Quantification of residual disease by RT-PCR

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Why is it molecular monitoring important for haematological malignancies?

- **The degree to which the bulk of disease is reduced is the most important prognostic indicator**
- **Poor primary response or relapse suggest the need to change therapy (or check compliance)**
- **Molecular monitoring by PCR-based methods provides:**
  - **A deeper assessment of response than other methods**
  - **The means to detect relapse earlier**

Hughes T, et al. Blood 2006;108:28-37.
Cross NCP. Best Pract Res Clin Haematol. 2009 Sep;22(3):355-65.

Why use RT-PCR?

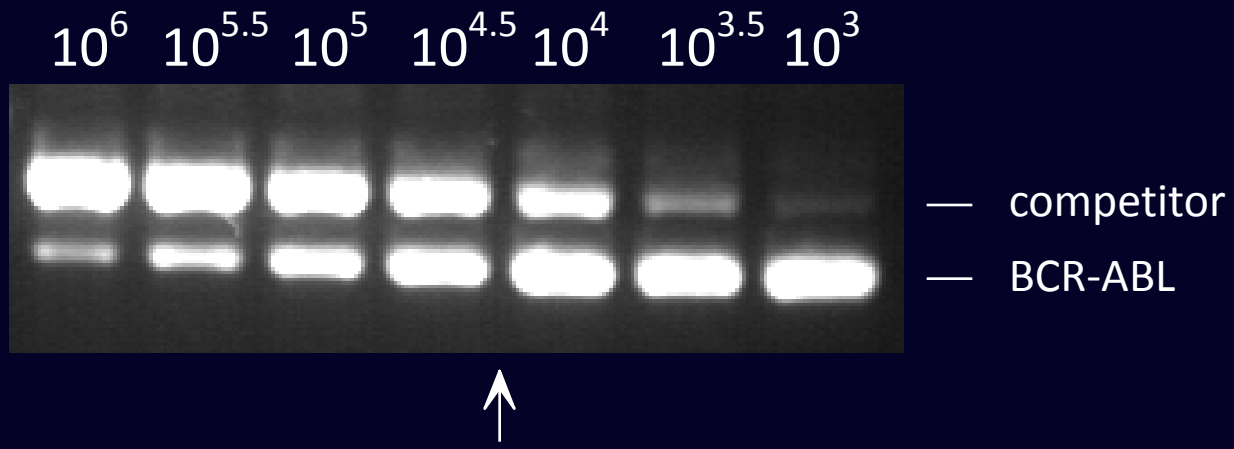
- **Fusion genes: breakpoints too dispersed at DNA level**
  - **BCR-ABL**
  - **PML-RARα**
- **Point mutations: sensitivity may be enhanced if gene expressed only in particular cell types or at elevated levels**
  - **NPM1**
  - **KIT**
- **Genes over-expressed in malignant cells**
  - **WT1**

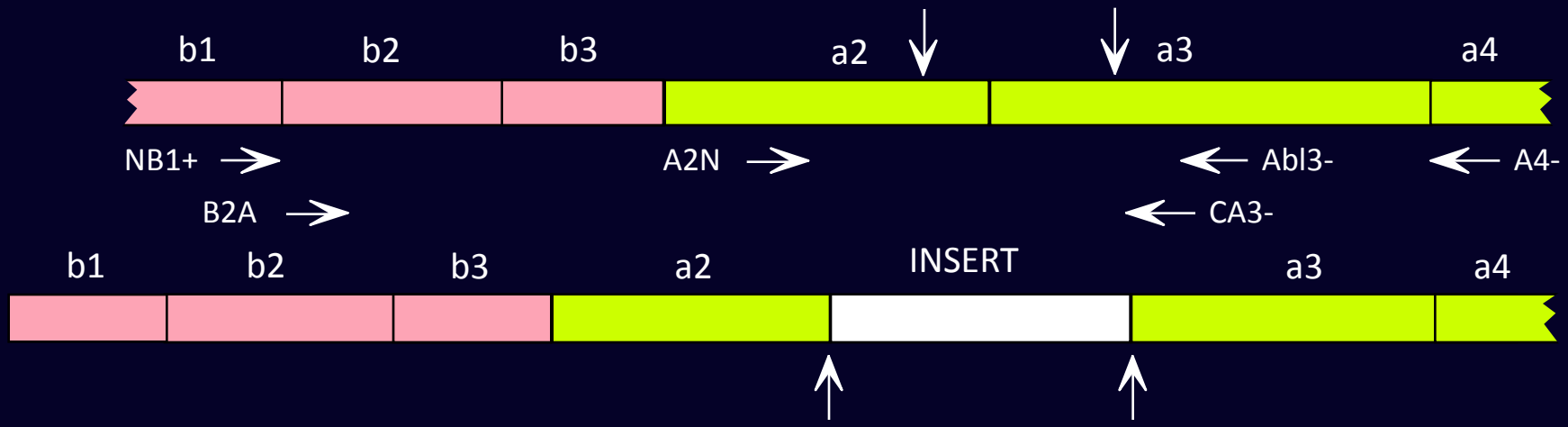
Disadvantages of RT-PCR

- **RNA susceptible to degradation**
- **Technically more challenging to make high quality cDNA than DNA**
- **More difficult to standardise diagnostic measurements across labs**
- **Transcript numbers cannot be easily related to numbers of cells**
- **No natural unit of measurement**

- History of molecular monitoring in CML
- The International Scale for BCR-ABL
- Development of accredited reference reagents

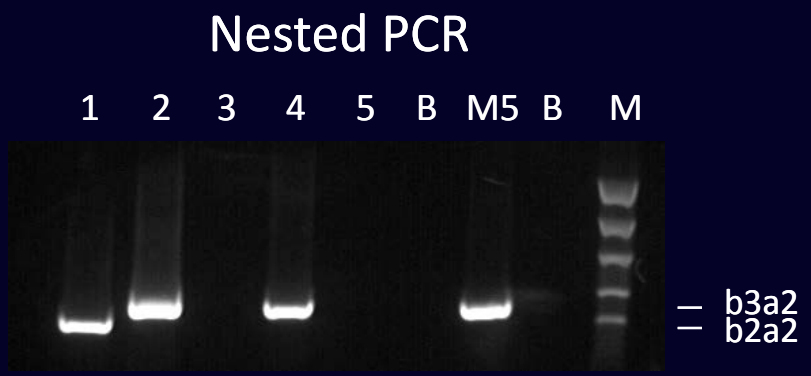
Competitive RT-PCR to quantify BCR-ABL mRNA

