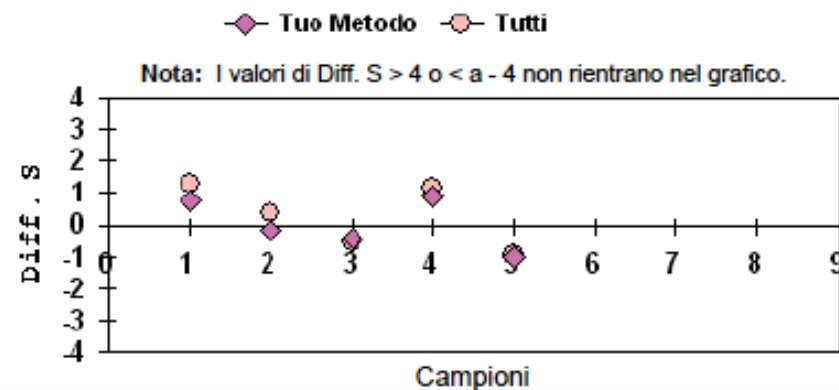
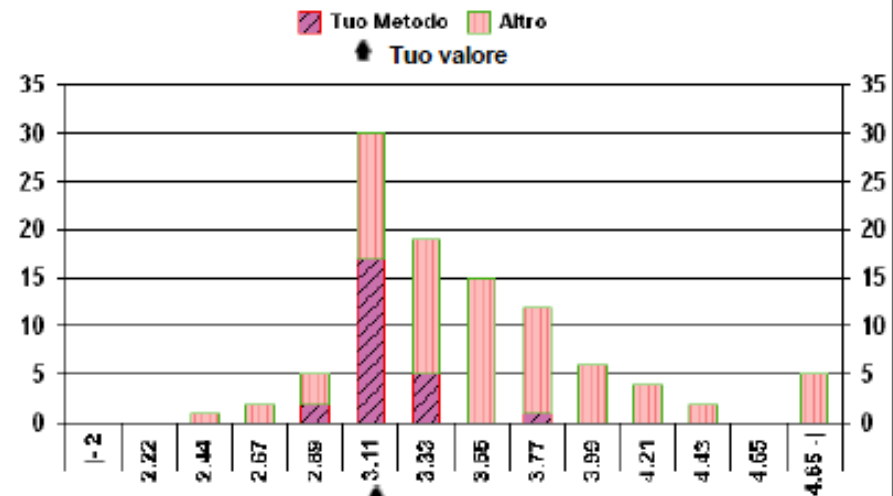
Campioni							
1	2	3	4	5	6	7	8
○	○	○	○	○			

○ = Interno X = Esterno rispetto ai limiti dichiarati

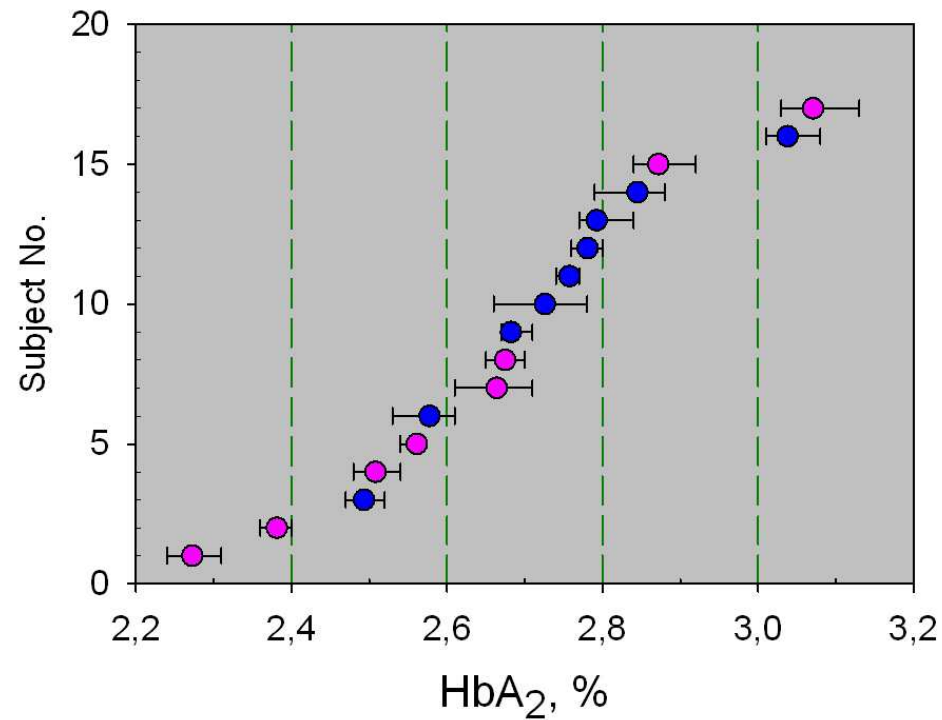
N. risultati numerici	101
N. risultati semiquantitativi/qualitativi	

Riepilogo x Metodo risultati numerici (> 7 Centri)				
Metodo	N.	Out	M.	C.V.
HPLC TOSOH G8	34	0	3.47	6.40
BIORAD VARIANT II DUAL KIT	25	1	3.03	4.41
SEBIA CAPILLARYS HEMOGLOBIN	13	0	2.88	6.68
HPLC	12	0	4.23	16.46

Tuo Metodo
BIORAD VARIANT II DUAL KIT



Analytical goals for the determination of HbA₂



Quality level	Imprecision, %	Bias, %	Total error, %
Optimal	0.2	1.0	1.5
Desirable	0.3	1.9	3.0
Minimal	0.5	2.9	4.5

Table 1 Analytical goals for HbA₂ measurement derived from data on biologic variation.

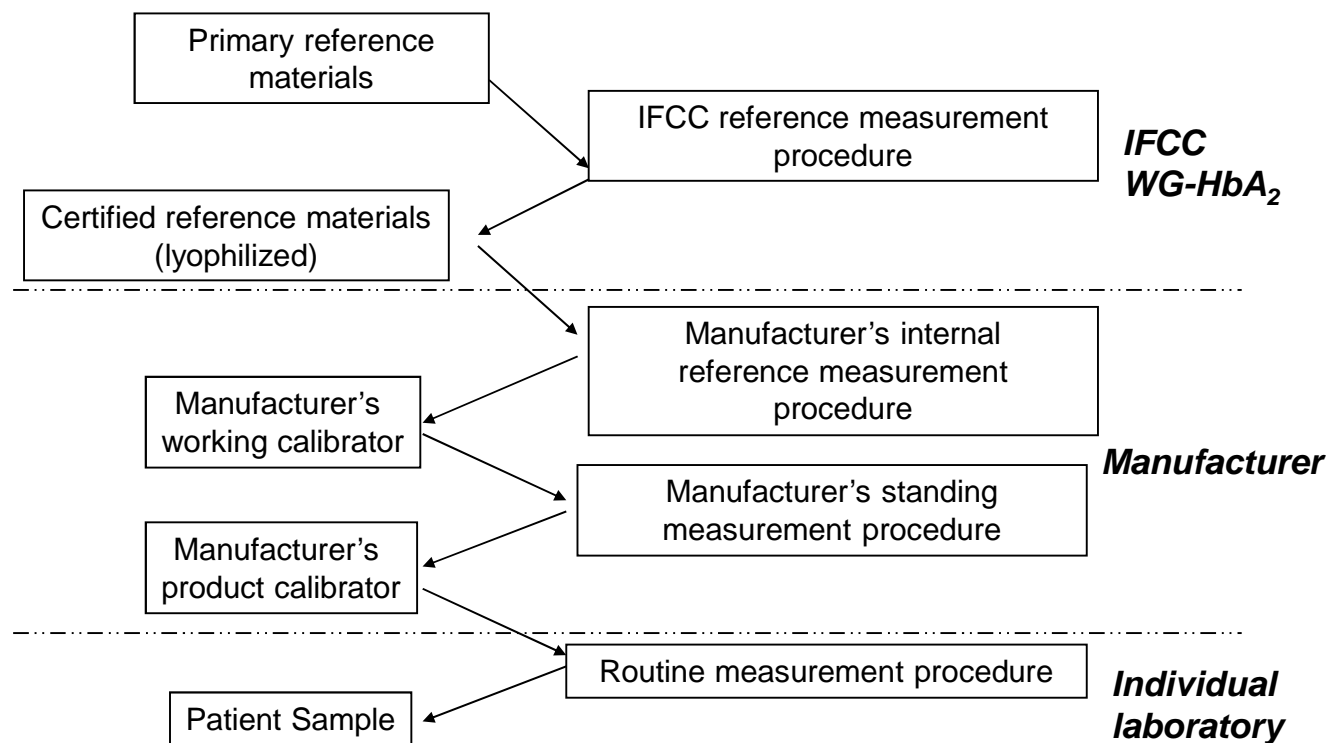
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IFCC Reference System for HbA₂

