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# Standardization of hemoglobin A2: does HbA1c history repeat itself?

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# Outline

- Clinical relevance
- State-of-the-art of $HbA_2$ measurements
- IFCC $HbA_2$ standardization
- Conclusions

# Clinical relevance

1.7 % of the world's population is carrying thalassemic genes

- β-thal
  - Mediterranean regions: up to 8 %
  - Middle East: up to 10 %
  - India: 3 – 15 %
  - Southeast Asia: up to 9 %

## Hb $A_2$ reference intervals (2SD, Tietz)

normals: 1.5 - 3.5 %
β-thal trait: 3.7 - 7.0 %

## Hb $A_2$ reference intervals (Menarini HA8160)

normals: < 3.2 %
borderline: 3.3 - 3.8 %
β-thal trait: >3.8 %

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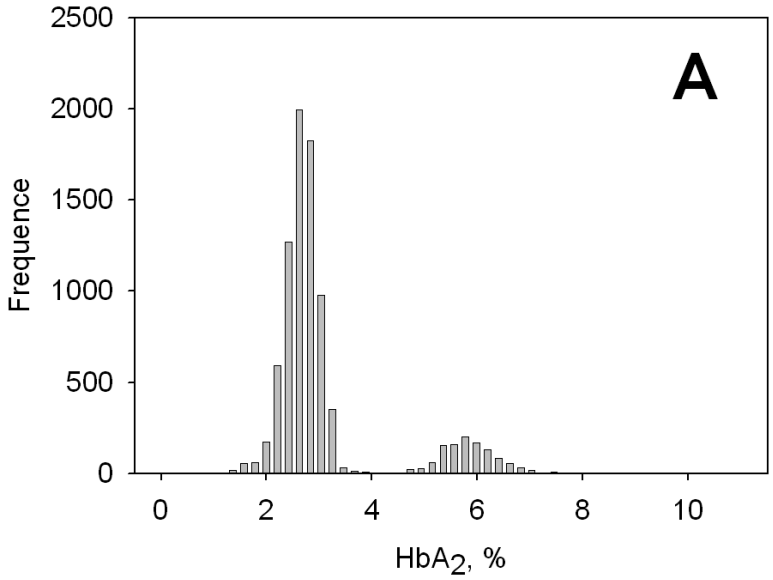


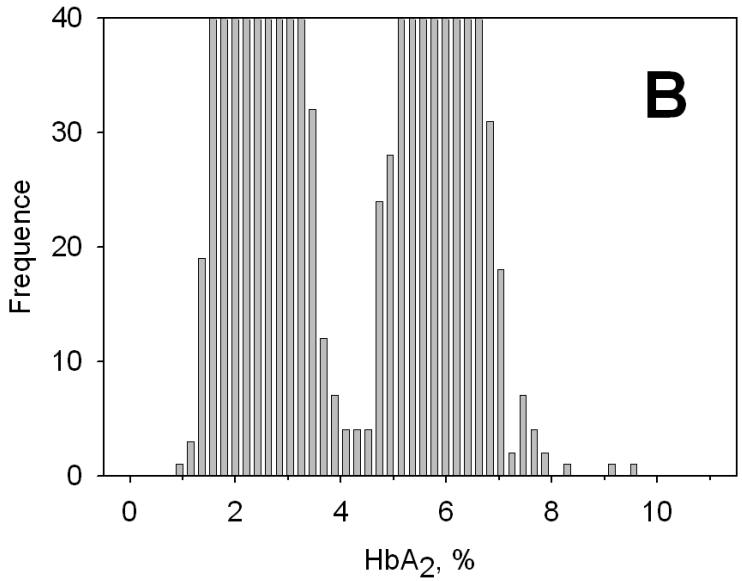


Incidence of $HbA_2$ "borderline" (between 3.3 and 3.7 %)
N = 194 over 8514 (2,3 % )

MCV <80 fL and Hb below the reference interval:
**156 over 8514 (1,8 %)**

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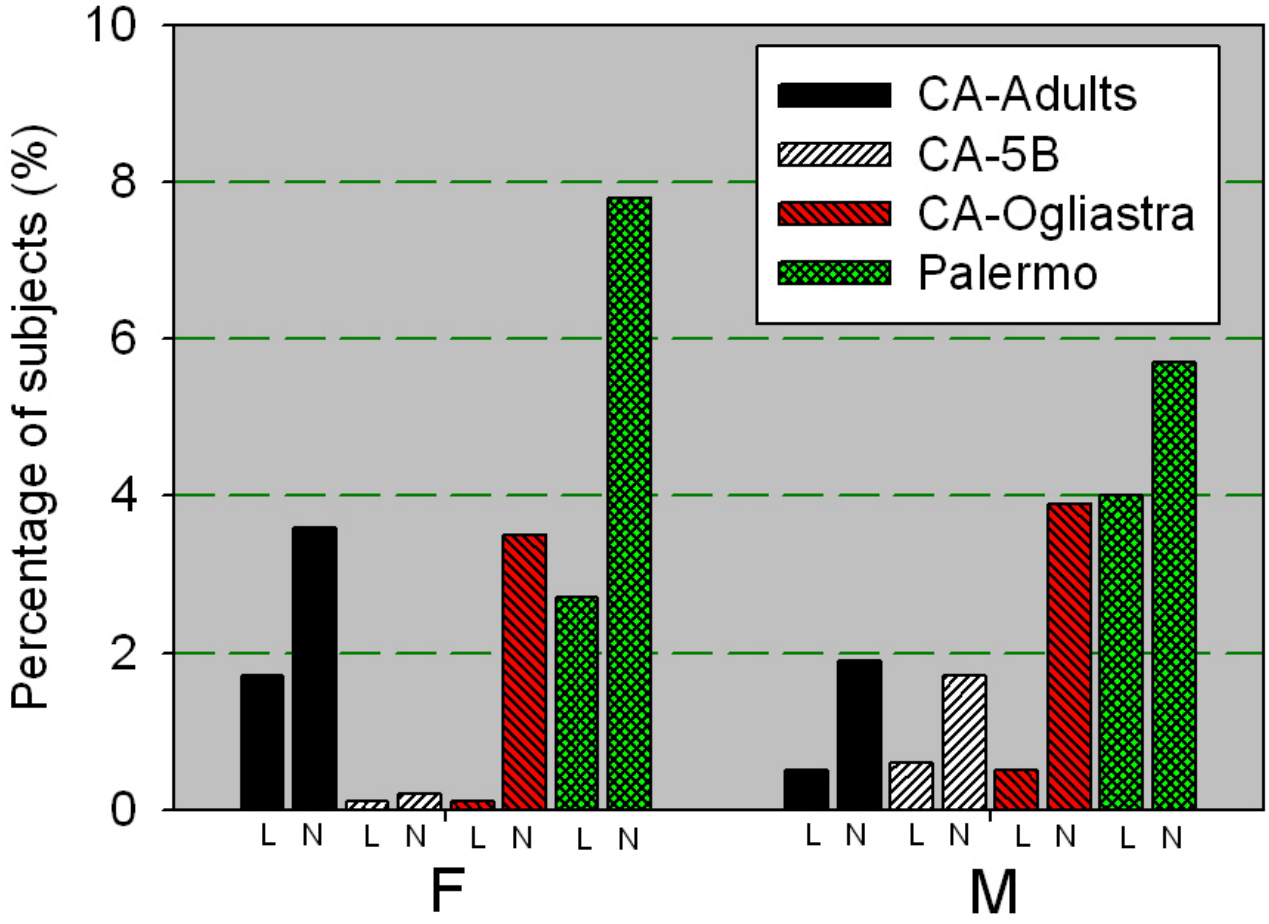