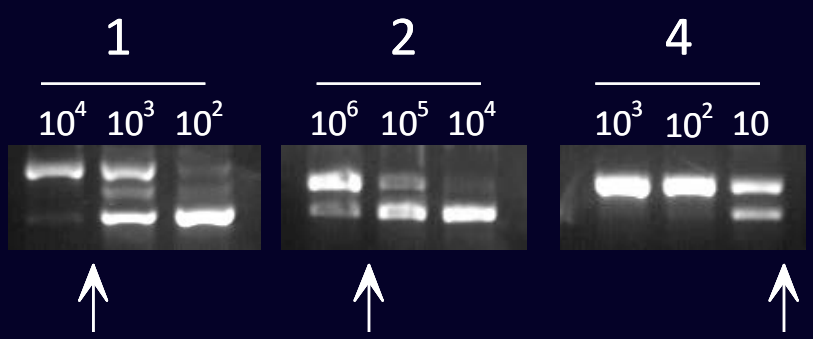


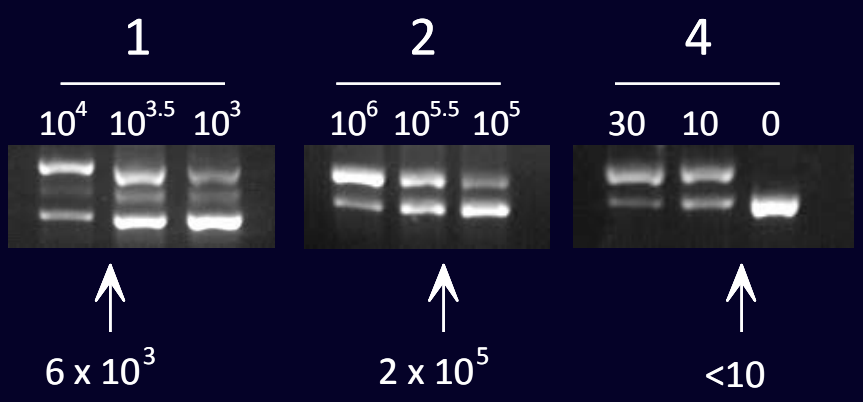


Cross et al. Blood. 1993;82(6):1929-36

Competitive RT-PCR to quantify BCR-ABL mRNA

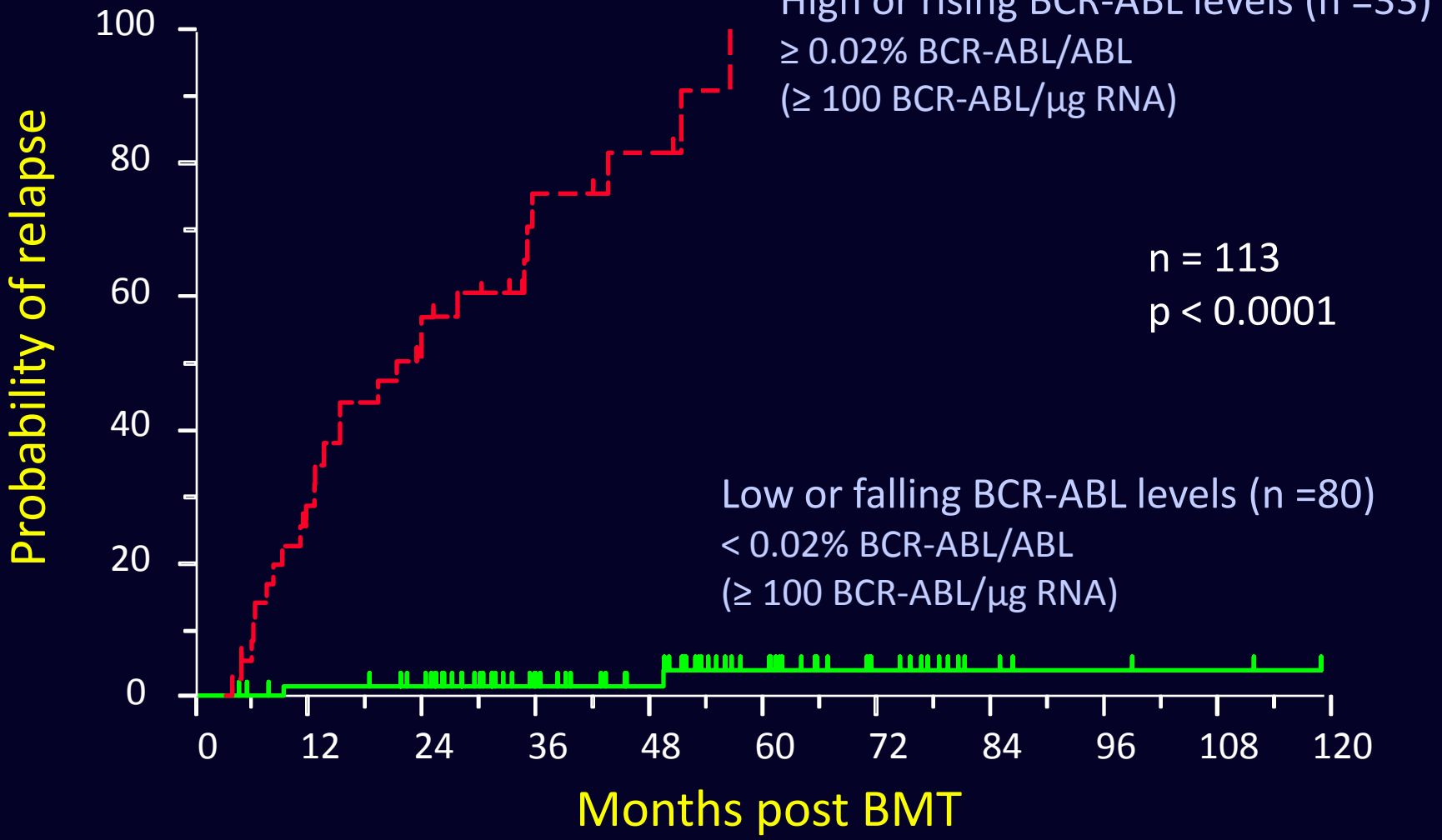






Cross et al. Blood. 1993;82(6):1929-36

Sequential quantitative PCR and subsequent relapse post BMT



1996: imatinib and qRT-PCR

© 1996 Nature Publishing Group http://www.nature.com/naturemedicine

Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells

BRIAN J. DRUKER[1], SHU TAMURA[1], ELISABETH BUCHDUNGER[2], SAYURI OHNO[1], GERALD M. SEGAL[1], SHANE FANNING[1], JÜRG ZIMMERMANN[2] & NICHOLAS B. LYDON[2]

[1]*Division of Hematology and Medical Oncology, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon, USA*
[2]*Ciba Pharmaceuticals Division, Oncology Research Department, Ciba-Geigy Limited, CH-4002, Basel, Switzerland*
Correspondence should be addressed to B.J.D.

Nat Med. 1996;2:561-6

GENOME METHODS

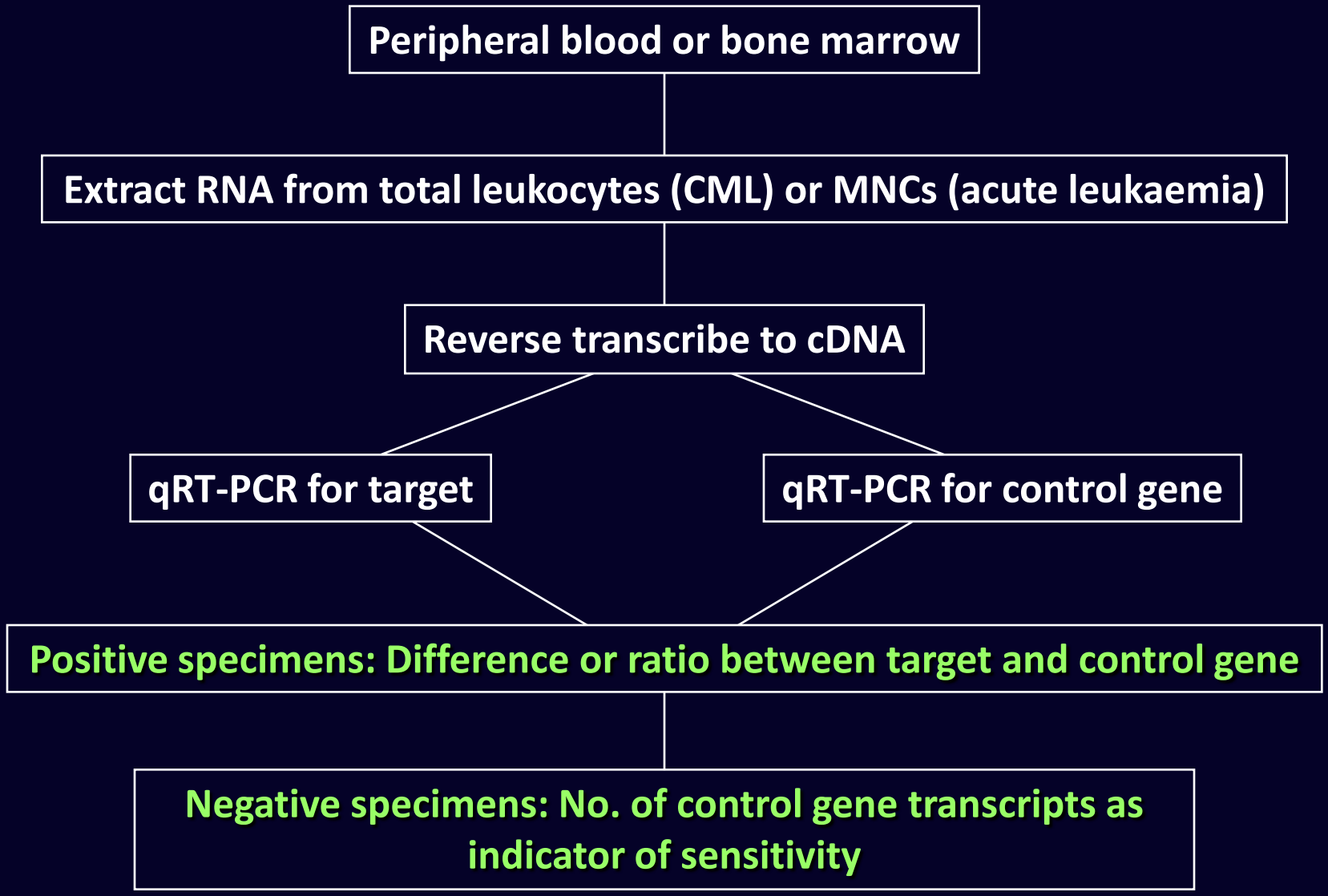
Real Time Quantitative PCR

Christian A. Heid,[1] Junko Stevens,[2] Kenneth J. Livak,[2] and P. Mickey Williams[1,3]

[1]BioAnalytical Technology Department, Genentech, Inc., South San Francisco, California 94080;
[2]Applied BioSystems Division of Perkin Elmer Corp., Foster City, California 94404

Genome Res. 1996;6: 986-994

Outline of quantitative RT-PCR analysis



Cross NCP. Best Pract Res Clin Haematol 2009;22:355-365.

What is the best internal control gene?

- **Internal control gene should give an accurate indication of quantity and quality of cDNA for each specimen and control for:**
  - **Variation in amount of RNA**
  - **RNA integrity**
  - **Efficiency of reverse transcription**
- **Ideal control gene would be:**
  - **expressed uniformly in different cell types**
  - **Expressed at same levels in blood and bone marrow**
  - **unaffected by therapeutic regimens**
  - **invariant between individuals**
  - **expressed a level similar to target mRNA**
  - **degrades at the same rate as target mRNA**

What is the best internal control gene?