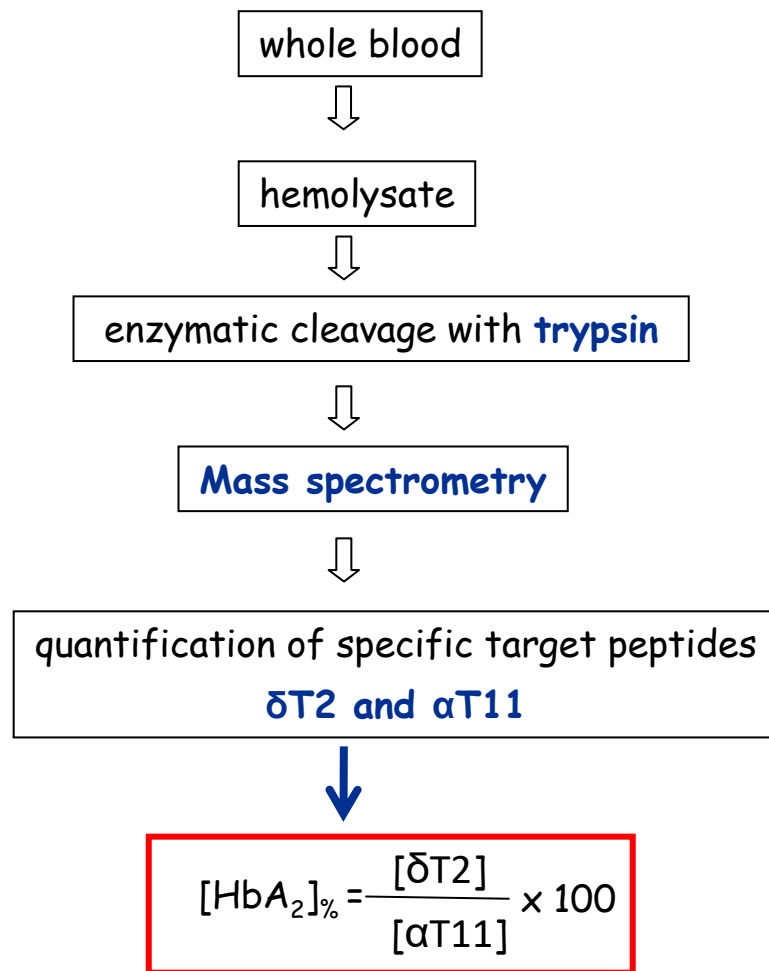
Metrological traceability chain





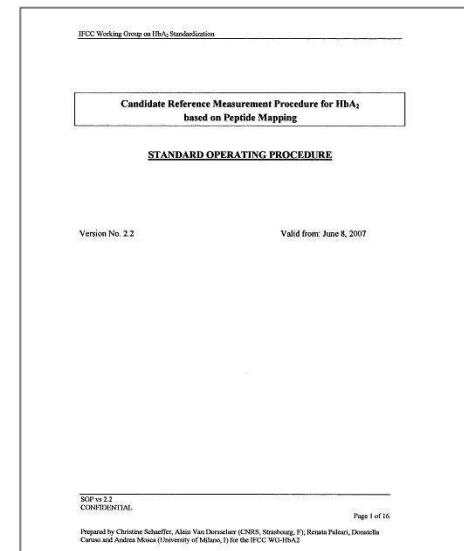
1. Definition of a reference measurement procedure using mass spectrometry associated with proteolytic degradation

➤ Based on the quantification of target peptides of δ and α chains



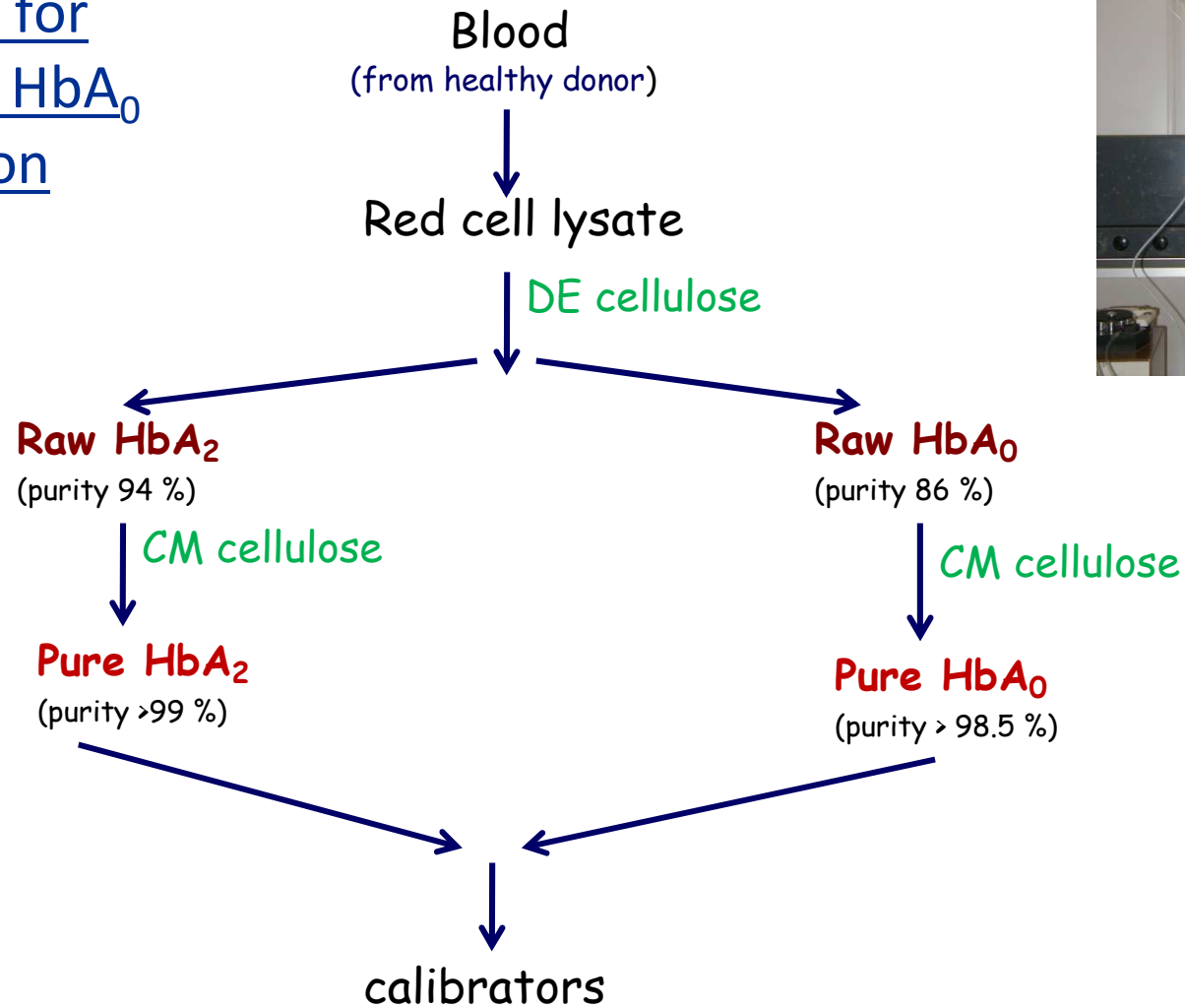
Calibration

- External calibration
- Calibrators consisting of mixtures of highly purified HbA₂ and HbA₀
- Target values assigned volumetrically on the base of their purity