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# Genotype of 234 (over 1743) subjects with $HbA_2$ borderline

| | |
|---|---|
| **mutation defect** | **25.6 %** |
| **no defect** | **74.4 %** |

| | |
|---|---|
| NEG/-α3.7 | 2 |
| NEG/IVS 1 nt 6 | 20 |
| β*+δCd 27 | 7 |
| NEG/αααanti3,7 | 10 |
| Hb Variants** | 3 |
| Cap +1570 | 1 |
| β prom. (-101; -92) | 10 |

* β-thal mutations: β 039, IVS I nt 1, IVS I nt 110

** Hb Variants: Hb Acharnes (cd 53 GCT>ACT); Hb Kokomo (cd 74 GGC>AGC), Hb Ernz (cd 123 ACC>AAC)

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# Genetic counselling

- $HbA_2$ borderline subjects should be always investigated in couples at risk

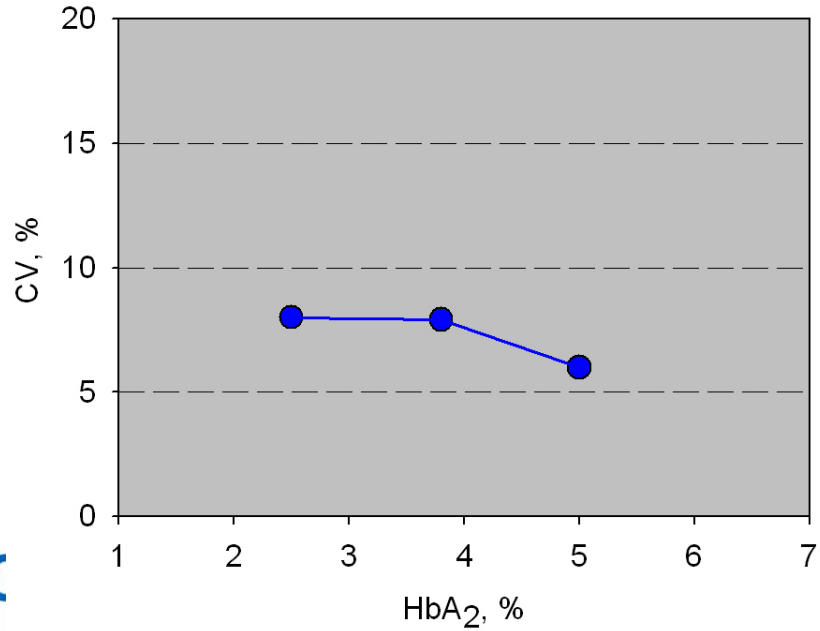
# State-of-the-art of $HbA_2$ measurements

- EQAS data

EQAS – So.S.T.E.

N = 48 HPLC
April-June 2005

Variabilità totale




SOSTE, VEQ $HbA_2$
(distrubuzione delle misure, maggio 2005)

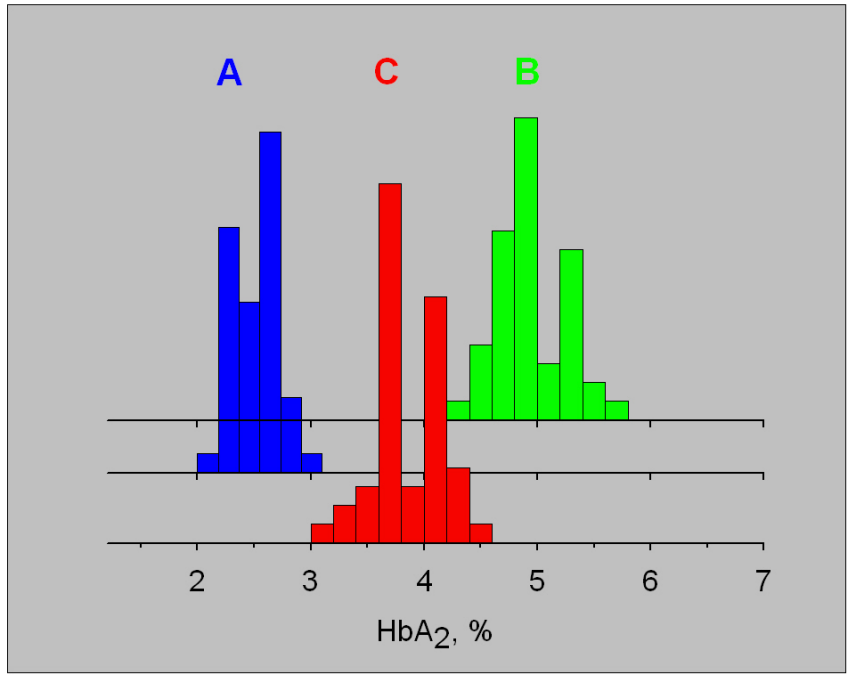


**Table 2** $HbA_2$ results reported in the study and grouped for method.

| HPLC systems | Sample | $HbA_2$, % | | | | | CV | n |
|---|---|---|---|---|---|---|---|---|
| | | *Mean* | *SD* | *Min* | *Max* | *Median* | % | |
| *D10* | A | 2.6 | 0.2 | 2.3 | 2.7 | 2.6 | 7.7 | 4 |
| | B | 5.1 | 0.3 | 4.6 | 5.3 | 5.2 | 5.9 | 4 |
| | C | 3.9 | 0.2 | 3.6 | 4.0 | 3.9 | 5.1 | 4 |
| *Variant* | A | 2.6 | 0.2 | 2.0 | 3.1 | 2.6 | 7.7 | 24 |
| | B | 5.0 | 0.2 | 4.5 | 5.4 | 5.0 | 4.0 | 23 |
| | C | 3.9 | 0.2 | 3.4 | 4.2 | 3.9 | 5.1 | 23 |
| *Variant II beta-thal* | A | 2.6 | 0.2 | 2.2 | 2.8 | 2.6 | 7.7 | 8 |
| | B | 4.9 | 0.4 | 4.2 | 5.3 | 5.0 | 8.2 | 8 |
| | C | 3.8 | 0.3 | 3.2 | 4.2 | 3.9 | 7.9 | 8 |
| *Variant II dual kit* | A | 2.4 | 0.1 | 2.2 | 2.7 | 2.3 | 4.2 | 11 |
| | B | 5.0 | 0.3 | 4.6 | 5.7 | 4.9 | 6.0 | 12 |
| | C | 3.8 | 0.3 | 3.1 | 4.4 | 3.7 | 7.9 | 12 |
| *All methods* | A | 2.5 | 0.2 | 2.0 | 3.1 | 2.6 | 8.0 | 47 |
| | B | 5.0 | 0.3 | 4.2 | 5.7 | 5.0 | 6.0 | 47 |
| | C | 3.8 | 0.3 | 3.1 | 4.4 | 3.8 | 7.9 | 47 |

# $HbA_2$, analytical goals

$$TE = 1.65*CV_a + 1/4(CV_I^2 + CV_G^2)^{1/2}$$

**$CV_I$= 2.8 – 3.4 % → 3.1 %**
**$CV_G$ = 20 %** → **TE = 7.6 %**

**$CV_a$ = 1.4 – 1.7 % → 1.6 % (goal for imprecision)**

$HbA_2$ "true value": 3.0 %
"acceptable" measured value: 2.8 – 3.2 %

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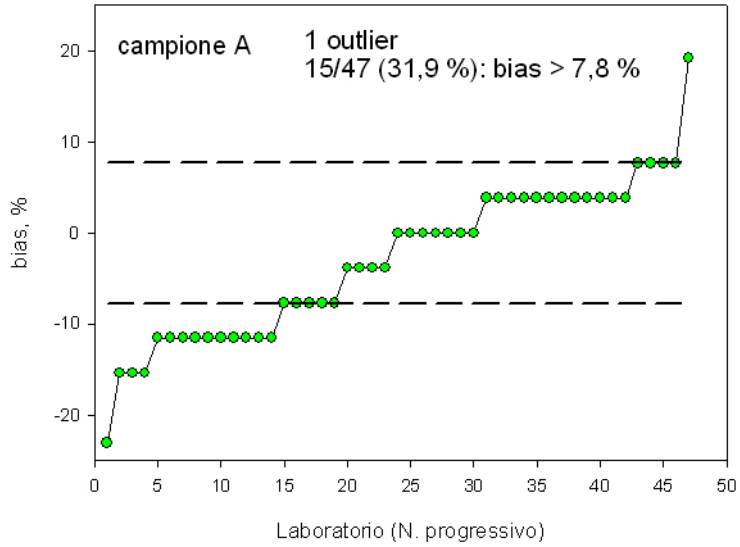