- **EAC: recommended *ABL*, *GUSB* or β*2M***

Leukemia (2003) 17, 2474–2486
© 2003 Nature Publishing Group All rights reserved 0887-6924/03 $25.00
www.nature.com/leu

Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) – a Europe against cancer program

E Beillard[1,10,11], N Pallisgaard[2,10], VHJ van der Velden[3], W Bi[4], R Dee[5], E van der Schoot[5], E Delabesse[6], E Macintyre[6], E Gottardi[7], G Saglio[7], F Watzinger[8], T Lion[8], JJM van Dongen[3], P Hokland[2] and J Gabert[1,9,11]

- **CML expert group: recommended *ABL*, *GUSB* and *BCR***

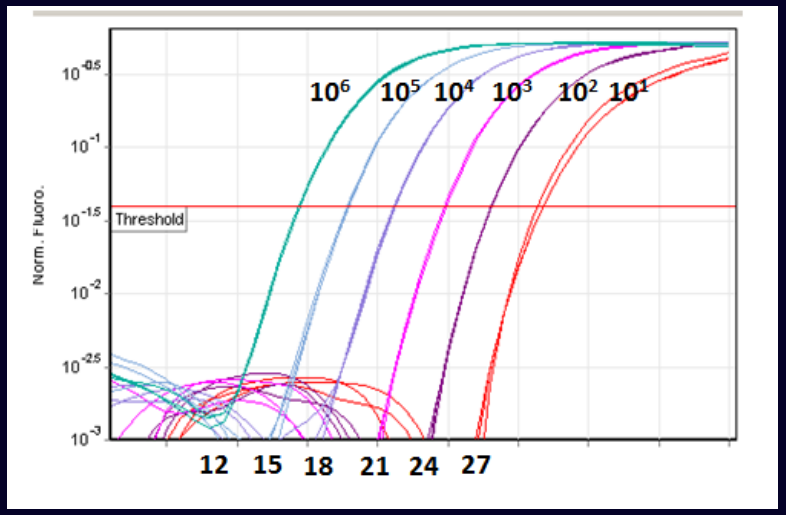
Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting *BCR-ABL* transcripts and kinase domain mutations and for expressing results

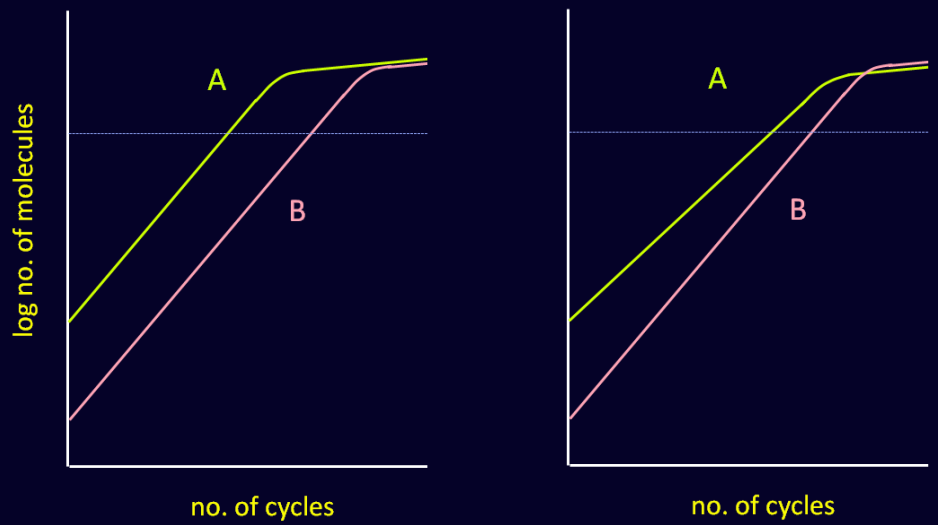
Timothy Hughes, Michael Deininger, Andreas Hochhaus, Susan Branford, Jerald Radich, Jaspal Kaeda, Michele Baccarani, Jorge Cortes, Nicholas C. P. Cross, Brian J. Druker, Jean Gabert, David Grimwade, Rüdiger Hehlmann, Suzanne Kamel-Reid, Jeffrey H. Lipton, Janina Longtine, Giovanni Martinelli, Giuseppe Saglio, Simona Soverini, Wendy Stock, and John M. Goldman

BLOOD, 1 JULY 2006 • VOLUME 108, NUMBER 1

How to define sensitivity of detection and log reductions on treatment

- **Standard approach**
  - **Measurements made pre and post treatment**
  - **Compared by ΔΔCt method**
  - **$(Ct\ TARGET - Ct\ CG)_{diagnosis}$ - $(Ct\ TARGET - Ct\ CG)_{treatment}$**
  - **For samples where target is undetectable, sensitivity based on expression of target at diagnosis and expression of CG in test sample (EAC criteria; Gabert et al., Leukemia 2003)**

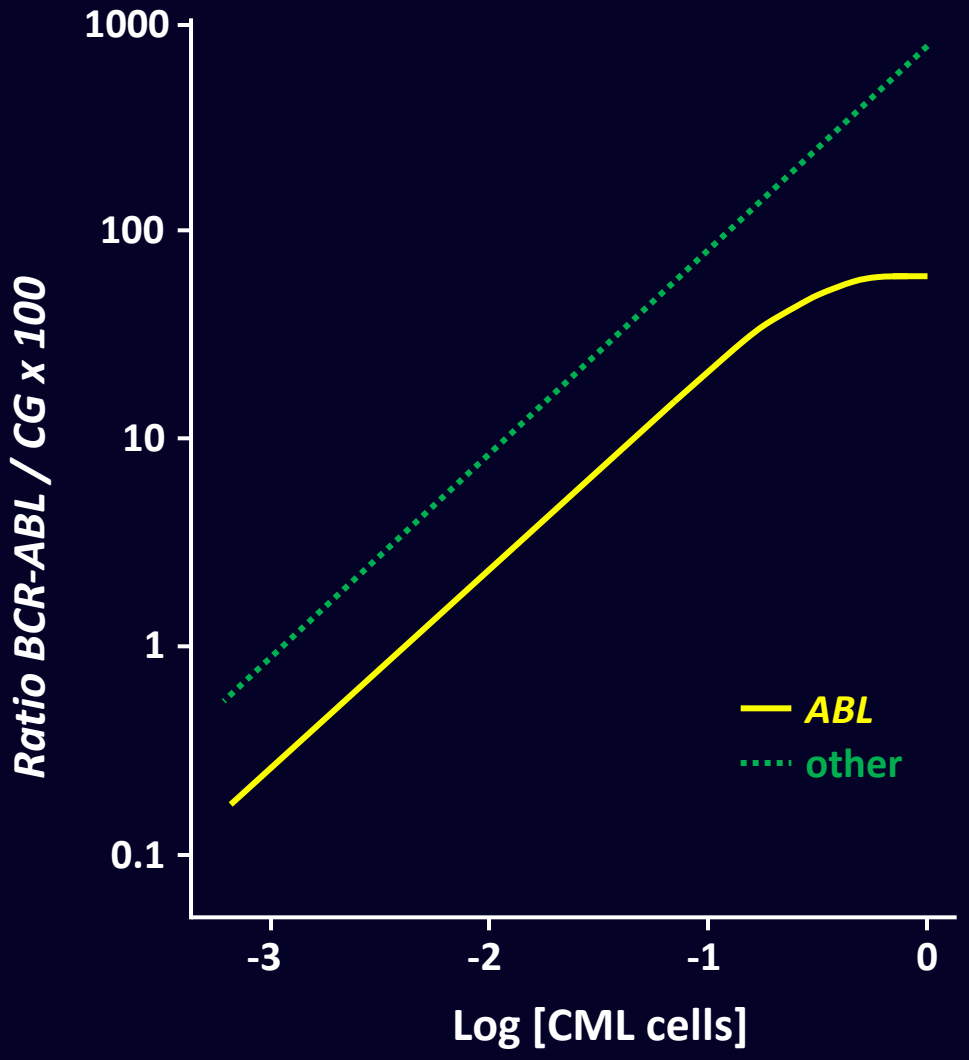




Definitions do not work for CML using ABL or BCR as control genes

- Primers used to amplify ABL actually amplify BCR-ABL+ABL
- Primers used to amplify BCR are specific but only one copy of BCR in CML cells and two copies in normal cells

- Results typically expressed as %BCR-ABL/CG without reference to pretreatment levels
- Some labs have used other units, eg K562 equivalents



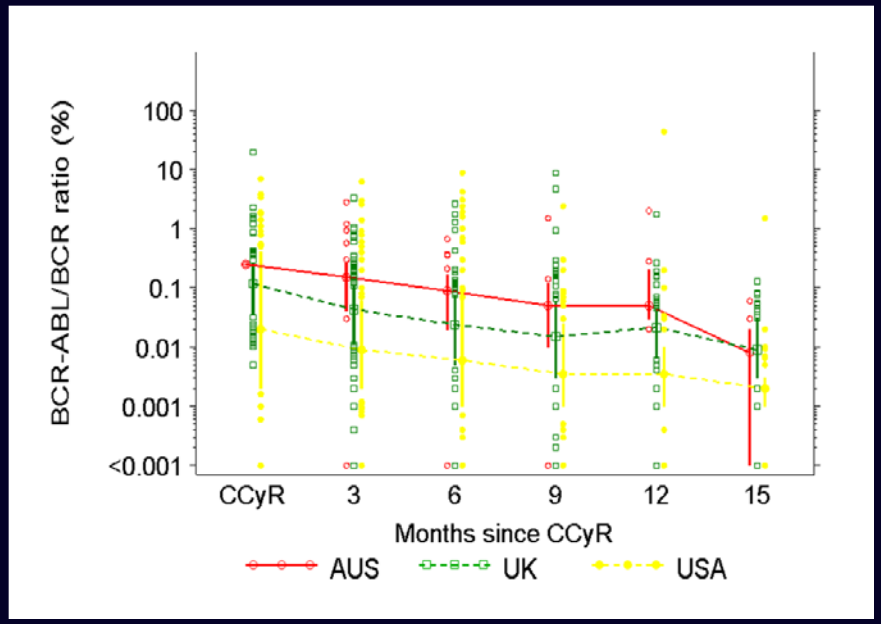
IRIS Trial: Standardization by normalisation of results to 30 shared pre-treatment samples