First approach
(2005-2009)

❖ Development of the protocol for HbA₂ and HbA₀ purification



❖ Interlaboratory exercizes

2006: 6 calibrators, 29 samples

2007: 6 calibrators, 20 samples
(2 digestions, 2 replicates/digested)

2008: 4 calibrators, 3 samples
(3 digestions, 3 replicates/digested)

2009: 1 calibrators, 1 samples
(centralized digestion, measurements over 5 days)

➔ **Inter-laboratory variability**

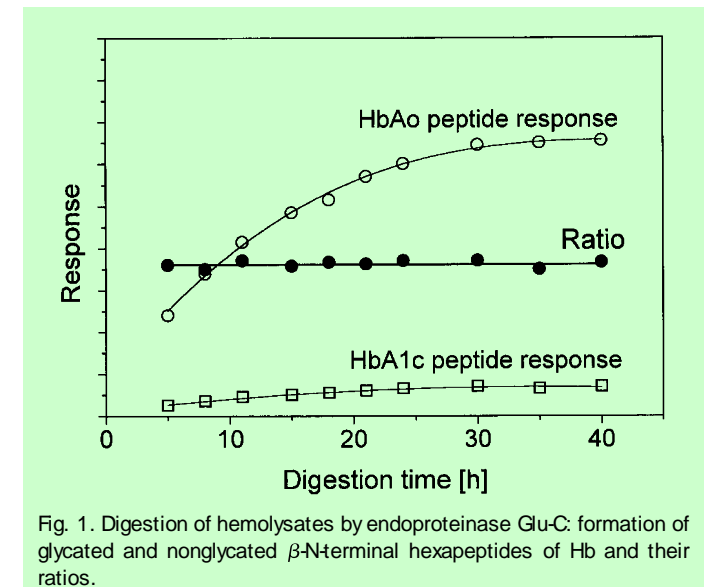
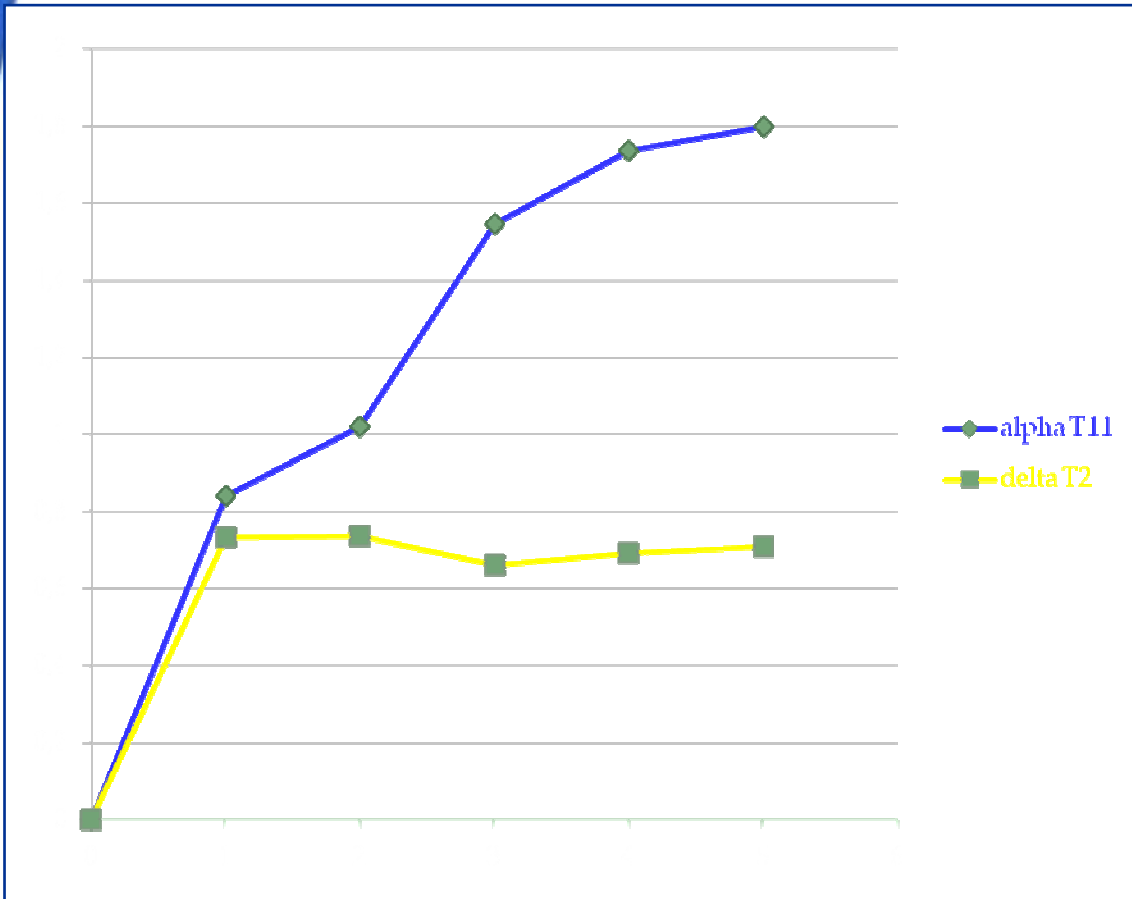


Fig. 1. Digestion of hemolysates by endoproteinase Glu-C: formation of glycated and nonglycated β -N-terminal hexapeptides of Hb and their ratios.

Problems:

- Digestion not completed
- Not defined and reproducible yield for tryptic digest (different kinetic for α T11 and δ T2 peptides)

