Il processo di standardizzazione dell'emoglobina A2

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INCONTRO DI AGGIORNAMENTO SCIENTIFICO

"EMOGLOBINE: DIAGNOSTICA, STANDARDIZZAZIONE, PROSPETTIVE"

AULA MAGNA OSPEDALE
DESENZANO DEL GARDA (BS)
Loc. Montecroce

9 MAGGIO 2009

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

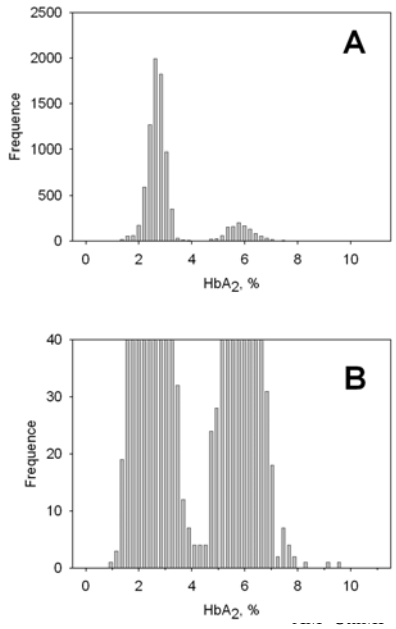
- β-thal
  - Mediterranean regions: up to 8 %
  - Middle East: up to 10 %
  - India: 3 – 15 %
  - Southeast Asia: up to 9 %

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 %



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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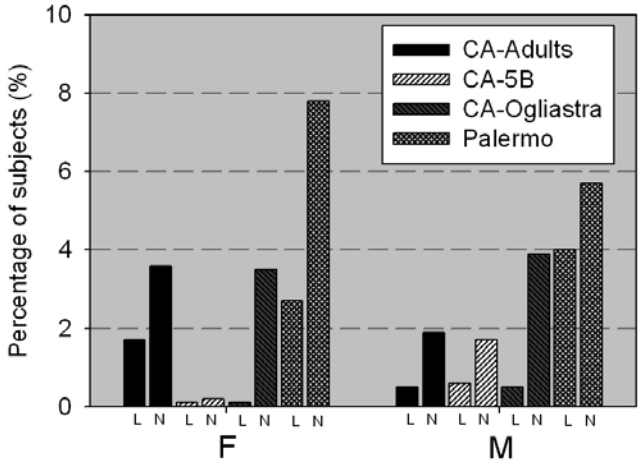

AM -UniMI

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Genotype of 234 (over 1743) subjects with HbA_2 borderline

mutation defect	**25.6 %**
no defect	**74.4 %**

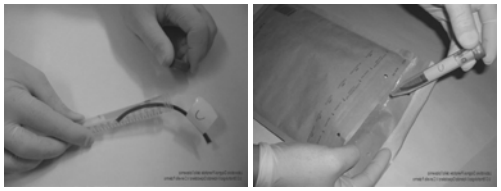
NEG/-α3.7	**2**
NEG/IVS 1 nt 6	**20**
β*+δCd 27	**7**
NEG/ααα^{anti3,7}	**10**
Hb Variants**	**3**
Cap +1570	**1**
β prom. (-101; -92)	**10**

* 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)

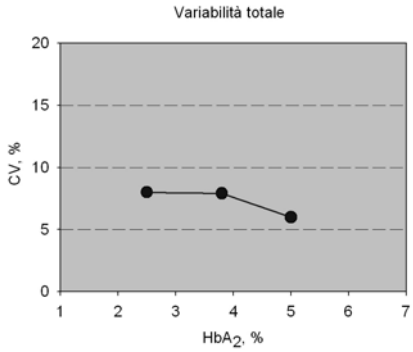
State-of-the-art of HbA_2 measurements

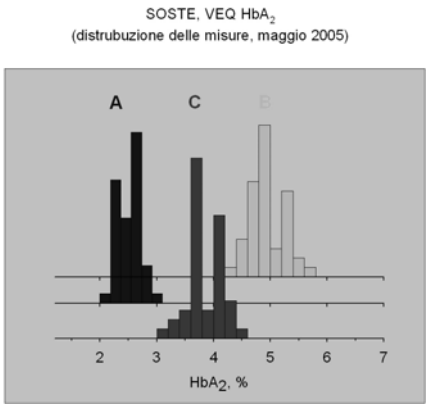
- EQAS data



EQAS – So.S.T.E.