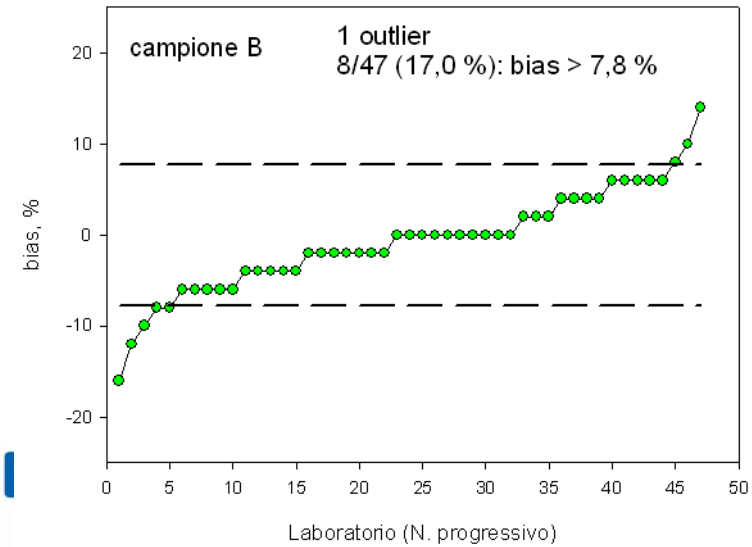


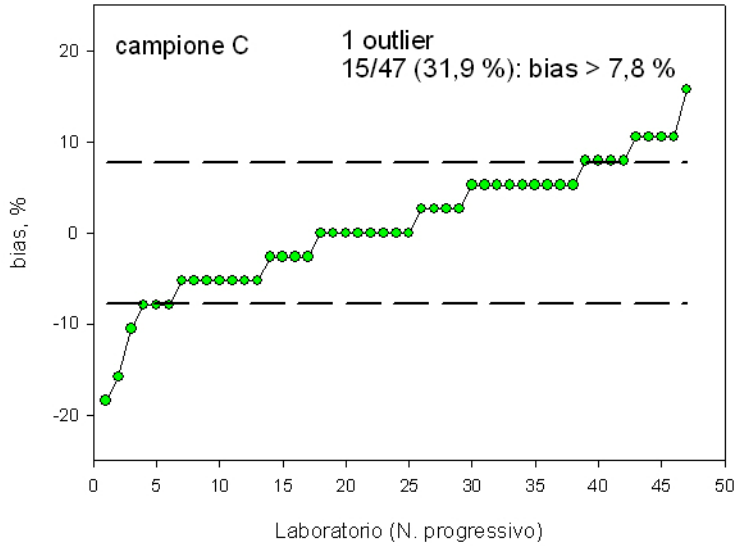


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# IFCC $HbA_2$ standardization

- To prepare a reference material for hemoglobin $A_2$ in conjunction with IRMM.

# IFCC WG-$HbA_2$ Membership

| Name | Position | Country | Term | Time in Office |
|---|---|---|---|---|
| A. Mosca | Chair | IT | 1st | 2004 01 – 2006 12 extended |
| E. Bissé | Member | DE | | |
| D. Caruso | Member | IT | | |
| B. Green | Corp. Rep. | UK | | |
| A. Van Dorsselaer | Member | FR | | |
| B. Wild | Member | UK | | |

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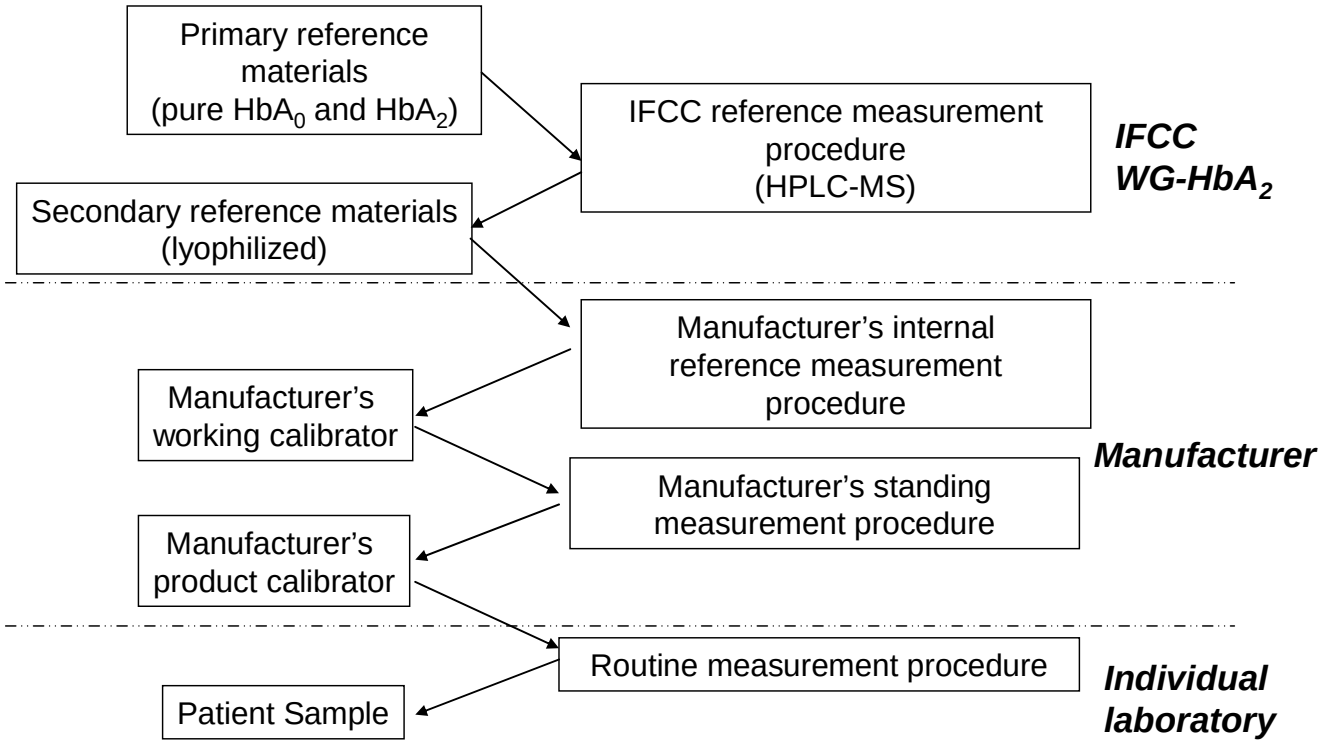
# IFCC Reference System for $HbA_2$

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# Primary Reference Materials

- Pure $HbA_0$ and $HbA_2$ (three batches produced so far)
- Liquid solutions (buffer with sucrose) at -80 °C
- Available c/o Dept. Science and Biomedical Technology, University of Milano
- Small aliquots will be transferred to IRMM