Alpha chain

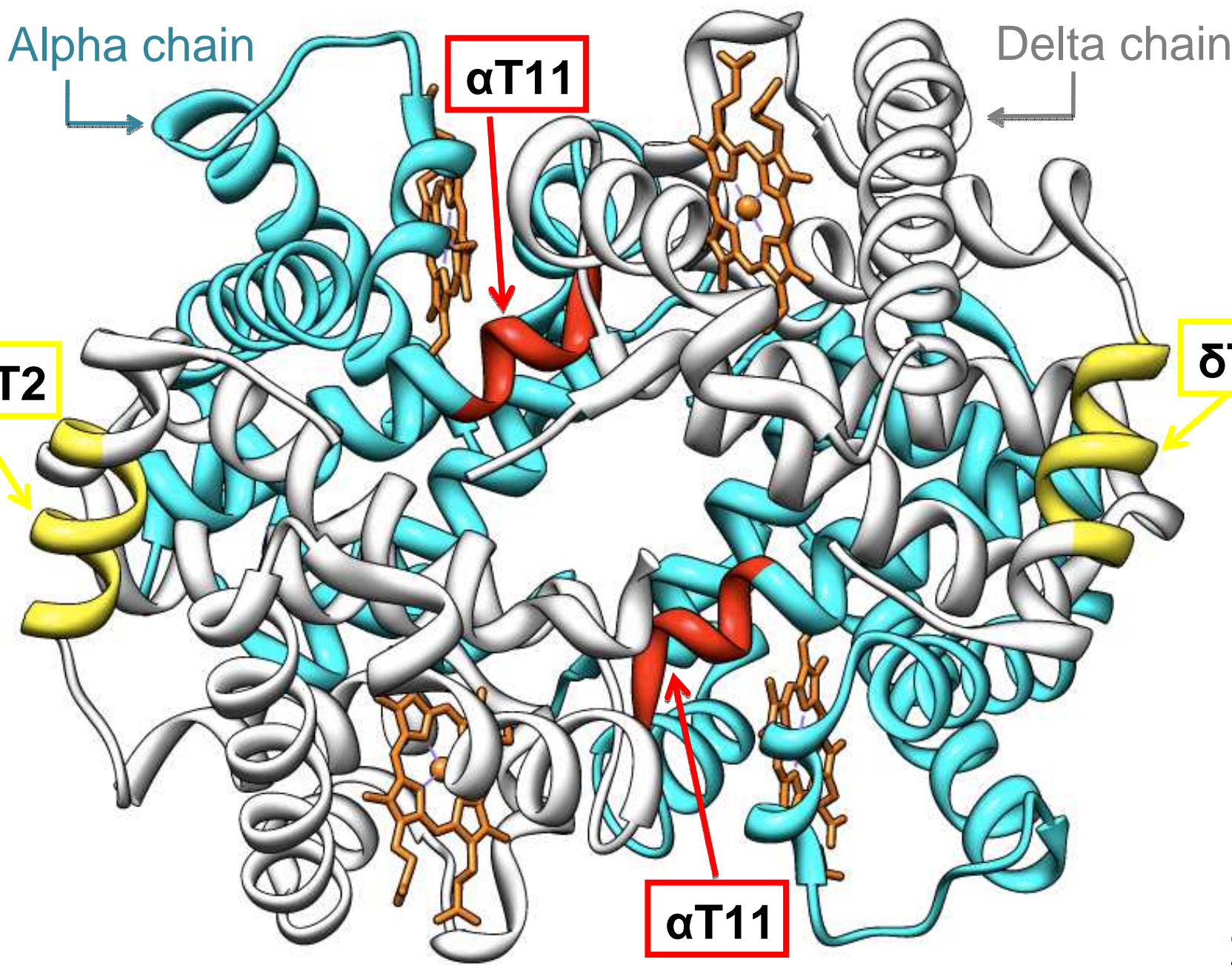
Delta chain

α T11

δ T2

δ T2

α T11




❖ The new approach is based on the use of

- Isotope dilution-mass spectrometry
- Recombinantly expressed, intact
HbA₂ and ¹⁵N-labeled HbA₂
HbA₀ and ¹⁵N-labeled HbA₀
- Target peptides specific for δ and α chains:
 δ T2, **α T5**

New approach
P. Kaiser, C. Arsene (Istanbul 2014)

❖ HbA₂ and HbA₀ intact proteins


 **trenzyme**
life science services

Product specification sheet


Protein: His-TEV-HbA₂ (~ 17.6 kDa)
HbA₂ (~ 16.0 kDa)

Date of production: 15. 01. 2014
Project number: 1836
Lot no.: D12
Sequence: see next page
Source: Recombinantly expressed in *E.coli*
Purification: Affinity chromatography and subsequent buffer exchange.
Prepared in: 50 mM Tris pH 7.5, 100 mM NaCl, stored and shipped at 4 °C.
Purity: > 95 % (measured by densitometry of Coomassie stained gels)
Concentration: 0.47 mg/ml (total protein, measured by BCA-Assay)
Quantity purified: 6.3 mg (total protein) Quantity shipped: 5.17 mg (total protein)
Long-term storage: no recommendations


FPLC chromatogram




SDS-PAGE/Coll.Coomassie:



Histogram (if marked line in gel picture):



 **trenzyme**
life science services


Product specification sheet

Protein: His-TEV-HbA₀ (~ 17.6 kDa)
HbA₀ (~ 16.0 kDa)

Specials: ¹⁵N-labeled

Date of production: 15. 01. 2014
Project number: 1837
Lot no.: D13
Sequence: see next page
Source: Recombinantly expressed in *E.coli*
Purification: Affinity chromatography and subsequent buffer exchange.
Prepared in: 50 mM Tris pH 7.5, 100 mM NaCl, stored and shipped at 4 °C.
Purity: > 97 % (measured by densitometry of Coomassie stained gels)
Concentration: 0.36 mg/ml (total protein, measured by BCA-Assay)
Quantity purified: 4.9 mg (total protein) Quantity shipped: 3.06 mg (total protein)
Long-term storage: no recommendations

FPLC chromatogram



SDS-PAGE/Coll.Coomassie:



Histogram (if marked line in gel picture):

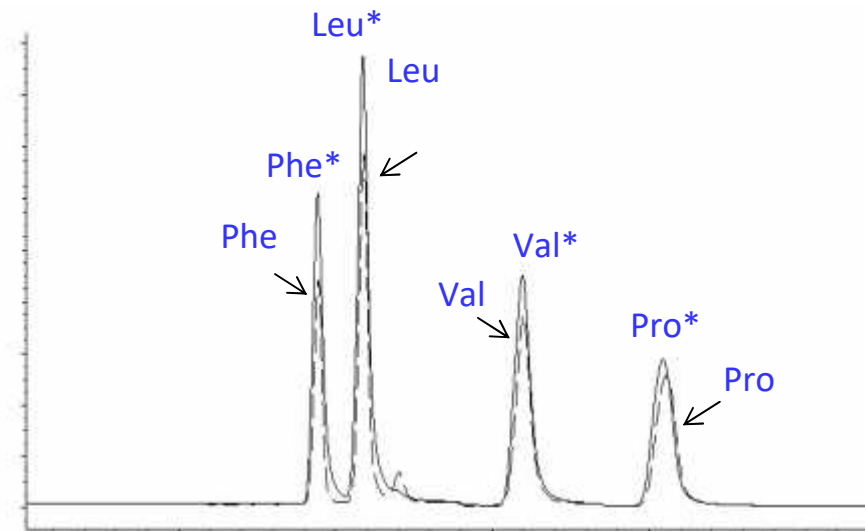
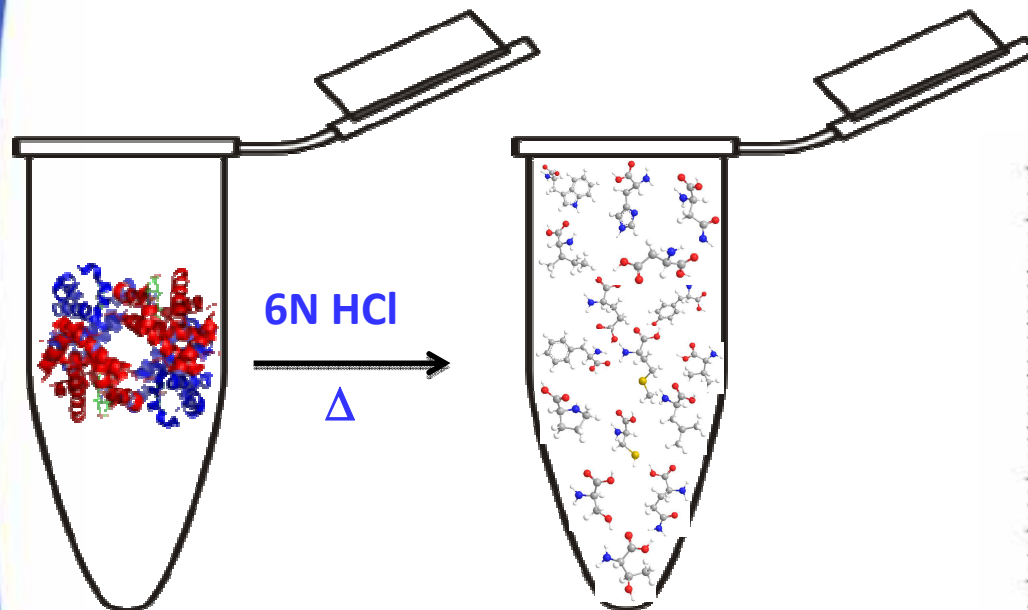


❖ Metrological traceability

The metrological traceability of measurement using the HbA₂ and HbA₀ protein standards is ensured by:

1. determination of content of peptide by LC-ID-MS (amino acid analysis)
2. determination of purity by LC-TOF -MS