N = 48 HPLC
April-June 2005





HbA_2, analytical goals (1)

- biological variablity -

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

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 %

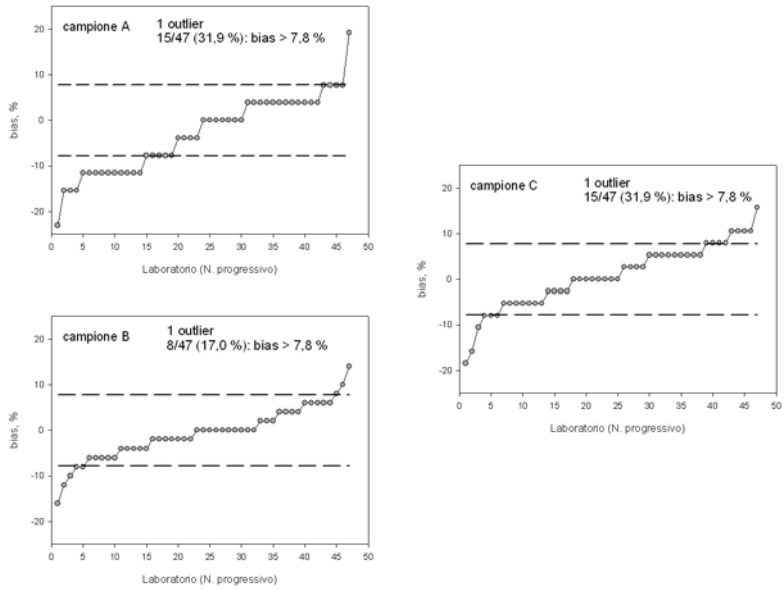
HbA_2, analytical goals (2)

- clinical needs -

HbA_2 = 3.3 % (upper normal)
HbA_2 = 3.8 % (low β-thal carrier)

HbA_2 = 3.55 %→ ?

TE = 0.25/3.55 x 100 = 7.0 %



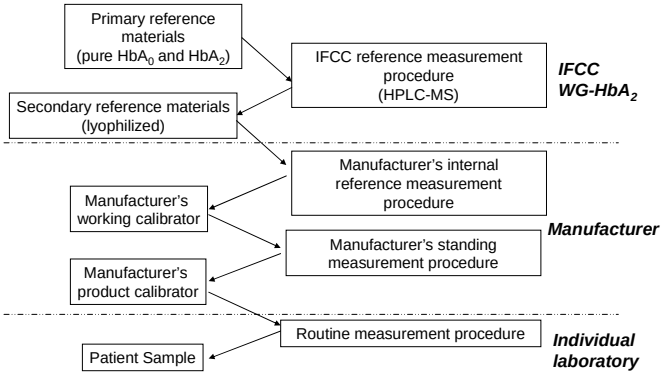
IFCC HbA_2 standardization

- 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		

IFCC Reference System for HbA_2



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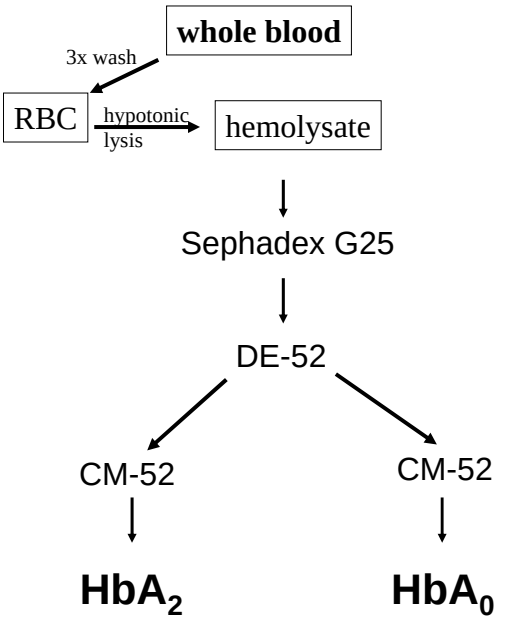
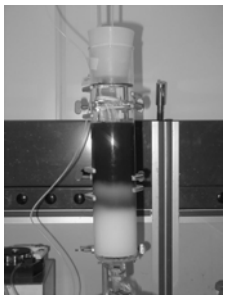
Primary Reference Materials