ORIGINAL ARTICLE

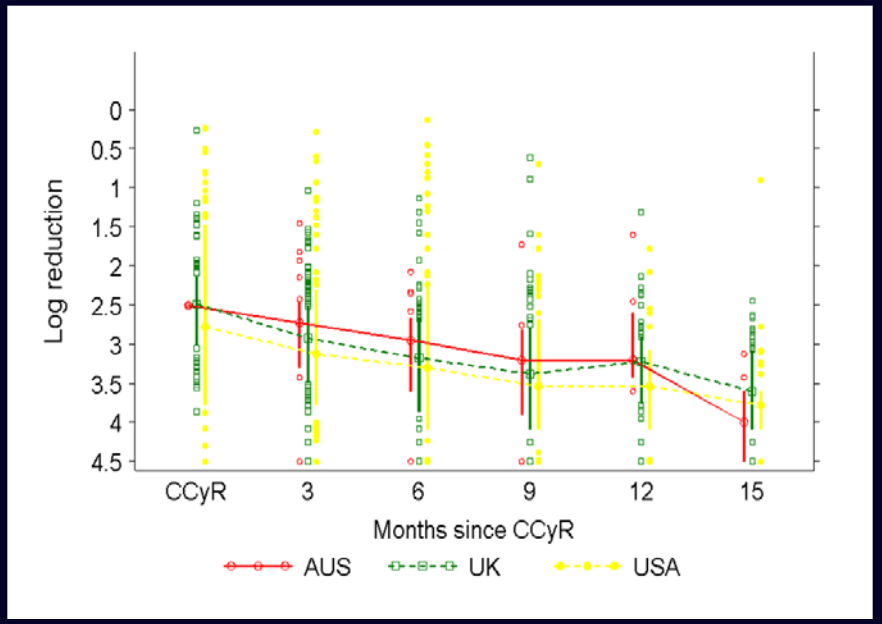
Frequency of Major Molecular Responses to Imatinib or Interferon Alfa plus Cytarabine in Newly Diagnosed Chronic Myeloid Leukemia

Tim P. Hughes, M.D., Jaspal Kaeda, Ph.D., Susan Branford, Zbigniew Rudzki, Ph.D., Andreas Hochhaus, M.D., Martee L. Hensley, M.D., Insa Gathmann, M.Sc., Ann E. Bolton, B.Sc.N., Iris C. van Hoomissen, B.Sc.N., John M. Goldman, D.M., and Jerald P. Radich, M.D. for the International Randomised Study of Interferon versus STI571 (IRIS) Study Group