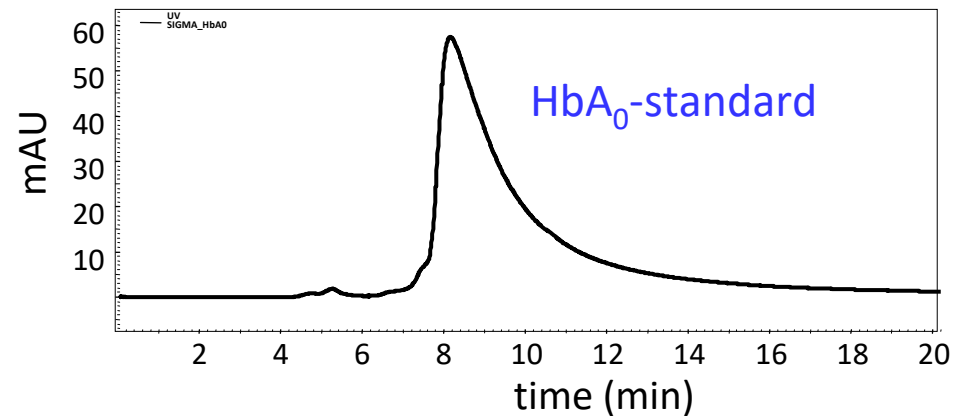
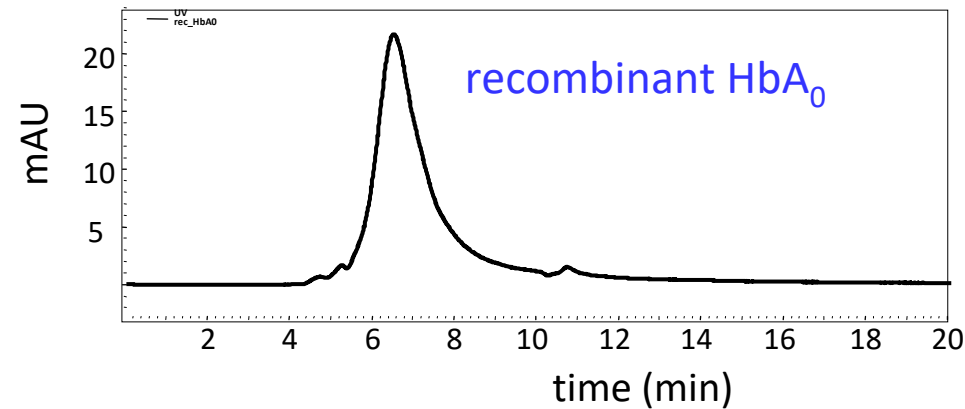
❖ ID-MS-based amino acid analysis of Hb-calibrator material



amino acid	Leu	Phe	Val
concentration of Hb-reference solution [nmol/g]	14.96	15.23	15.19
mean: 15.13 nmol/g			
U= 2.6%			

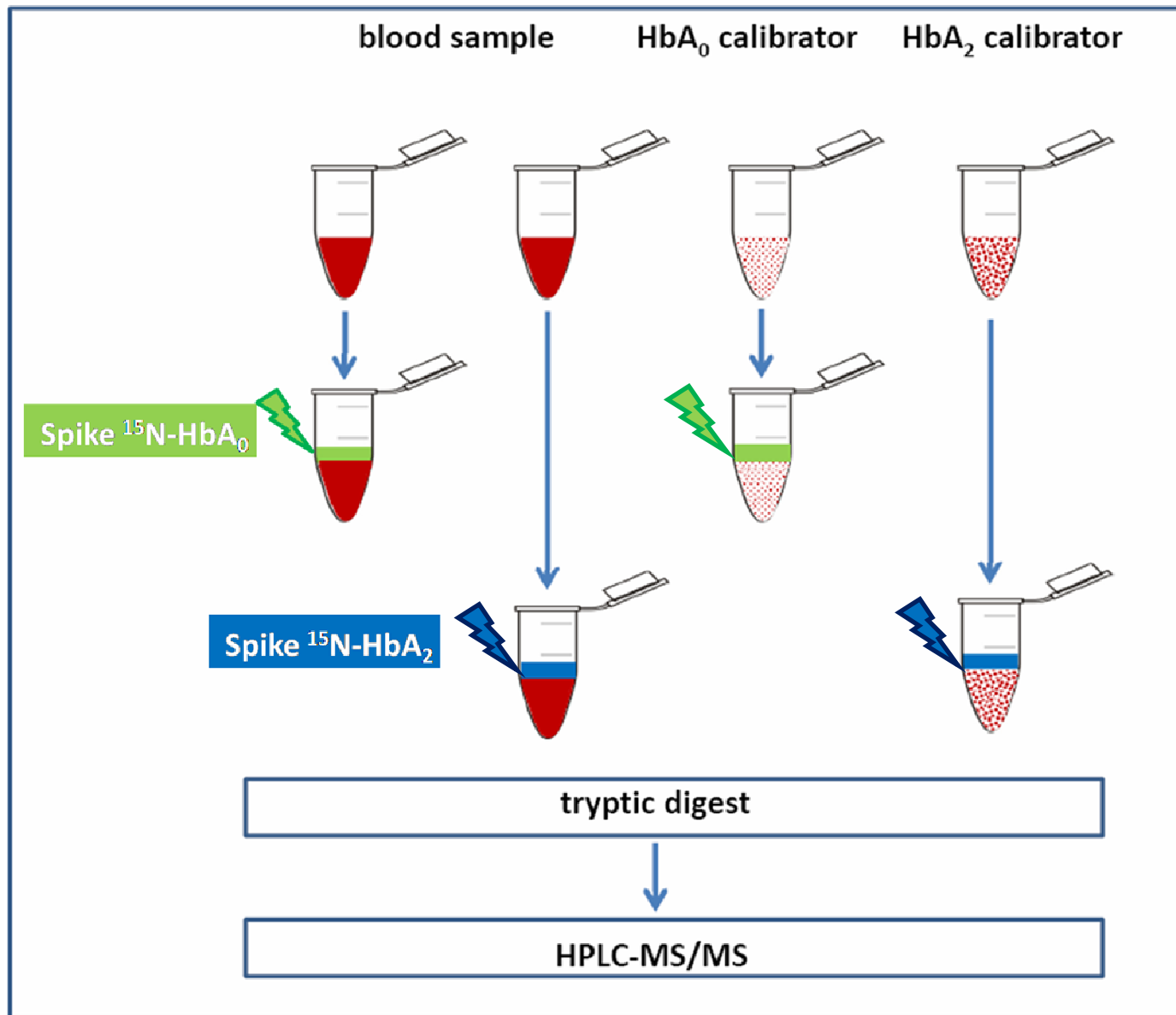
❖ Purification of HbA₀ reference material

purification of
Hb A
by centrifugal
filtration
using filter
units with
molecular
weight cut-off
(MWCO): 50
kDa



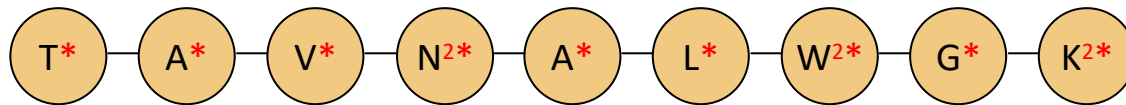
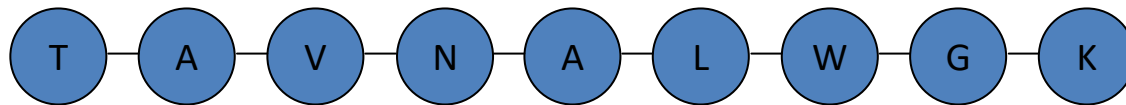
size-exclusion-chromatography of Hb A₀