- 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



Preparation of pure HbA_0 and HbA_2

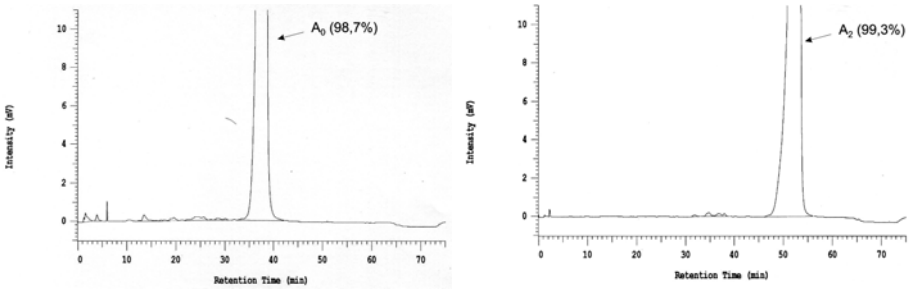


- HbA_0 purified January 2008 (350 mg)
- HbA_2 purification (under progress)

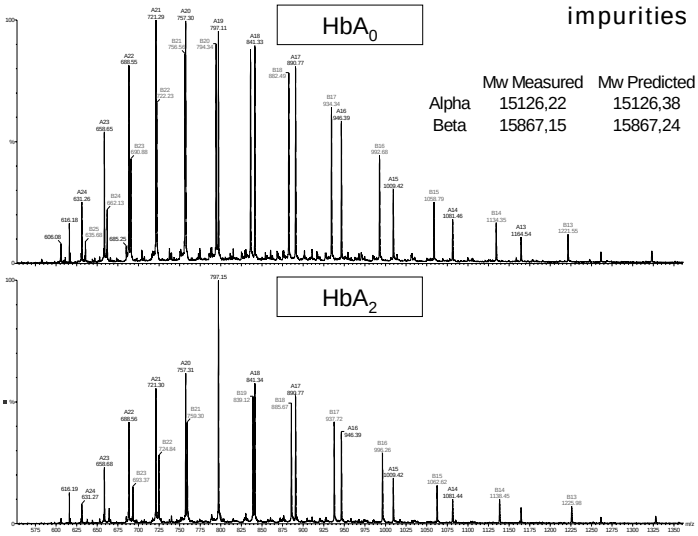
Tests for assessing purity of the primary calibrators

- HPLC
- IEF
- Capillary EF
- ESI-MS

PolyCATA HPLC analysis (EB)