N Engl J Med 2003; 349:1423-1432 | October 9, 2003

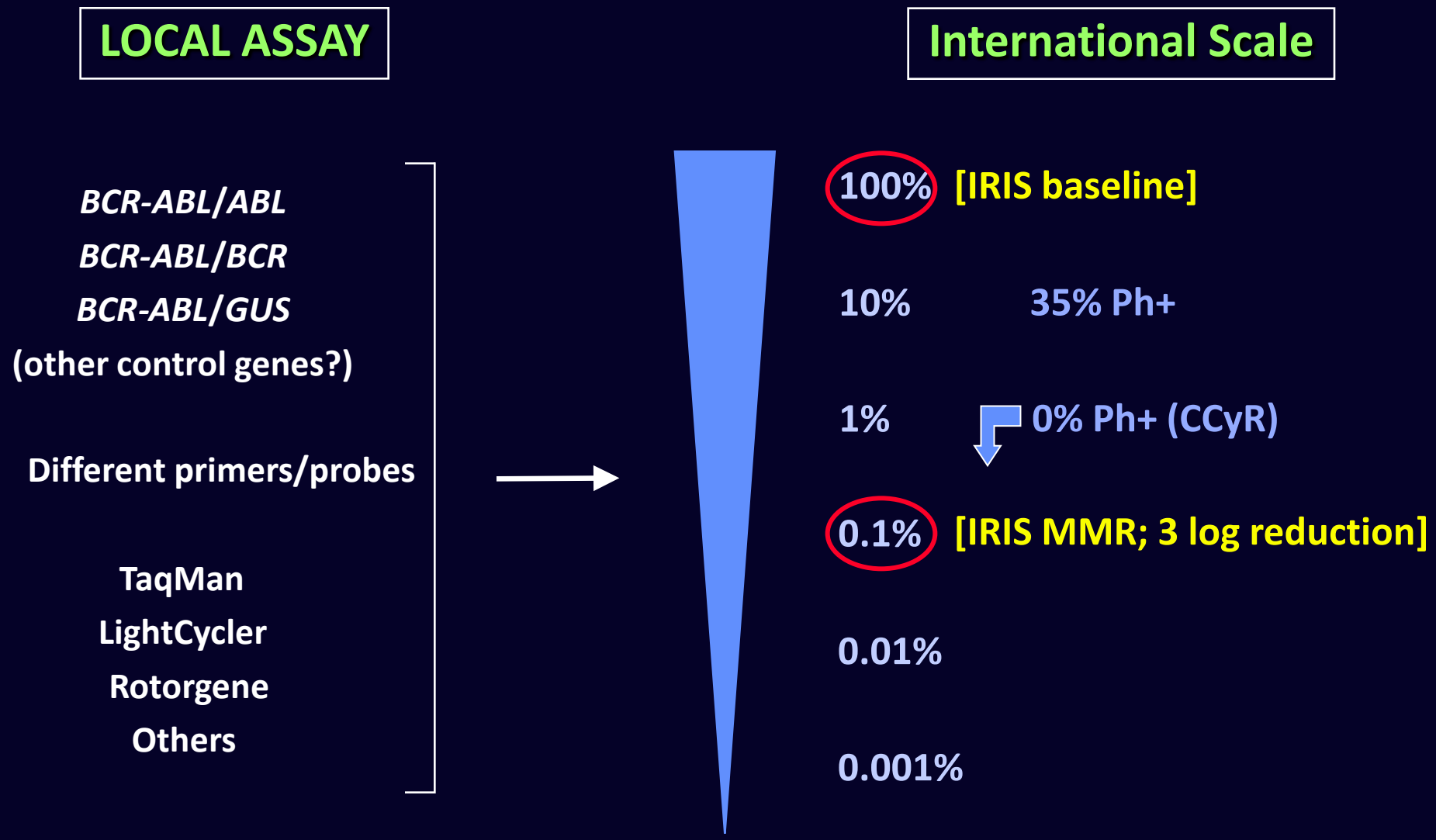


Before normalization



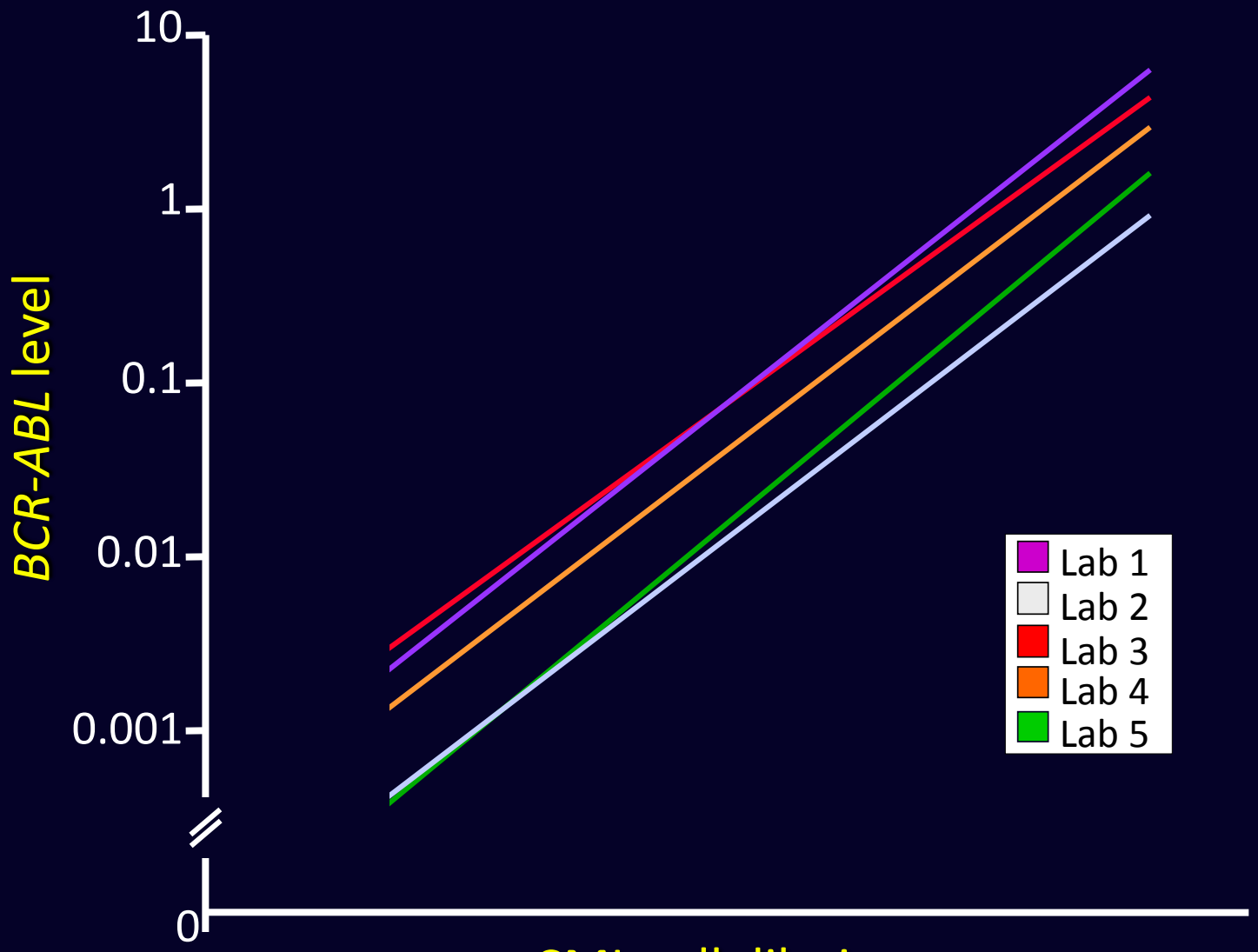
After normalization

Standardisation of qRT-PCR for BCR-ABL: The International Scale (IS)



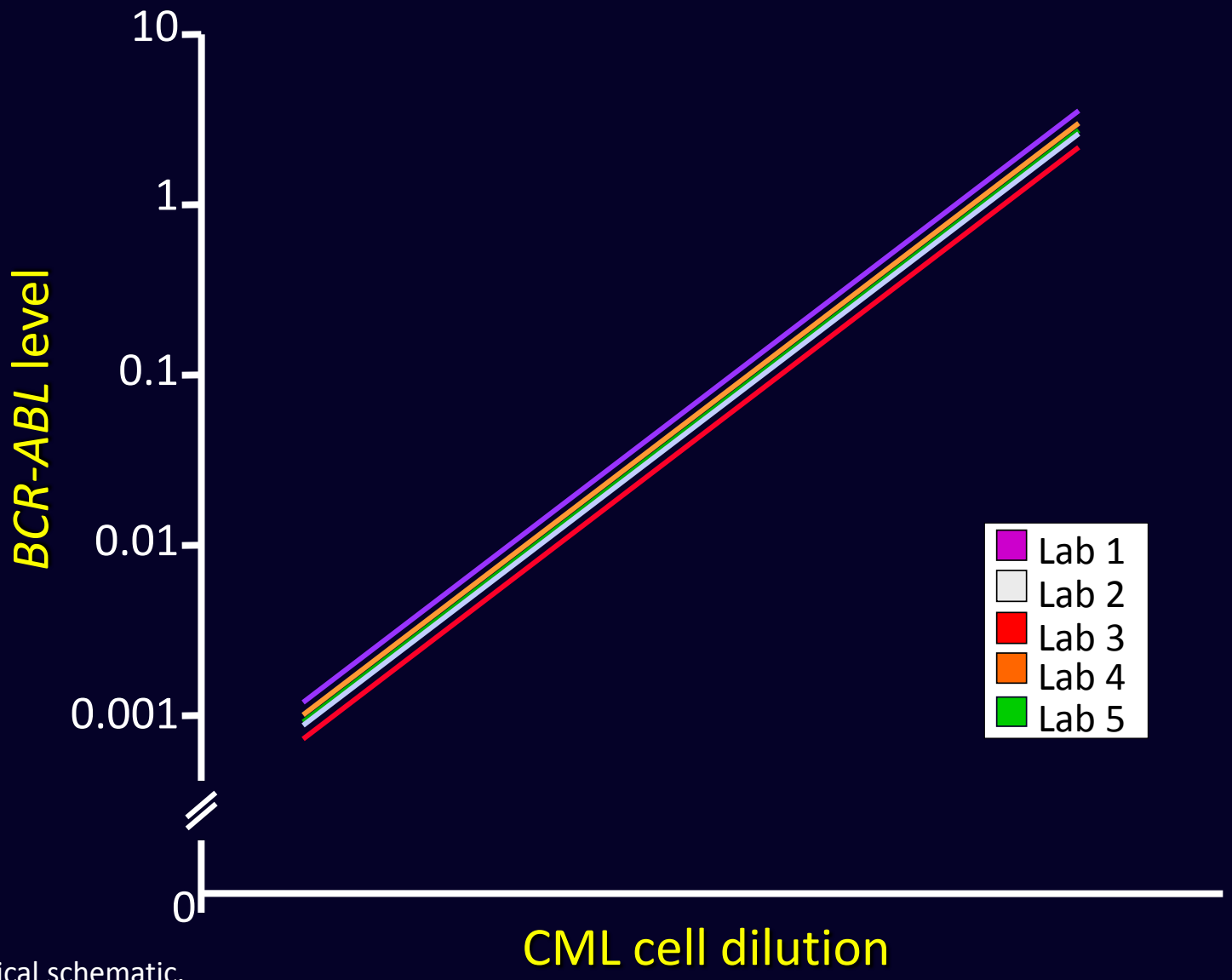
Hughes T, et al. Blood. 2006;108:28-37

The International Scale for BCR-ABL: Before standardisation



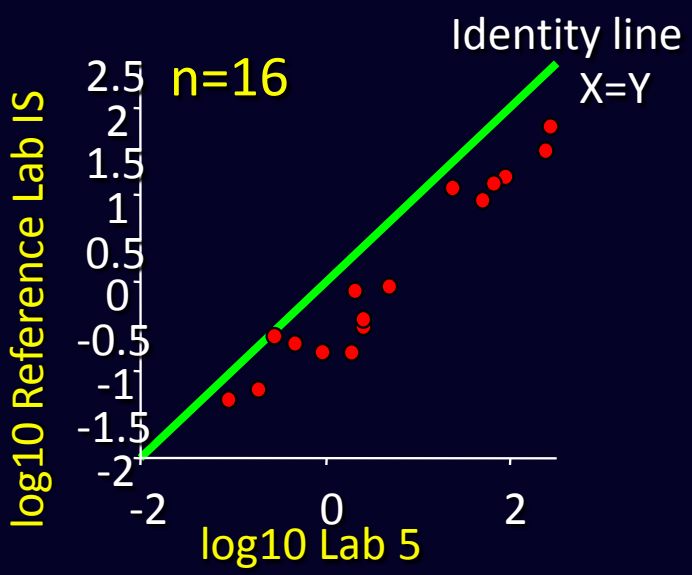
Hypothetical schematic.

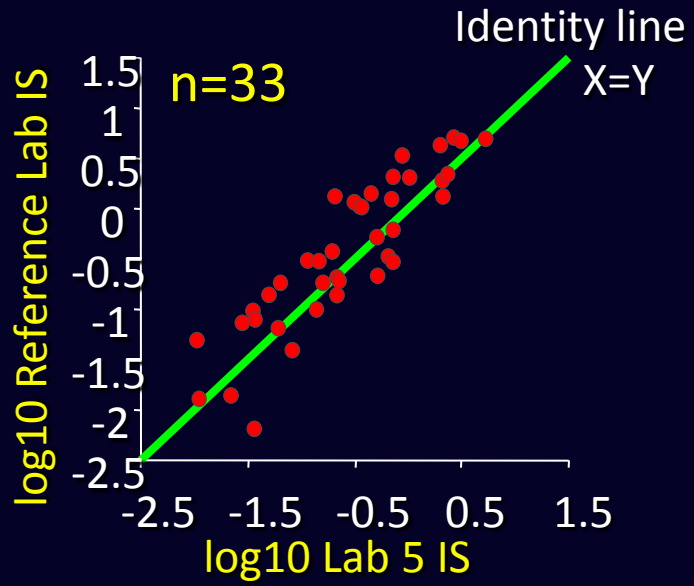
The International Scale for BCR-ABL: After standardisation



Hypothetical schematic.