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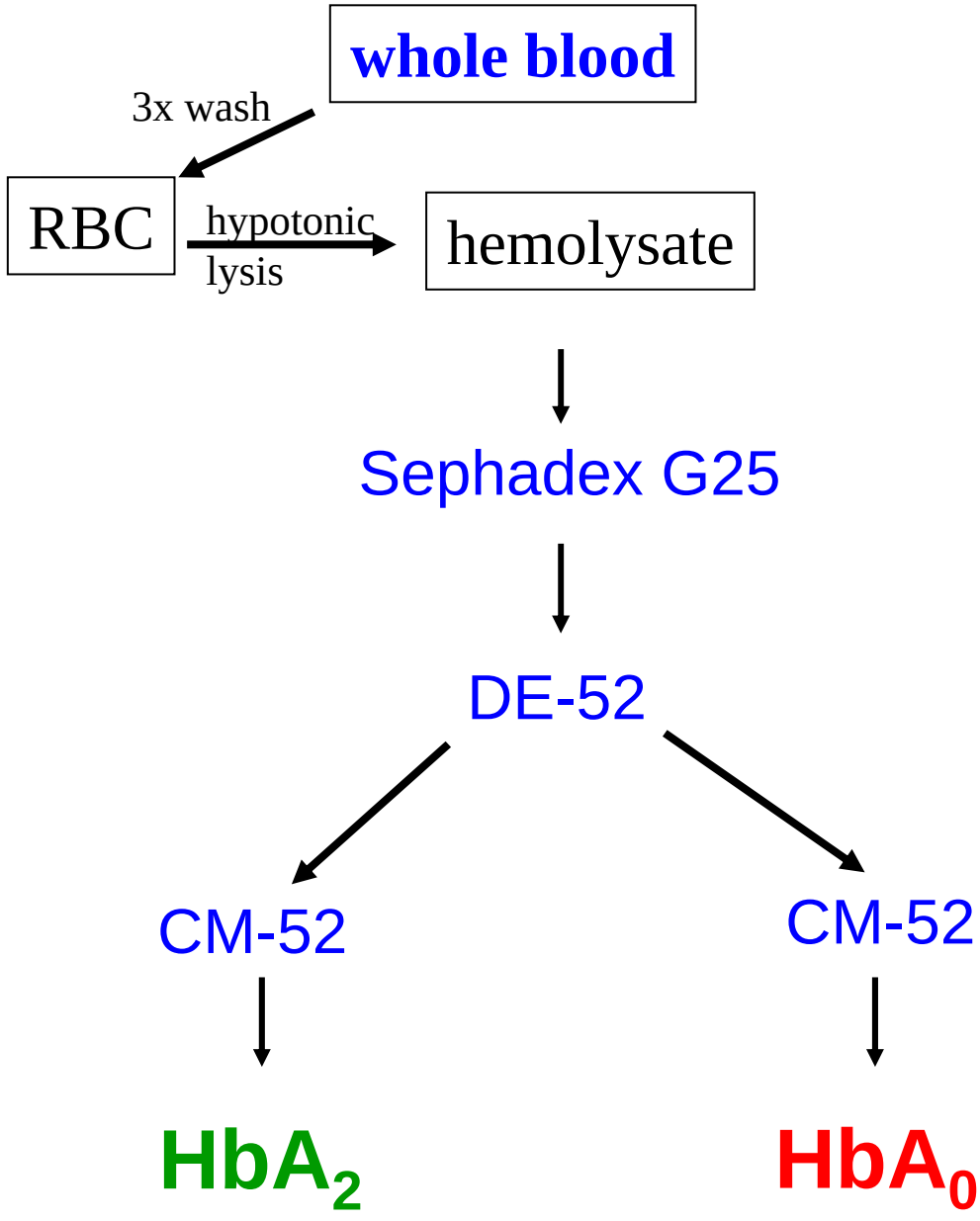
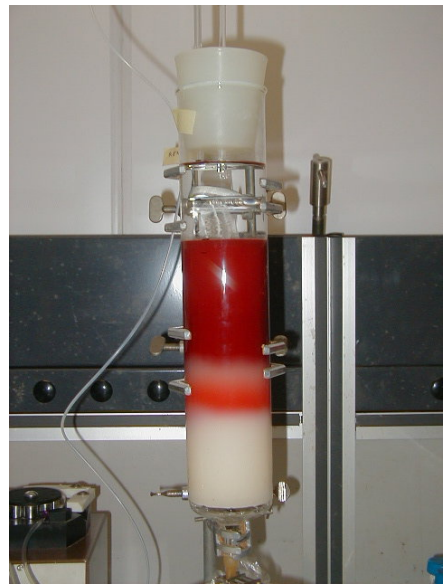
## Preparation of pure $HbA_0$ and $HbA_2$



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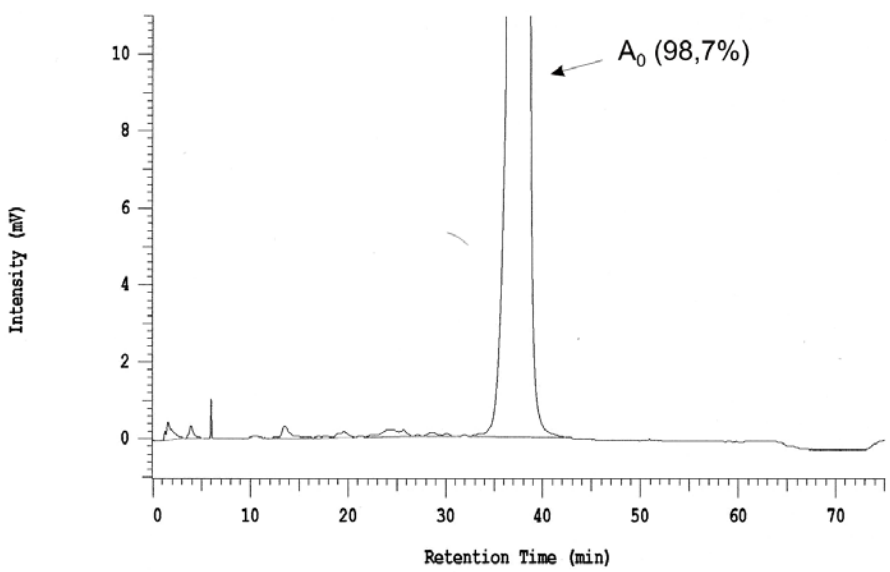
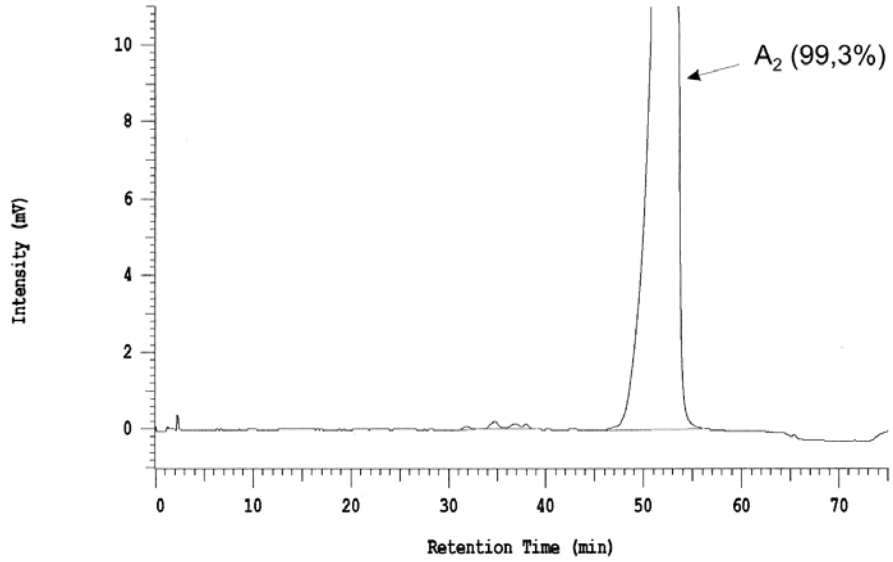
# Tests for assessing purity of the primary calibrators