❖ Workflow



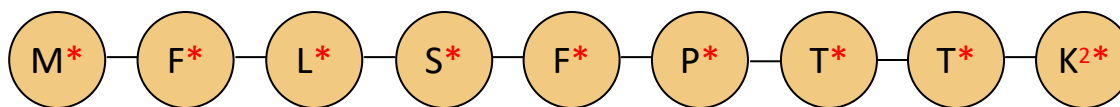
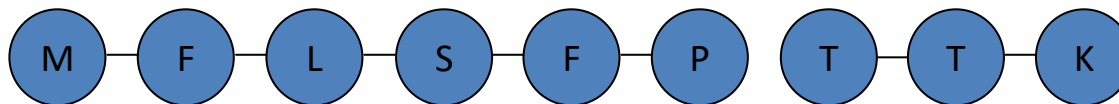
❖ Peptides selected for LC-MSMS measurements

deltaT2



¹⁵N Internal standard

alphaT5



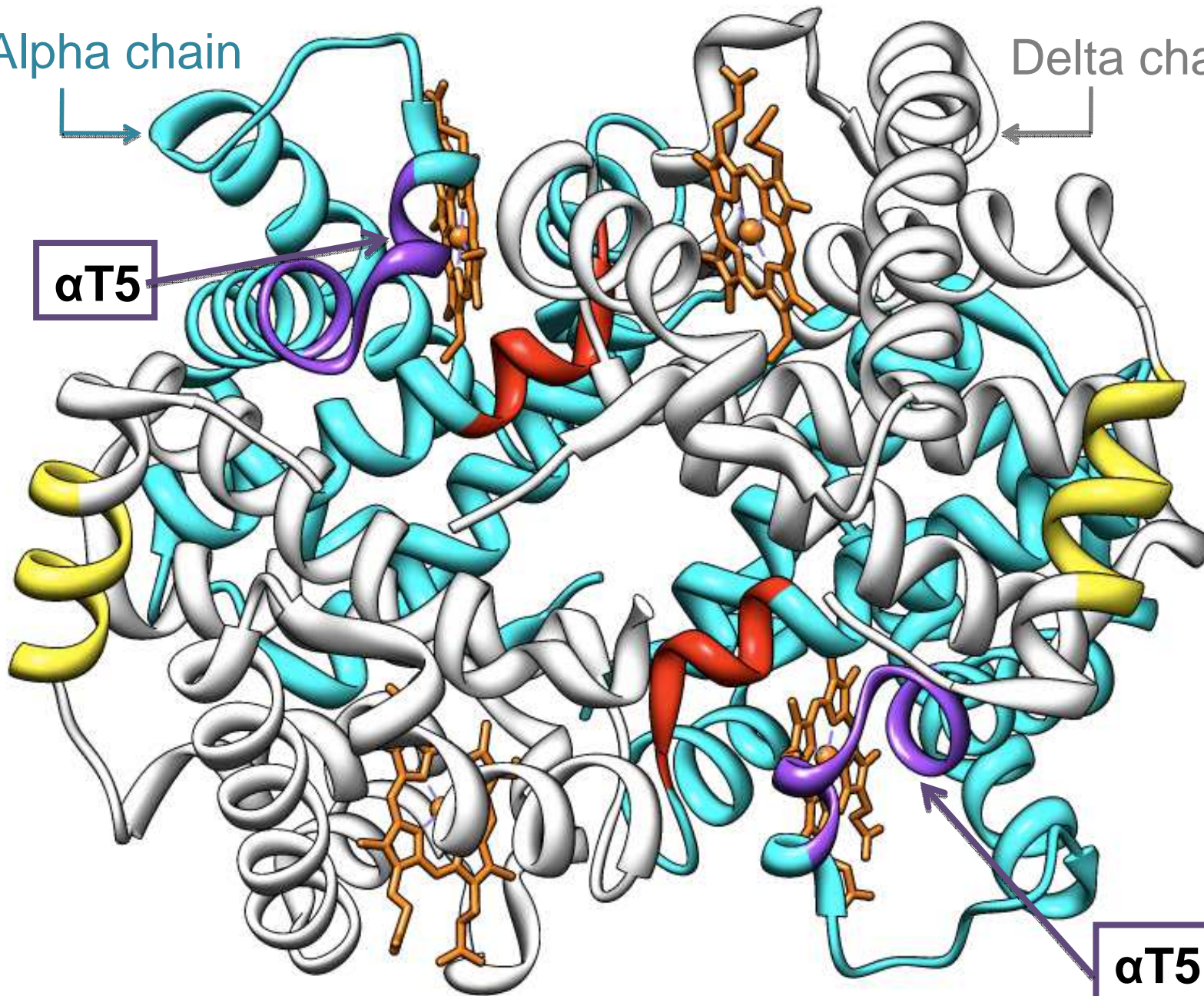
¹⁵N Internal standard

Alpha chain

Delta chain

α T5

α T5



❖ HbA₂ measurement results on blood samples



2. Preparation of a certified reference material for hemoglobin A₂ (in cooperation of IRMM)

❖ Development of a candidate certified reference material (CRM)

- Lyophilized material

First pilot batch (April 2008)

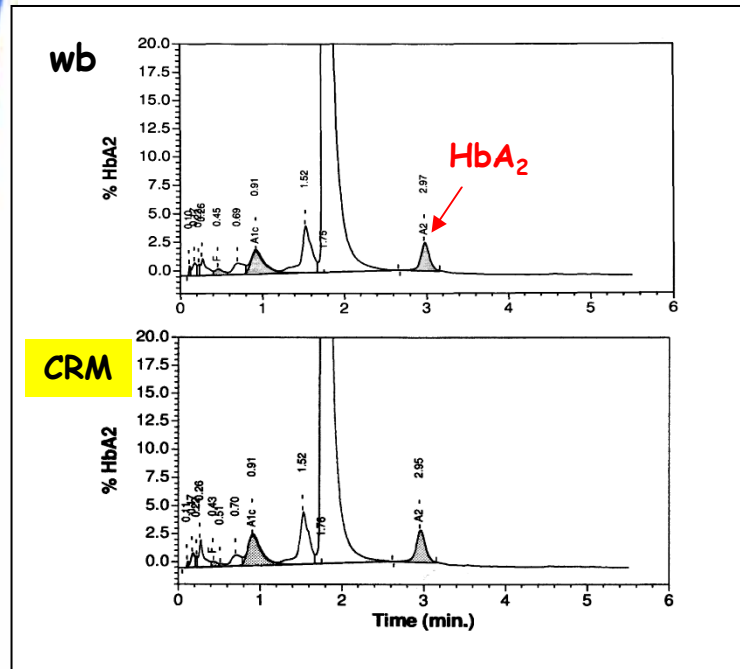
- homogeneity
- total Hb content
- MetHb
- stability at +4°/-20 °C
- Commutability



Second batch (November 2010)

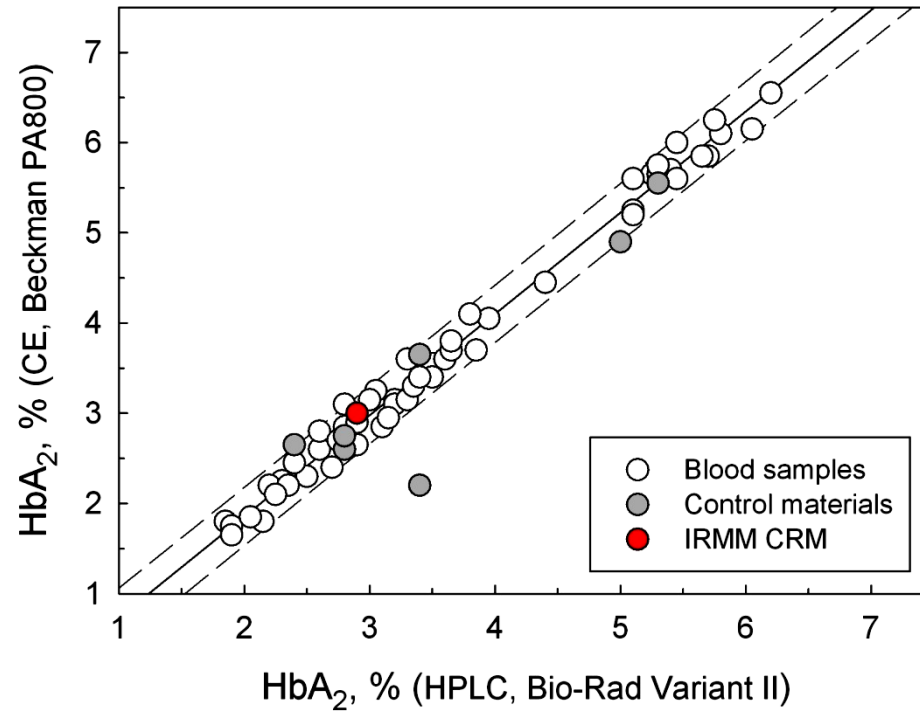
- Storage without O₂ to limit oxydation
- accelerated degradation experiments
- Long term stability