ESI-MS of purified HbA_0 and HbA_2



impurities < 1 %

	Mw Measured	Mw Predicted
Alpha	15126,22	15126,38
Beta	15867,15	15867,24

MS : ESI positive LCT, Micromass

Candidate Reference Measurement Procedure for HbA_2

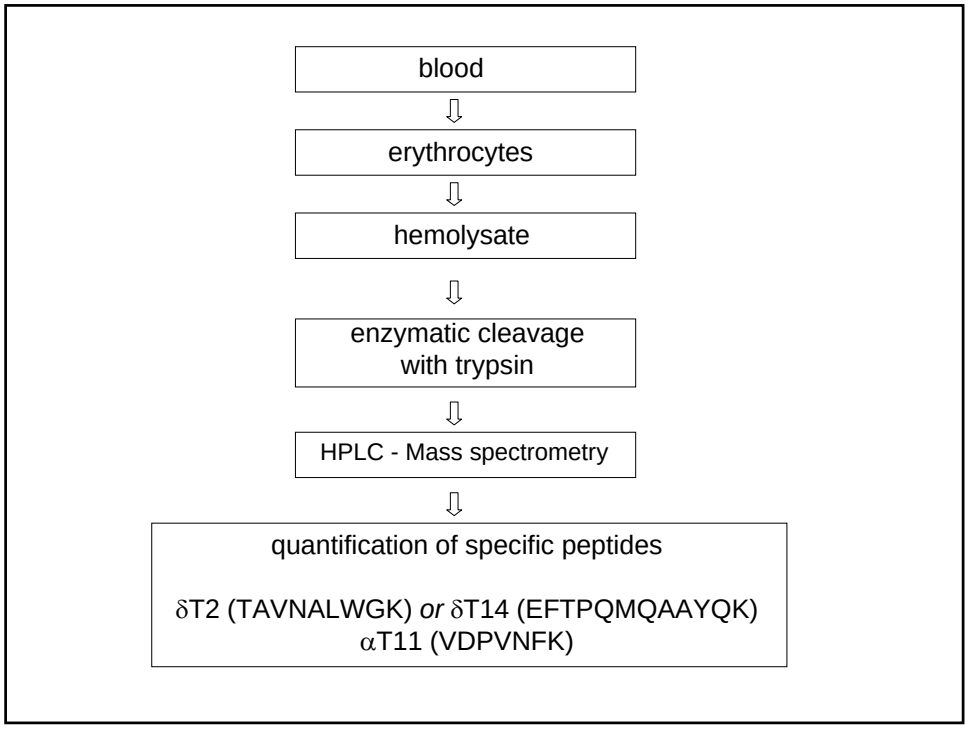
Principle

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.



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

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.)

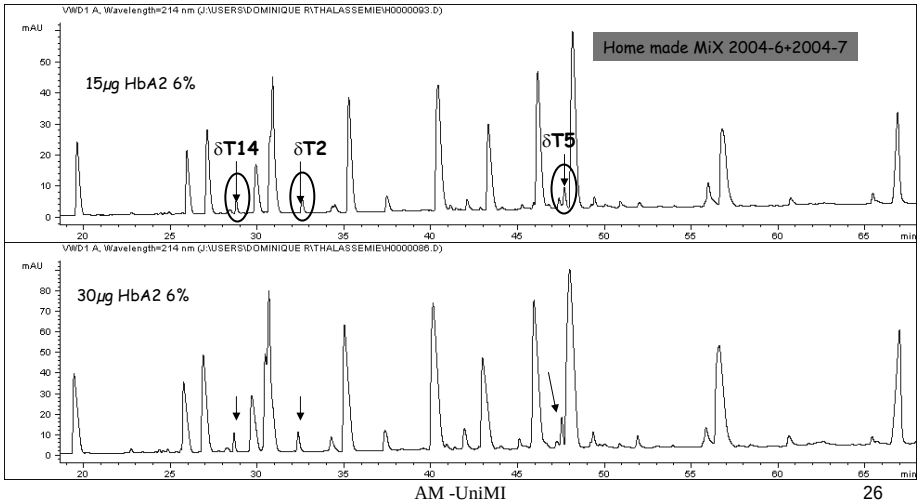
2) Two comparison between the results obtained in 2 MS labs has been performed in two steps during 2006 and 2007

3) The SOP has been defined

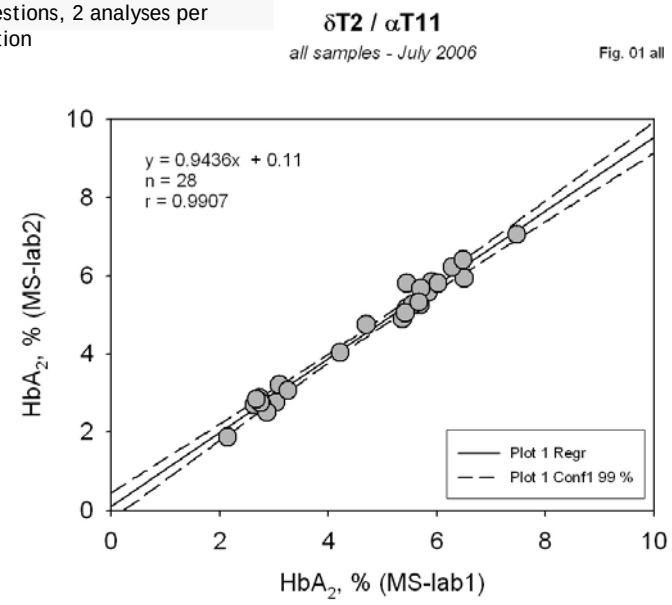
4) Another laboratory has been involved to reduce the uncertainty