- HPLC
- IEF
- Capillary EF
- ESI-MS

# PolyCATA HPLC analysis (EB)

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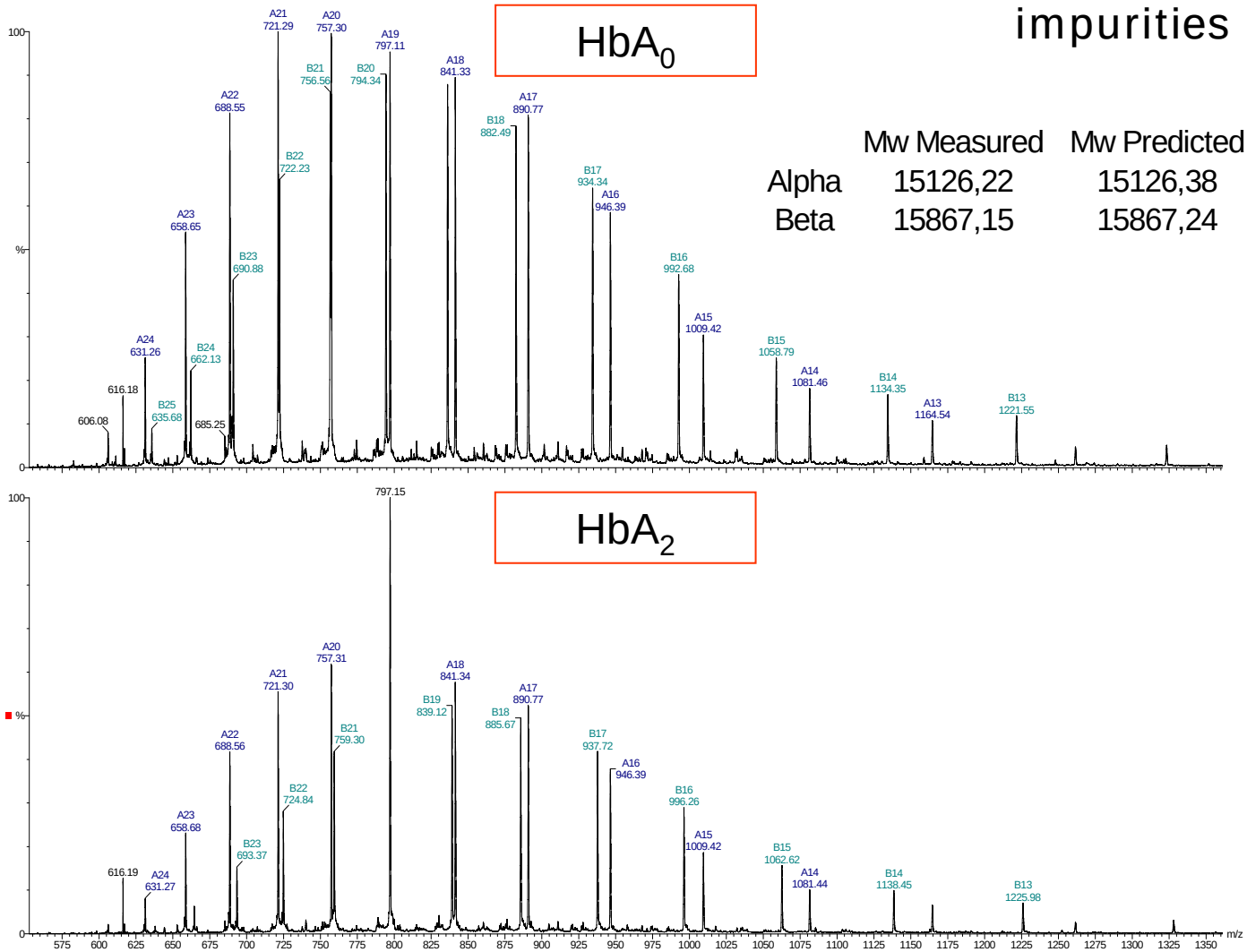
# ESI-MS of purified $HbA_0$ and $HbA_2$



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MS : ESI positive LCT, Micromass

# Candidate Reference Measurement Procedure for $HbA_2$

## Principle

**$HbA_2$ ratio to whole hemoglobin is determined as ratio of a delta chain specific peptide to an alpha chain specific peptide.**

**Peptides are obtained by treating total red blood cell lysate with trypsin.**

**Peptide mixture is analyzed by HPLC-ESI / MS.**

**Calibration is performed against primary calibrators made of mixtures of $HbA_2$ and $HbA_0$ primary reference materials.**

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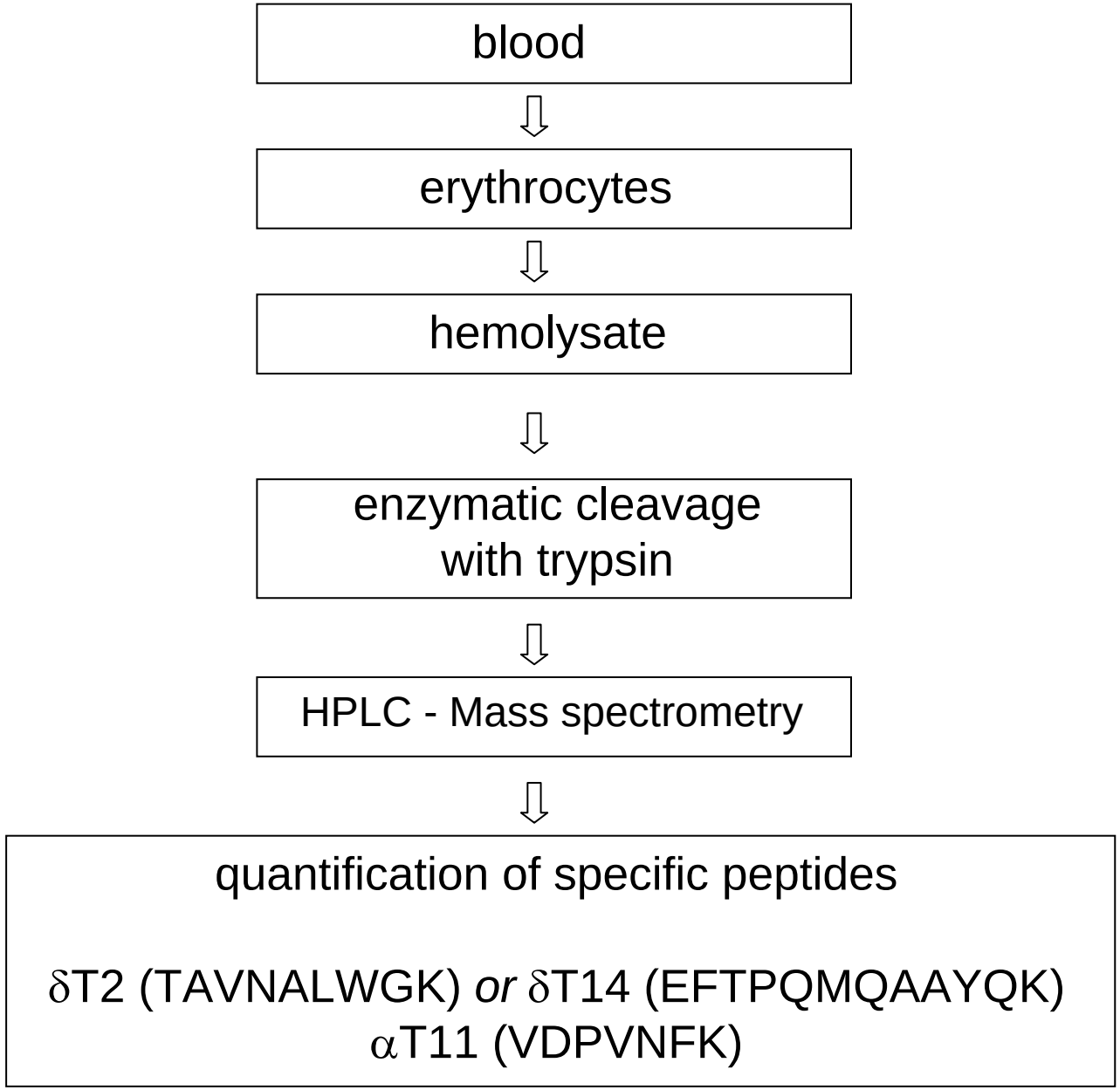