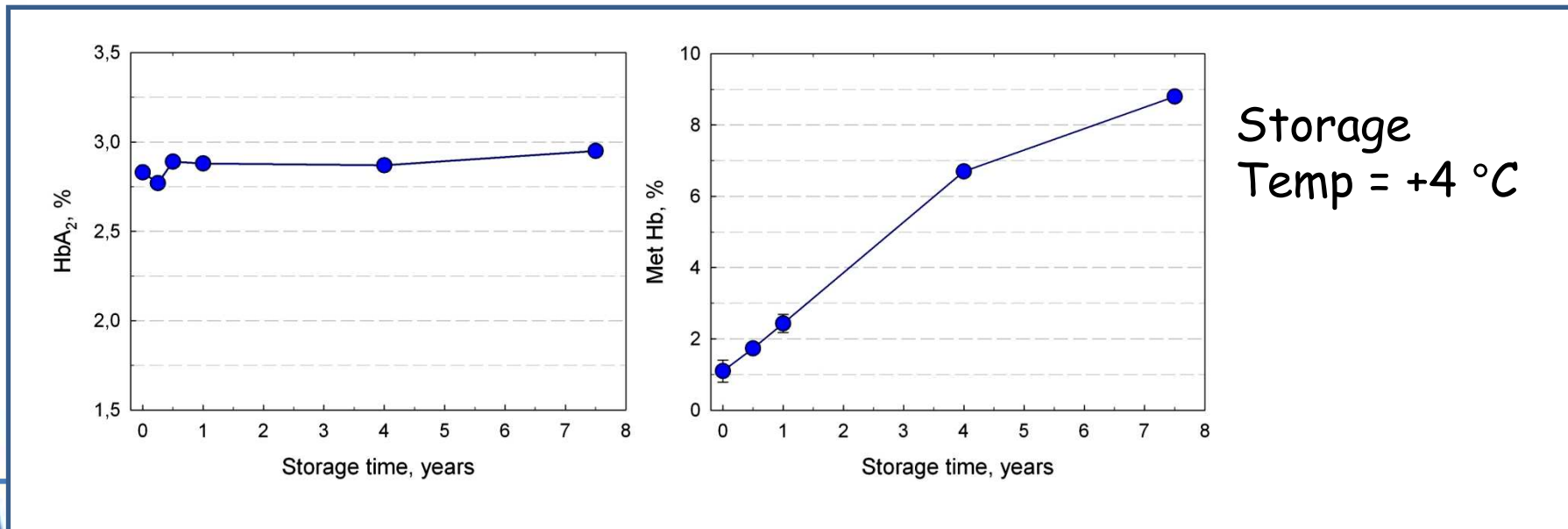
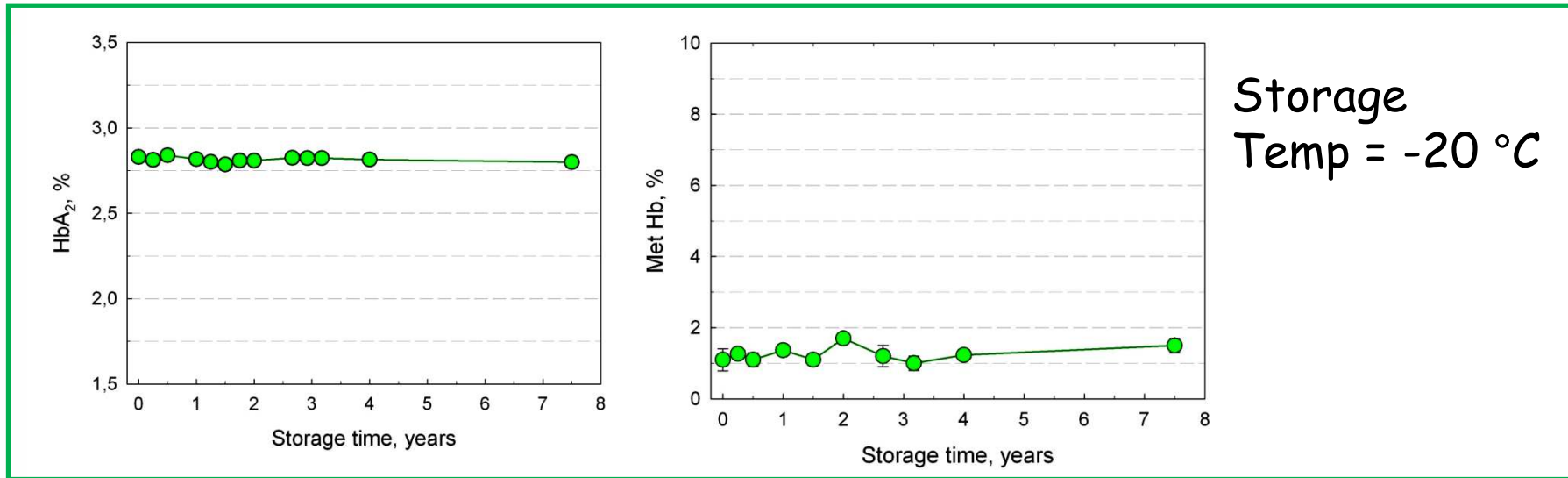


➔ No unexpected peaks due to preparation/lyophilizing process

Good commutability ➔



❖ Stability of the lyophilized material





- **Based on the quantification of intact globin chains by LC-ESI/MS**
(without protein digestion)

Modified from:

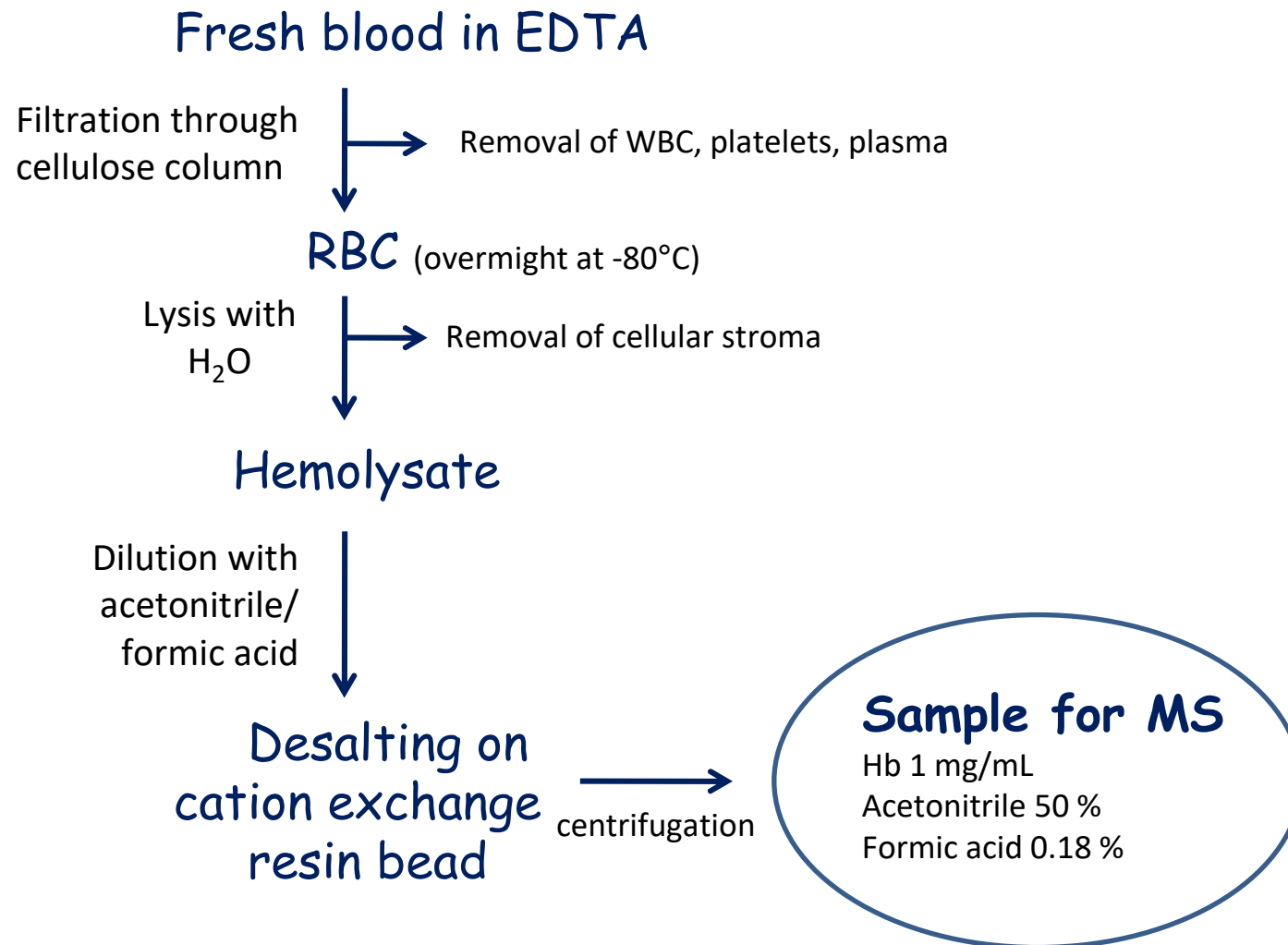
Fetal hemoglobin: assessment of glycation and acetylation status by electrospray ionization mass spectrometry