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

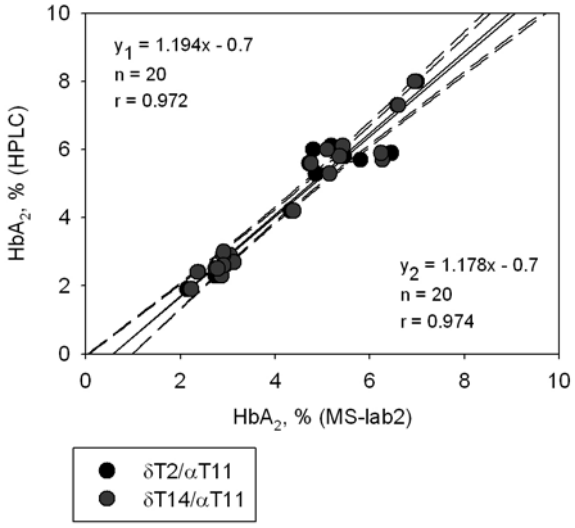
HPLC instrument: Agilent 1100 Chemstation, UV detector (MicroCell : 1µl)
Column : Tosoh TSKgel super ODS 2µm (2mmx10cm)
Gradient : 2% B à 40% B en 76 min Flow : **0.55ml/min** (T=40°C)



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



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



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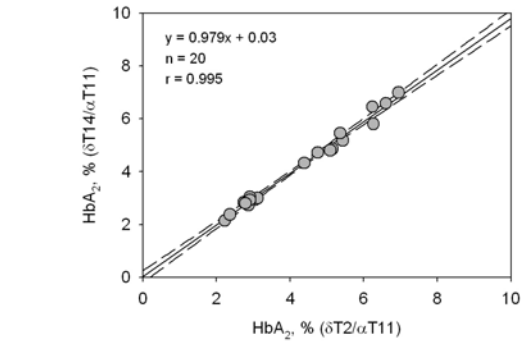
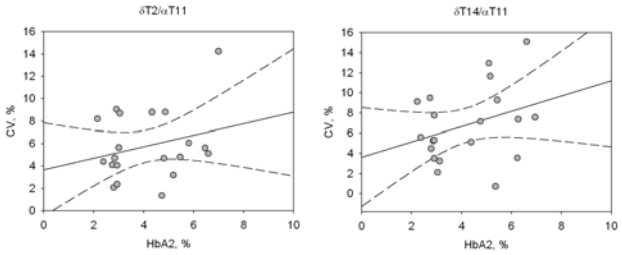




Secondary Reference Materials

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

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
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 %.

Secondary reference materials for HbA_2 (IRMM)

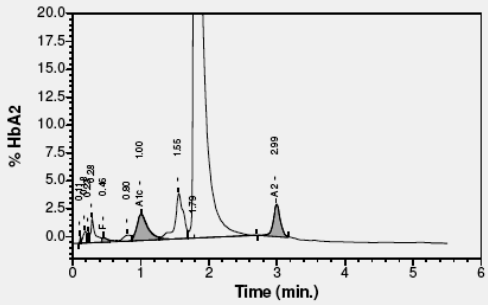
- Expression of interest
  - Analis
  - Bio-Rad Laboratories
  - Drew
  - Helena
  - Menarini
  - Sebia
  - Tosoh
- Pilot lyophilization (100 vials, 1 level): April 2008
- Working Lyophilization (1500 vials, 2 levels): 2009
- IRMM/reference labs activities
  - Homogeneity, stability, commutability
  - Certification

IRMM first lyophilization

(April 2008)

F Concentration = 0.6 %
A1c Concentration = 5.9 %
A2 Concentration = 2.9 %

Analysis comments:



Further developments

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

Outcomes

+ Improvement in genetic counselling
+ Improvement in analytical performance
+ Better service to patients and clinicians

References

- Mosca A. *Development of a reference system for HbA_2*. EARCR, 15th meeting, Muerten (CH), April 2005.
- Mosca A, Paleari R, Scimè-Degani V, Leone L, Ivaldi G. *Inter-method differences and commutability of control materials for Hb A_2 measurement*. Clin Chem Lab Med 2000;38:997-1002.
- Paleari R, Giambona A, Cannata M, Leto F, Maggio A, Mosca A for the IFCC WG-HbA_2. *External Quality Assessment of hemoglobin A_2 measurment: data from an Italian pilot study with fresh whole blood samples and commercial HPLC systems*. Clin Chem Lab Med 2007;45:88-92.
- IFCC WG-HbA_2. *Candidate reference method for HbA_2 based on peptide mapping. Standard Operating Procedure, vs. 2.0*, May 2007.
- Mosca A, Paleari R, Galanello R, Sollaino C, Perseu L, Demartis FR, Passarello C, Giambona N, Maggio A, for the IFCC WG-HbA_2. *New analytical tools and epidemiological data for the identification of HbA_2 borderline subjects in the screening for beta-thalassemia*. Bioelectrochemistry (in press).

Aknowledgments

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