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# Candidate Reference Measurement Procedure for $HbA_2$

## Optimization

**1) The method has been optimized with regard to several steps (digestion conditions, separation of tryptic peptides by RP-HPLC, determination of LOD and QOD, MS tuning, etc.)**

**2) A comparison between the results obtained in 2 MS labs has been performed in two steps during 2006**

**3) The SOP has been defined**

**4) Another comparison is planned in 2007**

**5) Further development to be discussed**

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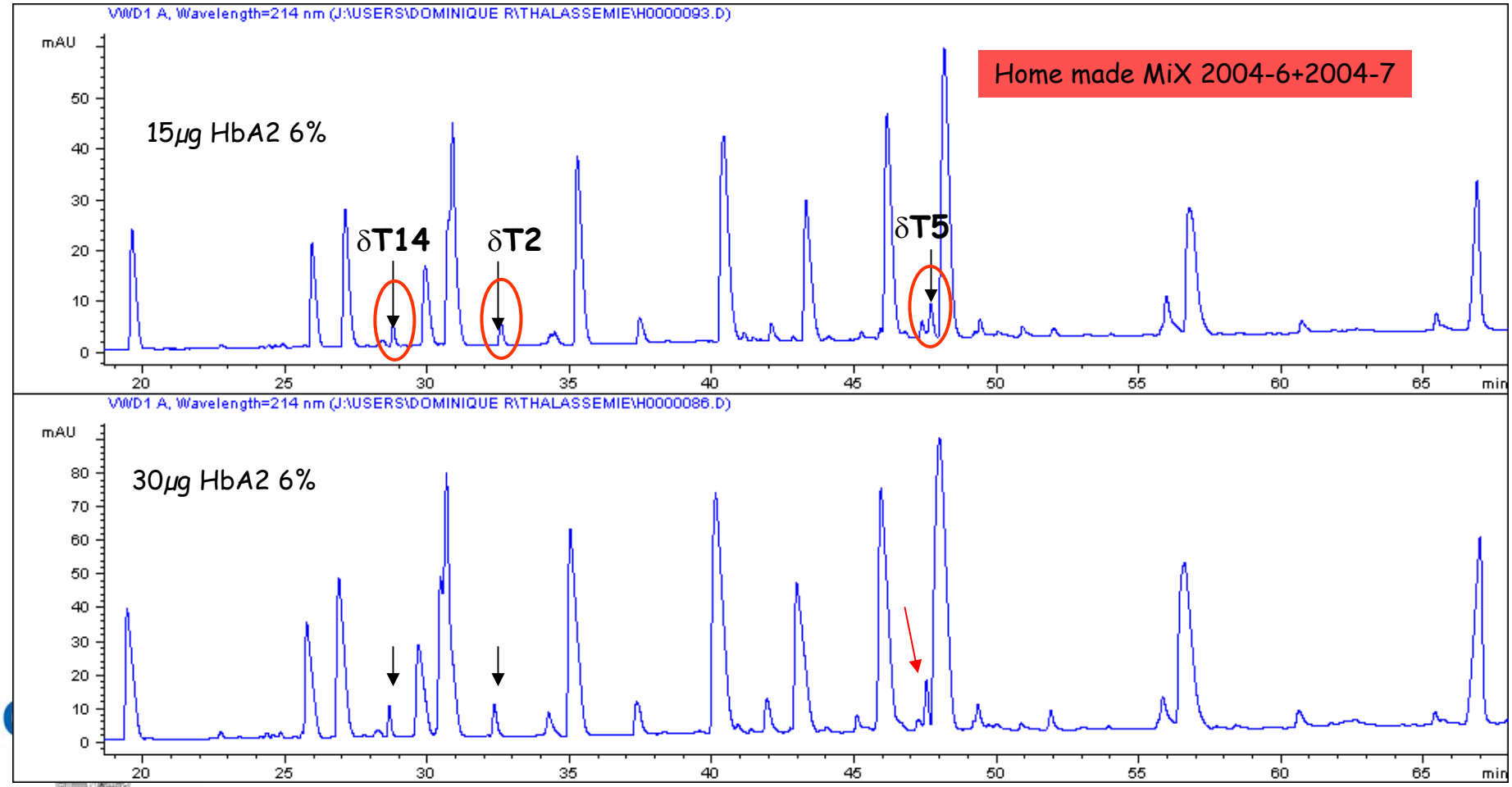
# HPLC conditions optimised to separate specific peptides for α and δ chains:

HPLC instrument: Agilent 1100 Chemstation, UV detector (MicroCell : 1µl)
**Column : Tosoh TSKgel super ODS 2µm (2mmx10cm)**
Gradient : 2% B à 40% B en 76 min Flow : **0.55ml/min** (T=40°C)



# Selected peptides

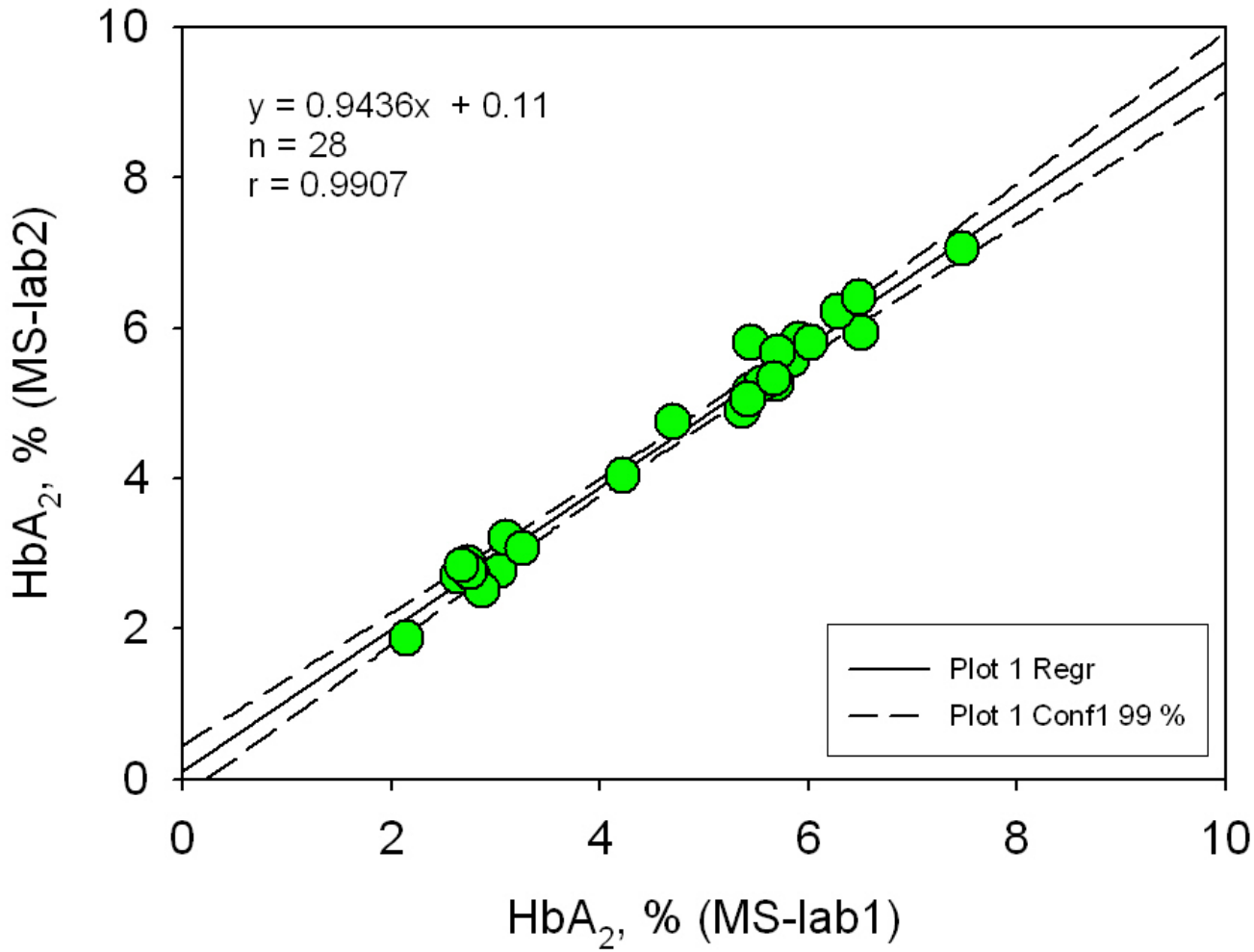
| Peptide | Sequence | RT | MH+ | M2H+ |
|---|---|---|---|---|
| αT11 | VDPVNFK | 14.02 min | 818.4 | 409.3 |
| δT2 | TAVNALWGK | 20.19 min | 959.5 | 480.6 |
| δT3 | VNVDAVGGEALGR | 18.59 min | 1256.7 | 629.1 |
| δT14 | EFTPQMQAAYQK | 16.56 min | 1441.4 | 721.5 |