Andrew S. Davison^{1,*}, Brian N. Green² and Norman B. Roberts¹

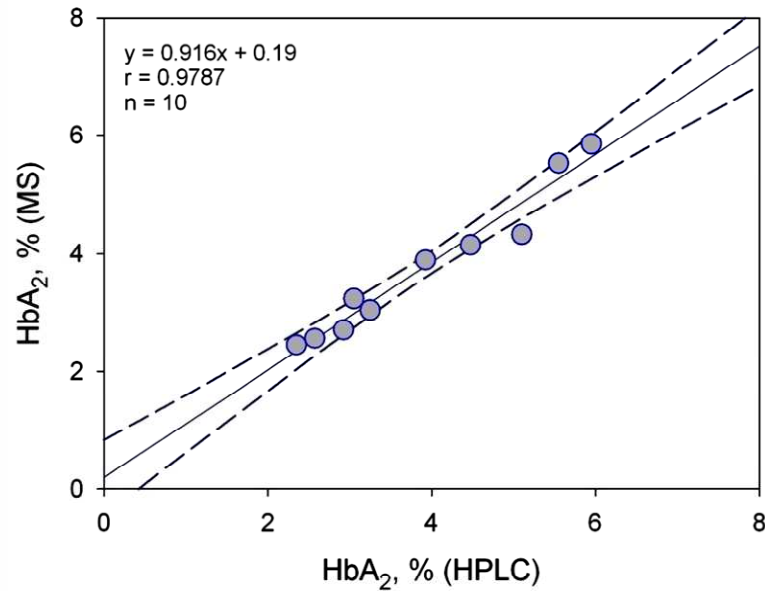
Clin Chem Lab Med 2008;46(9):1230–1238

Alternative approach
(harmonization)

❖ Protocol for sample preparation



❖ Results from experiment June 2014



Sample ID	HbA ₂ , %		CV, % (MS)
	HPLC	MS	
P2	3.05	3.23	2.7
P3	2.58	2.56	1.4
P7	3.25	3.04	1.5
P10	5.95	5.86	1.9
P12	2.35	2.45	2.5
P15	5.10	4.33	2.6
P16	5.55	5.54	2.2
P20	3.93	3.89	2.0
P21	4.48	4.15	1.1
P23	2.93	2.71	0.5

Close correlation with HPLC (except for 1 sample)
Improvement in the reproducibility

Harmonization, a possible model

Harmonization of Measurement Results of the Alcohol Biomarker Carbohydrate-Deficient Transferrin by Use of the Toolbox of Technical Procedures of the International Consortium for Harmonization of Clinical Laboratory Results

Cas Weykamp,^{1*} Jos Wielders,² Anders Helander,³ Raymond F. Anton,⁴ Vincenza Bianchi,⁵ Jan-Olof Jøppsson,⁶ Carla Siebelder,¹ John B. Whitfield,⁷ and François Schellenberg⁸ on behalf of the IFCC Working Group on Standardization of Carbohydrate-Deficient Transferrin

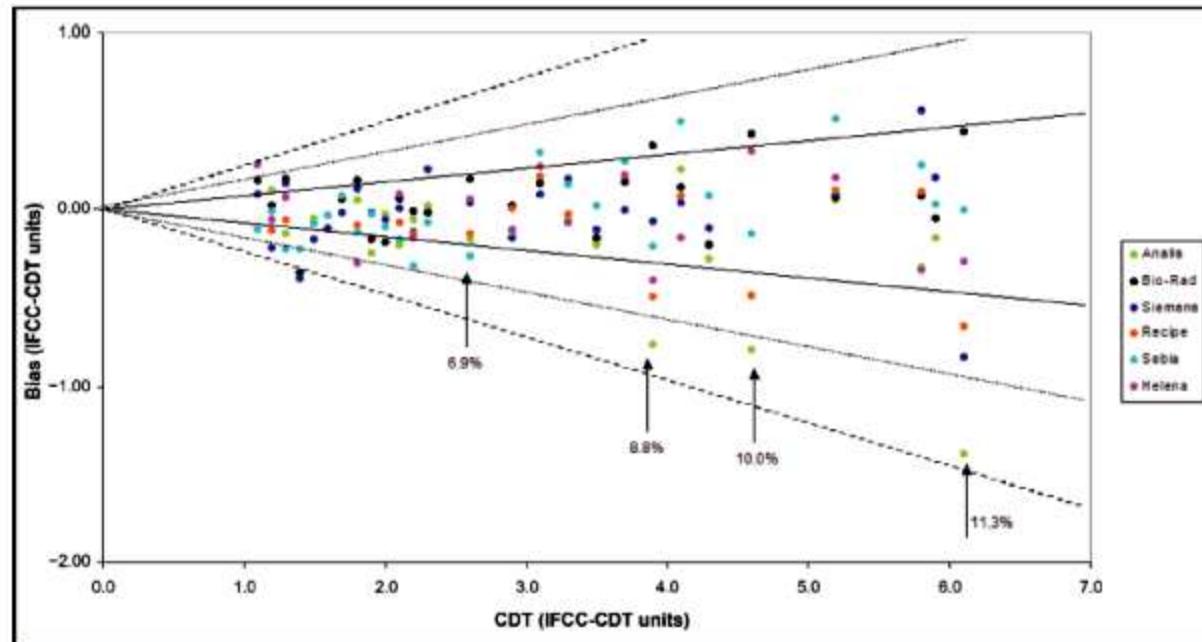


Fig. 2. Success of harmonization: bias for routine MPs in individual patient samples after calibration.

The bias of routine MPs of individual patients after calibration with the frozen cRMs in IFCC CDT units is on the y axis. The CDT concentration in IFCC CDT units is on the x axis. Samples with an increased trisialotransferrin concentration are indicated with an arrow and the percentage of trisialotransferrin. Solid, dotted, and broken lines are the limits for optimum, desirable, and minimum TEa, respectively.

Conclusions

- **Reference measurement procedure: under way to be finalized and validated**
- **Alternative reference method: to be validated**
- **Certified reference material**
 - Defined the optimal condition for sample preparation and lyophilization
 - Composition in Hb similar to that of blood (Hbtot, MetHb)
 - Good commutability (for the methods tested)
 - HbA₂ stable at least for 4 years at +4°C or -20°C (lyophilized form)

Next steps

- **Reference measurement procedure: to be approved by IFCC (ballot)**
- **Certified reference material**
 - to be prepared in at least one large batch
 - to be distributed and used (manufacturers)
- **State-of-the-art: to be monitored on a regular base by adequate EQAS studies and/or surveys**

Acknowledgments

IFCC WG on Standardization of HbA₂

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Cristian Arsene	DE
Emmanuel Bissè	DE
Barbara Wild	UK
Maria Ospina	US
Victor De Jesus	US

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