Realising the International Scale for BCR-ABL

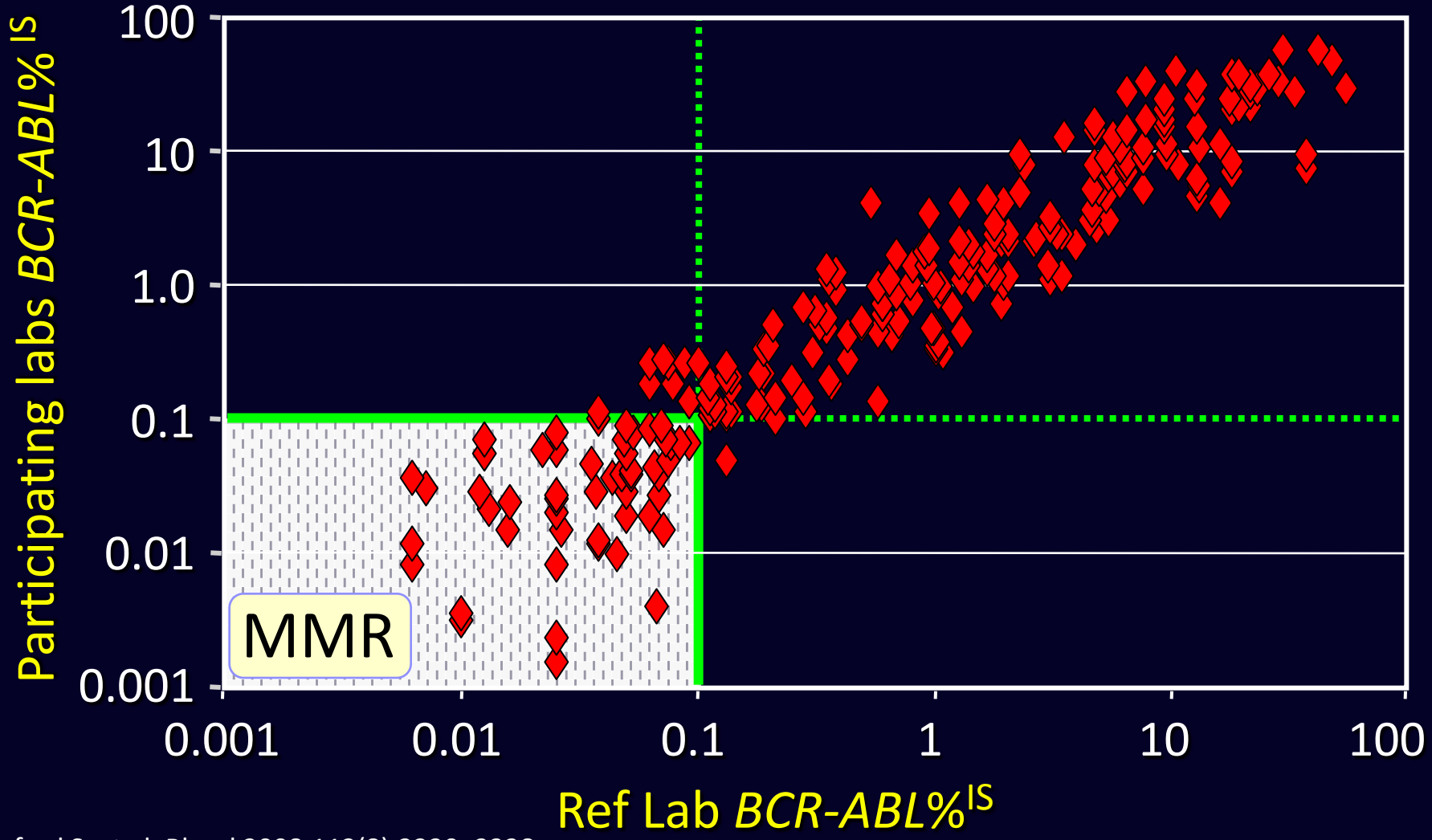




- **Common samples analyzed in test and reference labs**
- **Results compared; calculation of conversion factor**
- **Validation of conversion factor by analysis of further samples**
- **Re-validation at regular intervals**

Branford et al. Blood. 2008 Oct 15;112(8):3330-8

qRT-PCR results at low levels are inevitably more variable than those at high levels



Branford S, et al. Blood 2008;112(8):3330–3338.

qRT-PCR: ELN 2013 recommendations for first line TKI therapy

Time	Optimal	Warning	Failure
Dx	–	High risk CCA/Ph+: major route	–
3 mos	*BCR-ABL* ≤10% and/or Ph+ ≤35%	*BCR-ABL* >10% and/or Ph+ 36-95%	No CHR and/or Ph+ >95%
6 mos	*BCR-ABL* ≤1% and/or CCyR	*BCR-ABL* 1-10% and/or Ph+ 1-35%	*BCR-ABL* >10% and/or Ph+ >35%
12 mos	*BCR-ABL* ≤0.1% (MMR)	*BCR-ABL* 0.1-1%	*BCR-ABL* >1% and/or Ph+ ≥1%
Anytime later	*BCR-ABL* ≤0.1% (MMR)	CCA/Ph- (-7/7q)	Loss of CHR Loss of CCyR Mutations Loss of MMR*

* Confirmed loss of MMR: two consecutive tests, one with *BCR-ABL*^{IS} ≥1%

Baccarani M, et al. Blood in press

Primary reference standards WHO definition

- **Ideally be as close as possible to real samples.**
- **Should cover all steps of the process, including RNA extraction.**
- **Must be stable over several years (=freeze dried) and physically possible to prepare batches that last several years.**
- **Must be applicable to all or most existing methods**

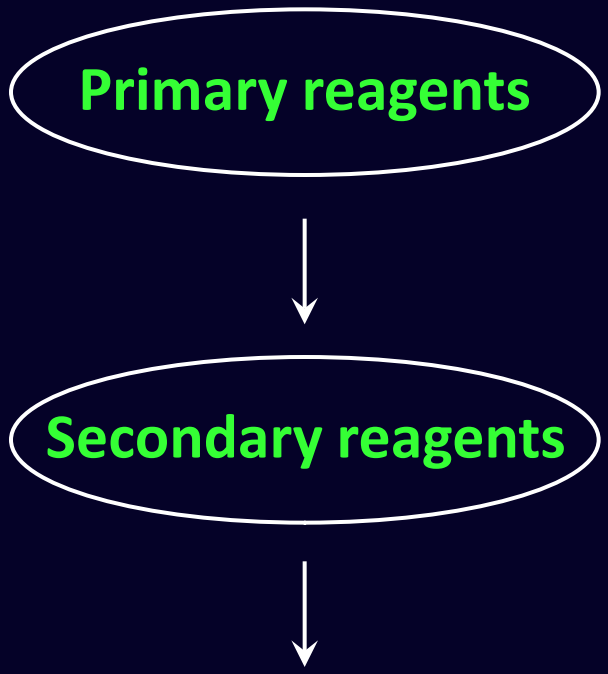
Reference Reagents: full scale production

- Several cell line combinations trialled: selected K562 and HL60
- Large scale grow ups of HL60 (40L)
- Mixtures made with K562 to yield approx 10%, 1%, 0.1%, 0.01% BCR-ABL^{IS}.
- 3000 vials at each dilution freeze dried by NIBSC
- Initial in house evaluations successful
- International field trial
- Assignment of IS values (*ABL*, *BCR*, *GUSB*)
- WHO accreditation

White et al. Blood. 2010 Nov 25;116(22):e111-7

Use of primary reference reagents

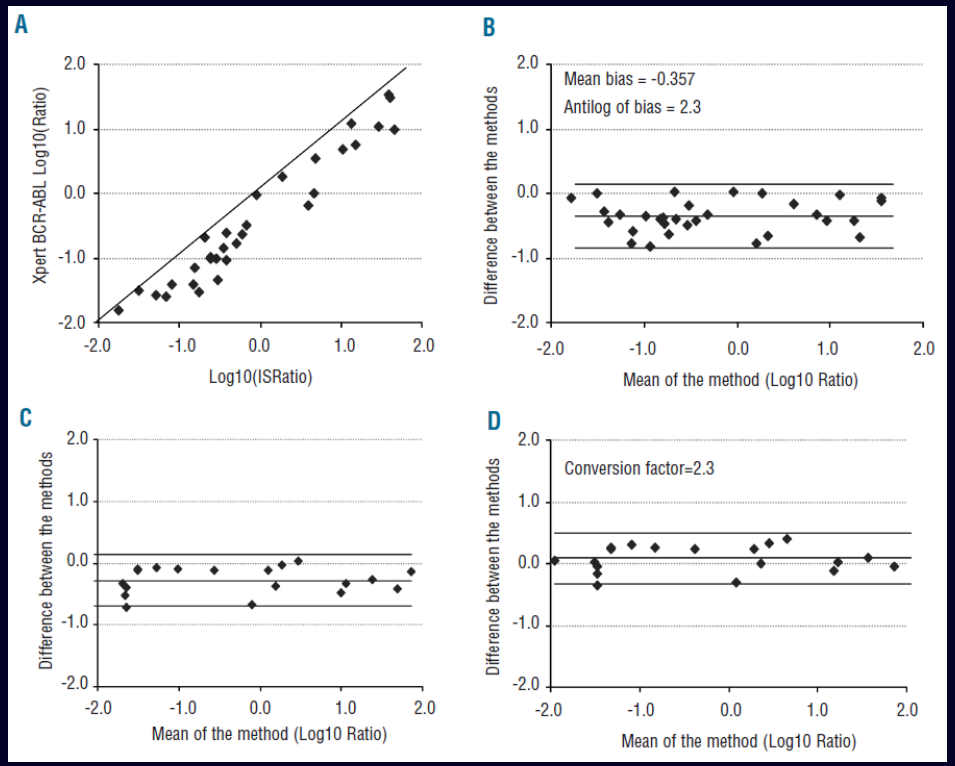
- ??500 labs worldwide
- Median 3 runs/week = 78,000 runs pa
- Four dilutions, each containing 10^6 cells
- Would need $3x10^{11}$ cells (>600 litres culture) pa to satisfy world wide demand for reference reagents to be included in each run