2006 exercise
6 calibrators
29 samples
2 digestions, 2 analyses per digestion

**δT2 / αT11**

*all samples - July 2006*

Fig. 01 all

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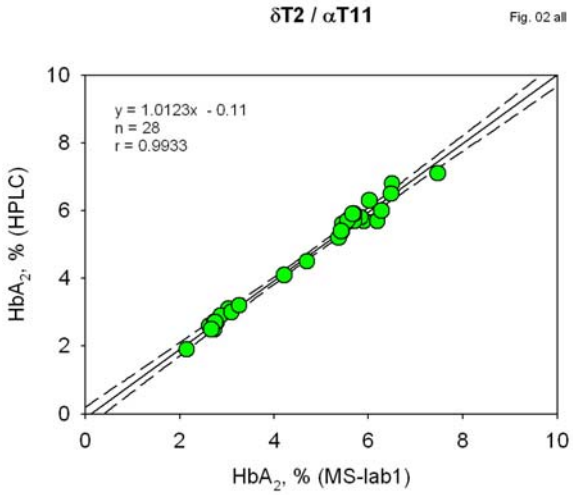


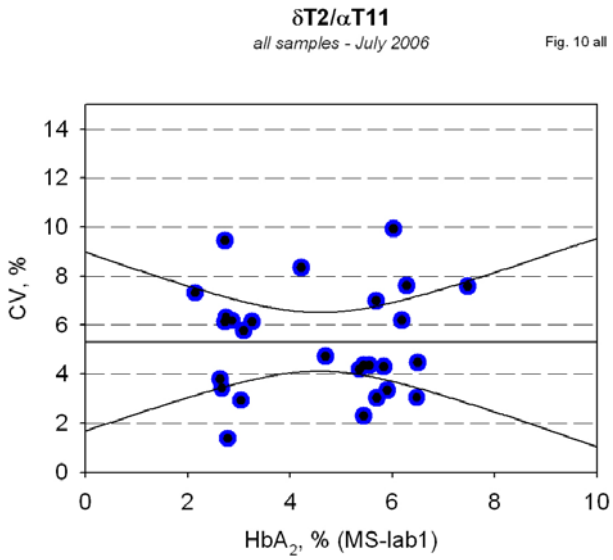


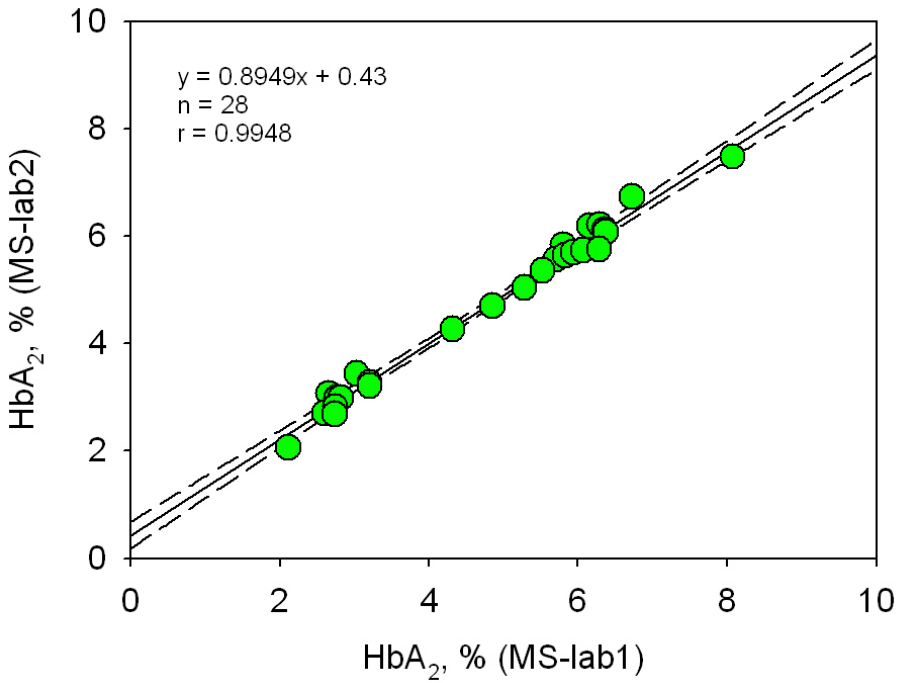


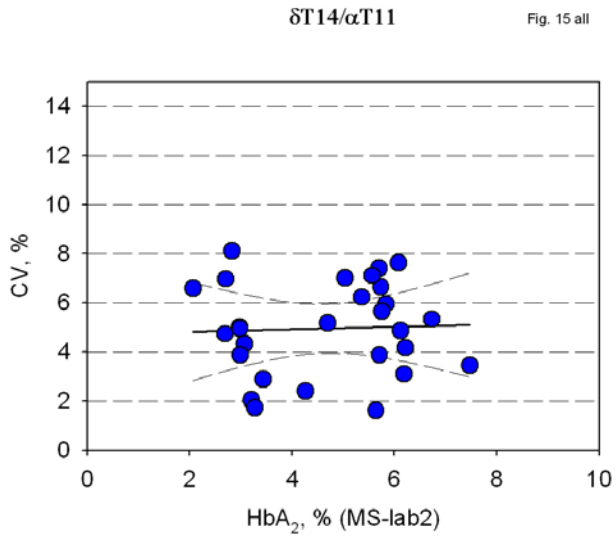


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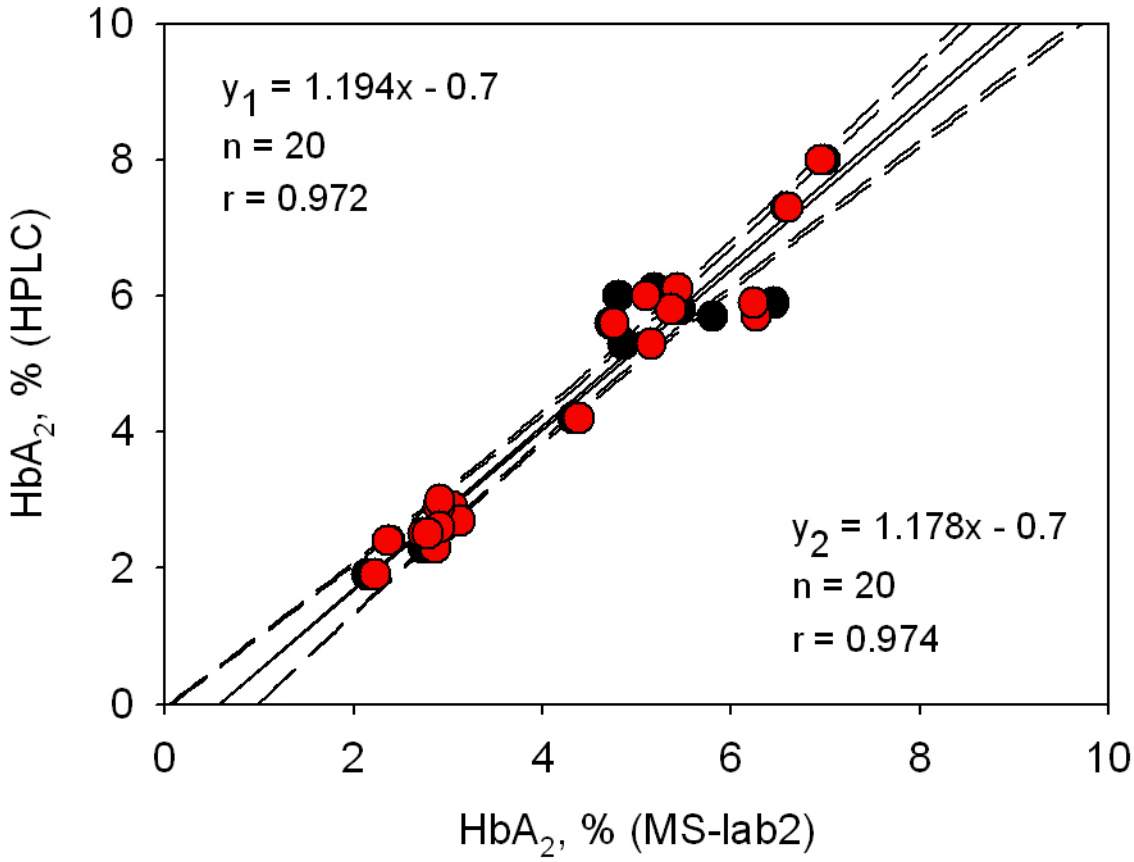
2007 exercise
6 calibrators
3 controls
20 samples
2 digestions, 2 analyses per digestion

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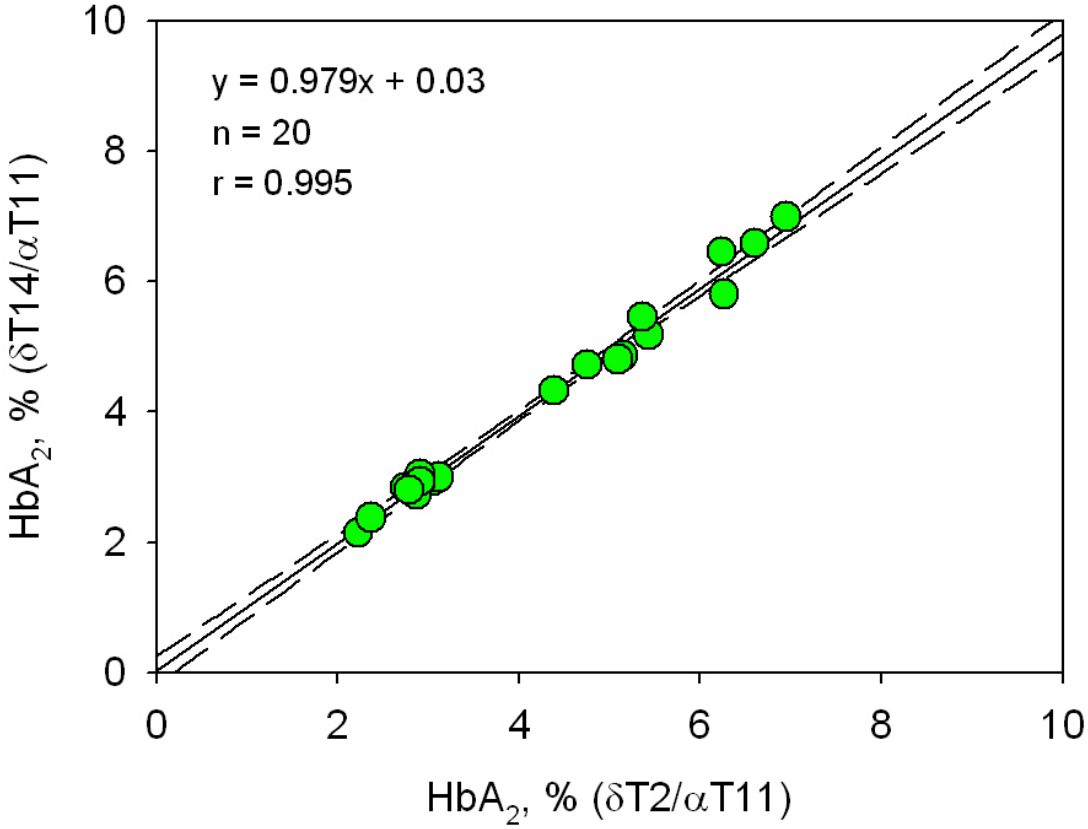


| Reproducibility | $HbA_2$, % (CV, %) | |
|---|---|---|
| δT2/αT11 | 2.7 (4.9) | 5.5 (6.3) |
| δT14/αT11 | 2.7 (6.3) | 5.6 (8.1) |

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# Secondary Reference Materials

- Lyophilized hemolysates
- Process started on May 15, 2007
- Previously tested for stability and commutability against 3 HPLC methods
- Probably available in 2008

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Institute for Reference Materials and Measurements

# CERTIFIED REFERENCE MATERIAL
# BCR® – 348R

## CERTIFICATE OF ANALYSIS

| HUMAN SERUM | | | | |
|---|---|---|---|---|
| | Concentration | | | |
| | Certified value [1] | | Uncertainty [2)] | |
| | [µg/L] | [nmol/L] | [µg/L] | [nmol/L] |
| Progesterone | 8.5 | 26.9 | 0.4 | 1.2 |

1) The certified value is the concentration of progesterone determined by Isotope Dilution Gas Chromatrography coupled to Mass Spectrometry (ID-GC-MS). This value is the unweighted mean of 4 sets of results, independently obtained from 4 laboratories. The material must be reconstituted according to the specified procedure (see below). The certified value is traceable to the International System of Units (SI).

2) The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM). It is expressed with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %.

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# Secondary reference materials for $HbA_2$ (IRMM)

- Expression of interest (2006)
  - Analis
  - Bio-Rad Laboratories
  - Drew
  - Helena
  - Menarini
  - Sebia
  - Tosoh
- Pilot lyophilization (100 vials, 1 level): November 2007
- Working Lyophilization (1500 vials, 2 levels): 2008
- IRMM/reference labs activities
  - Homogeneity, stability, commutability
  - Certification

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**Form RM F/0007**

**Revision 9, Decv. 2006**

Institute for Referenc
Measure

# Project Planning Form

## PROJECT TITLE

# IRMM/IFCC-xxx (HbA2)

not and ERM Material

| Project Information | |
|---|---|
| Action Number | 15012 |
| Action Leader | Stefanie Trapmann |
| Project Responsible | Amalia Muñoz-Piñeiro |

# Further developments

- Reference method
  - Isotopic dilution
  - More reference labs (4?)
  - Establishing a network (?)
- Implementation
  - Change of units (mmol/mol Hb) (?)

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# Standardization of hemoglobin A2: does HbA1c history repeat itself?

| | $HbA_{1c}$ | $HbA_2$ |
|---|---|---|
| Primary reference materials | IRMM | Milano |
| Reference method | IFCC official | under development |
| Secondary reference materials | under dev. | IRMM (PPF) |
| Network | implemented | -- |
| Implementation | under dev. | probably not dramatic |

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# Aknowledgments

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Christine Schaeffer

IFCC Scientific Division

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