Available to routine testing laboratories

Cepheid GeneXpert

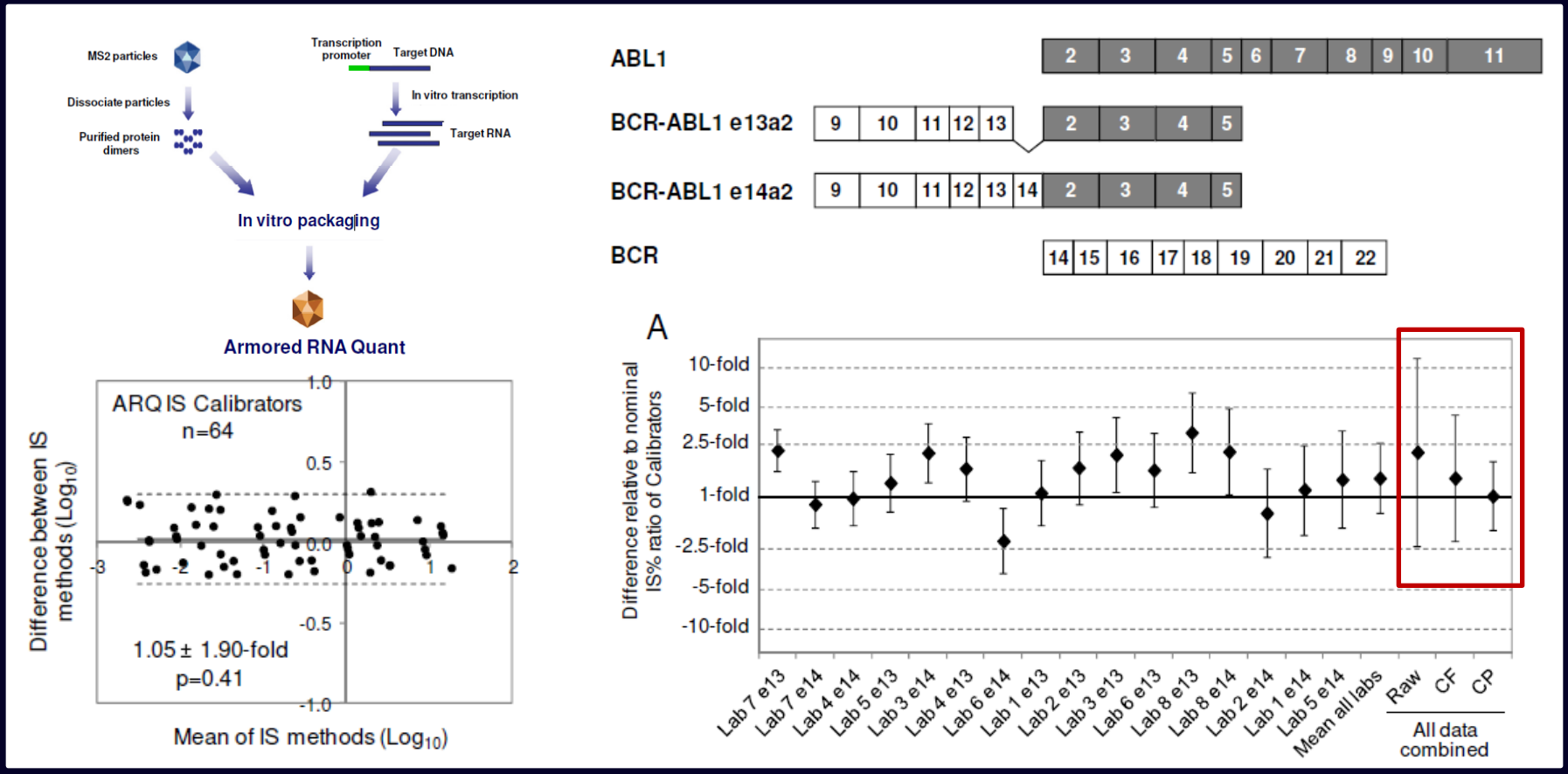
- Cartridge based system with potential for near-patient analysis
- Current version sufficiently sensitive to detect MMR
- More sensitive version in development



Winn-Deen E et al., Clin Chem. 2007 Sep;53(9):1593-600.
Cayuela J et al., Haematologica. 2011 May;96(5):664-71.
McNiven M & Talaulikar D. Pathology. 2012 Jan;44(1):55-7
López-Jorge C et al., Ann Hematol. 2012 Aug;91(8):1245-50.

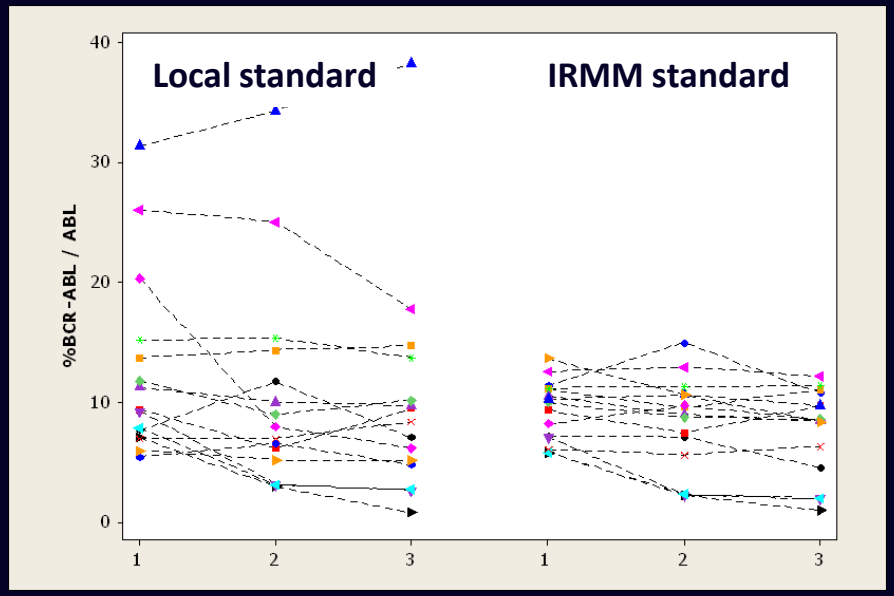
Armored RNA Quant calibrators (Asuragen)

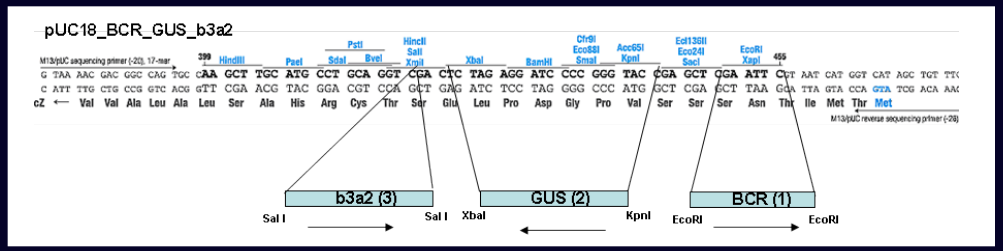
- ARQ reagents calibrated to WHO primary standards
- Used to derive correction parameter to convert results to IS



White H et al., Clin Chem. 2013 Mar 7. [Epub ahead of print].

Improved comparability using the traceable IRMM calibration plasmid





JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements

CERTIFICATE OF ANALYSIS

ERM®-AD623

SET OF PLASMID SOLUTIONS		
	Number of specific DNA fragments per plasmid	
	Certified value [b]	Uncertainty [c]
BCR-ABL b3a2 transcript [a]	1	negligible
BCR transcript [a]	1	negligible
GUSB transcript [a]	1	negligible

a) The sequence identity has been confirmed by dideoxy termination sequencing of the entire plasmid. The estimated error probability of the sequence identification of each fragment is lower than 0.0002 %.
b) The certified value is traceable to the International System of units (SI).
c) The uncertainty of the certified value refers to a standard uncertainty. It is estimated by a type B evaluation based on information provided in section 6 of the corresponding certification report.

	Copy number concentration of the plasmid [d]	
	Certified value [cp/µL]	Uncertainty [g] [cp/µL]
ERM®-AD623a	1.08×10^{6} [e]	0.13×10^{6}
ERM®-AD623b	1.08×10^{5} [f]	0.11×10^{5}
ERM®-AD623c	1.03×10^{4} [f]	0.10×10^{4}
ERM®-AD623d	1.02×10^{3} [f]	0.09×10^{3}
ERM®-AD623e	1.04×10^{2} [f]	0.10×10^{2}
ERM®-AD623f	10.0 [e]	1.5

d) The copy number concentration of the plasmid has been determined by digital PCR using experimental conditions as described in Annex A of the certification report.
e) Unweighted mean value of 17 independent measurements obtained in three different laboratories. The certified value and its uncertainty are traceable to the International System of units (SI).
f) Unweighted mean value of 18 independent measurements obtained in three different laboratories. The certified value and its uncertainty are traceable to the International System of units (SI).
g) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of approximately 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2009.

This certificate is valid for one year after purchase.
Sales date:
The minimum amount of sample to be used is 5 µL.
Accepted as an ERM®, Geel, September 2012

Signed:

Prof. Dr. Hendrik Emons
European Commission, Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111, B-2440 Geel, Belgium

Registration No. 268-RM
ISO Guide 34 for the production of reference materials

All following pages are an integral part of the certificate.
Page 1 of 3

White H and Cross N, unpublished data
https://web.jrc.ec.europa.eu/rmcatalogue/searchrmcatalogue.do

Rationale for standardisation of deeper levels of response

- **Second generation TKIs produce deeper molecular responses compared to imatinib**
- **Can CML be cured?**

- **Complete molecular response (CMR); ELN 2009 definition:**
  - **Undetectable *BCR-ABL* transcripts by qRT-PCR and/or nested PCR in 2 consecutive samples with a sensitivity >10^4**

Kantarjian H, et al. Lancet Oncol. 2011 Sep;12(9):841-51.
Mahon FX, et al. Lancet Oncol 2010; 11: 1029–35.
Baccarani M, et al. J Clin Oncol 2009; 27(35):6041–6051.

Definitions of Complete Molecular Response

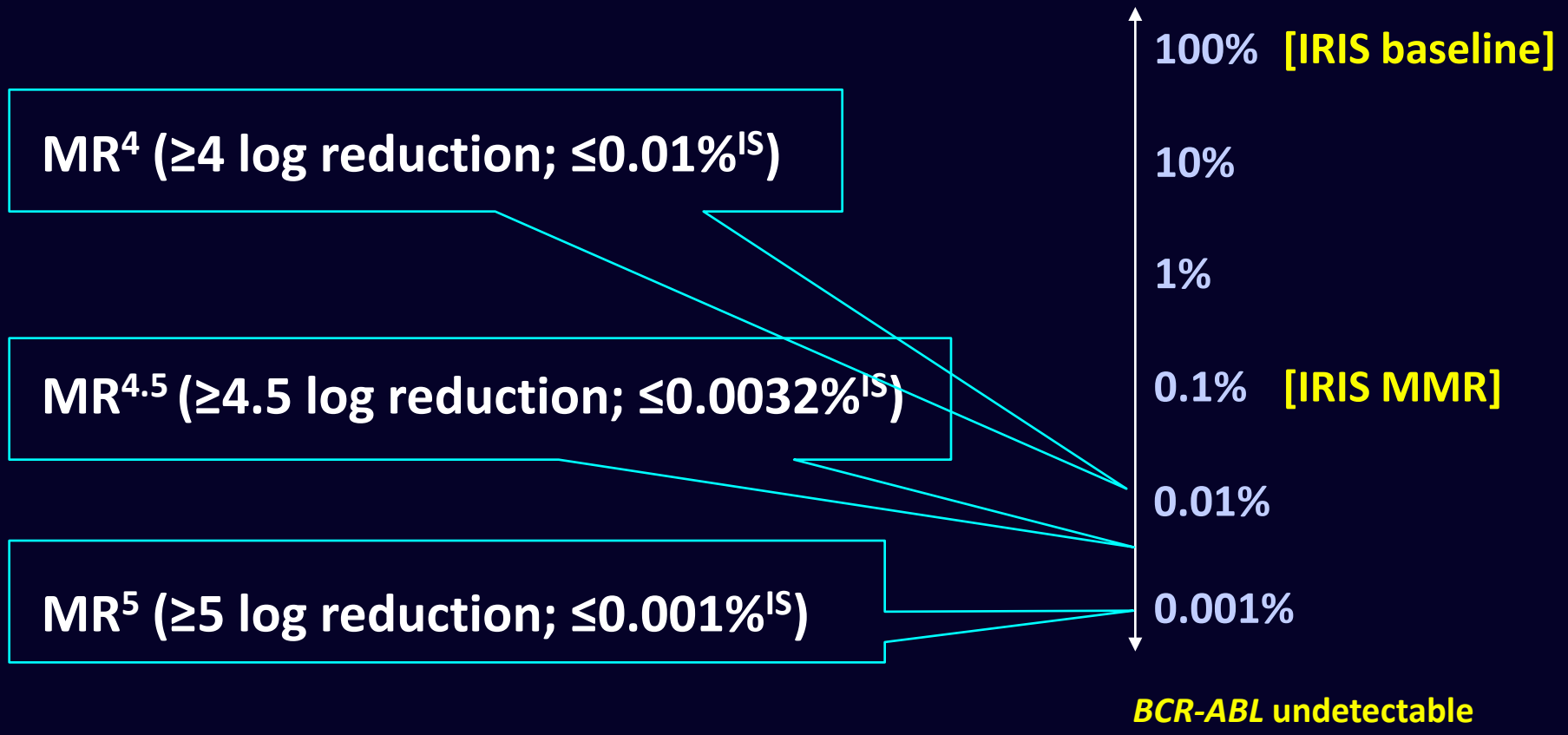
- **Problems with ELN 2009 definition**
  - **How to define sensitivity >10^4 in a reproducible way?**
  - **Sensitivity depends in part on how hard you look**

- **Need a robust definition of CMR that is:**
  - **reproducible between laboratories**
  - **takes into account intrinsic variables**
  - **is able to accommodate future technological improvements**

EUTOS for CML

European Treatment and Outcome Study

Definitions of Deep Molecular Responses



log reduction = reduction from IRIS baseline, not individual pretreatment levels

Cross et al. Leukemia. 2012;26:2172-5

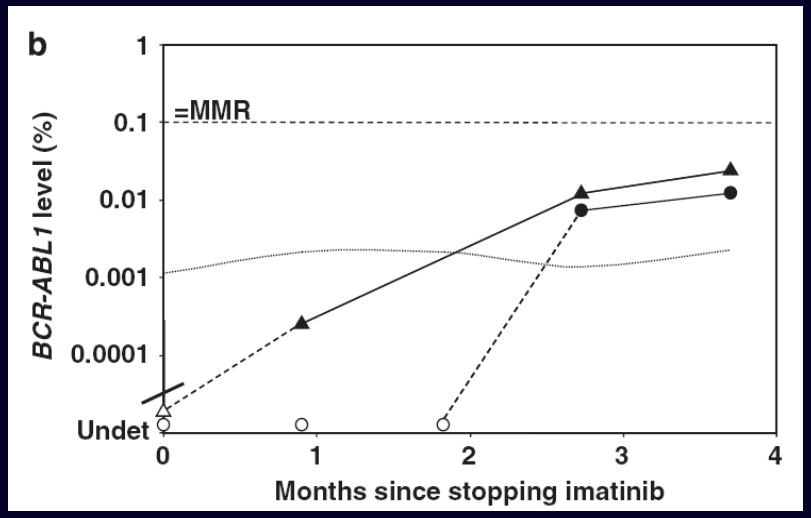
How well do labs perform currently? UKNEQAS data

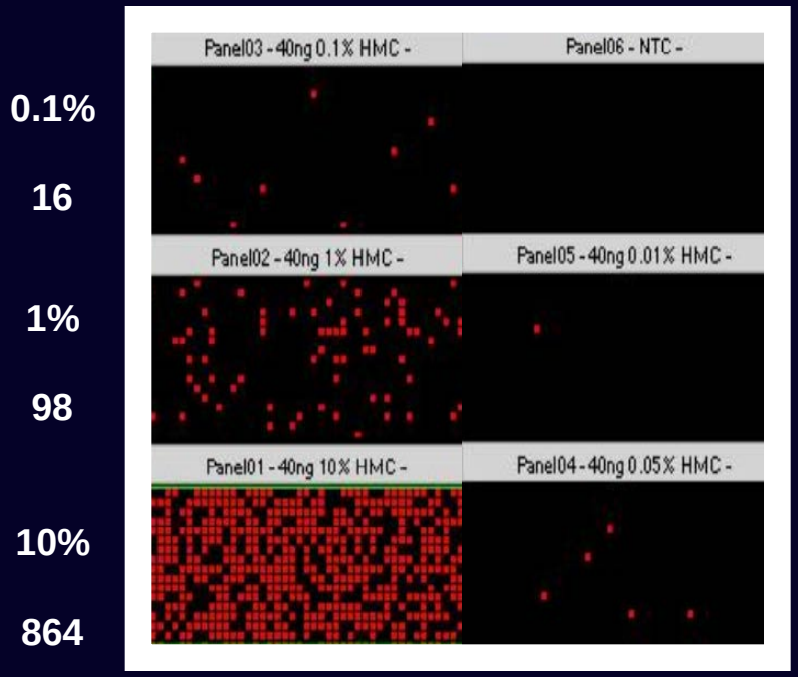
- **Recent round (2012) involved 135 labs worldwide**
- **Two samples with a median 28,000 ABL**
- **15% of labs reported <10,000 *ABL* and therefore would not be able to score MR^4**
- **40% of labs had *ABL* >32,000 and therefore would be able to score $MR^{4.5}$**

UKNEQAS

Alternative approaches? Will new technologies help?

- DNA-based approaches?
- High throughput sequencing?
- RNA-seq?
- Digital PCR?





Ross, et al. Leukemia. 2010;24(10):1719-24.
Sobrinho-Simões et al. Blood. 2010;116(8):1329-35.

Acknowledgements

Salisbury

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Andreas Hochhaus, Martin Müller

National Institute for Biological Standards & Control

Paul Metcalfe, Paul Matejtschuk

Hammersmith

John Goldman, Letizia Foroni, Jaspal Kaeda

International BCR-ABL Standardisation Group

Challenges for laboratories and clinicians

- **Testing laboratories need to:**
  - **Engage with the international standardisation process**
  - **Implement the International Scale**
  - **Minimise test variability**
  - **Maximise test sensitivity to enable detection of $MR^{4.5}$**
  - **Report results in a meaningful way**

- **Clinicians need to:**
  - **Understand the limitations of molecular monitoring**
  - **Appreciate that sensitivity is variable between samples**
  - **Understand that apparent fluctuations on sequential analysis at low disease levels in some patients is inevitable**