Monitoring CML patients by quantitative real time PCR on the International Scale

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University of Southampton, UK
Methods to specifically detect CML cells

- BCR-ABL protein
- BCR rearrangement
- BCR-ABL mRNA
- BCR/ABL juxtaposition
# Routine methods to specifically detect CML cells

<table>
<thead>
<tr>
<th>Method</th>
<th>Target</th>
<th>Tissue</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytogenetics</td>
<td>Ph-chromosome</td>
<td>BM</td>
<td>1 - 10</td>
</tr>
<tr>
<td>FISH</td>
<td>Juxtaposition of $BCR &amp; ABL$</td>
<td>PB/BM</td>
<td>0.2 - 5</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>$BCR-ABL$ mRNA</td>
<td>PB/BM</td>
<td>Up to 0.001</td>
</tr>
</tbody>
</table>
Assessment of residual disease

- Leukocytosis
- Ph-chromosome pos
- RQ-PCR positive
- RQ-PCR negative

Number of leukemia cells (log\(10\))

Decreasing residual leukemia
Why is it important to monitor molecular response to BCR-ABL inhibitors?

• The degree to which the bulk of disease is reduced is an important prognostic indicator

• Poor primary response or relapse after initial response both suggest the need to change therapy

• Molecular monitoring by RQ-PCR provides:
  – A deeper assessment of response
  – The means to detect relapse earlier than other methods

**RQ-PCR: strengths and weaknesses**

- **Strengths**
  - Only technique that can gauge molecular therapeutic milestones: major molecular response (MMR) and complete molecular response (CMR)
  - Routinely performed on peripheral blood

- **Weaknesses**
  - Technically challenging
  - Issues concerning comparability of results between centres
  - Several different methods / units of measurement
• History of molecular monitoring in CML

• The International Scale for BCR-ABL

• Ongoing efforts to standardize BCR-ABL testing
**BCR-ABL transcripts in CML**

**p210 BCR-ABL**
(98% of CML; 30-50% Ph-positive ALL)

**p190 BCR-ABL**
(>1% of CML; 50-70% Ph-positive ALL)
Minor BCR-ABL mRNA variants in CML detected by multiplex RT-PCR

- 98%: e13a2 or e14a2 (~10% express both e13a2 and e14a2)
- 1%: e13a3 or e14a3
- 1%: e1a2, e6a2, e8a2, e19a2, others

Cross et al. Leukemia 1994;8(1):186-9
RT-PCR for BCR-ABL in the 1980s and 1990s

- Diagnosis of CML and Ph-positive ALL
- Early detection of relapse after allogeneic bone marrow transplant.
- Response of patients treated for relapse by donor lymphocyte infusion (DLI).
- Evaluation of leukaemia cell contamination in stem cell collections.
- Prognostic significance in complete cytogenetic responders to IFN-α.
Early uses of RT-PCR to detect BCR-ABL mRNA

Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro
(Philadelphia chromosome/BCR-ABL/t(9;22) chromosomal translocation/polymerase chain reaction)

ERNEST S. KAWASAKI‡*, STEVEN S. CLARK‡, MAZIE Y. COYNE*, STEPHEN D. SMITH‡, RICHARD CHAMPLIN‡,
OWEN N. WITTE‡, and FRANK P. MCCORMICK‡

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Detection of Minimal Residual bcr/abl Transcripts by a Modified Polymerase Chain Reaction

By Ming-Sheng Lee, Kun-Sang Chang, Emil J. Freireich, Hagop M. Kantarjian, Moshe Talpaz, Jose M. Trujillo, and Sanford A. Stass

Blood, Vol 72, No 3 (September), 1988; pp 893-897

CONCISE REPORT
Detection of the Molecular Abnormality in Chronic Myeloid Leukemia by Use of the Polymerase Chain Reaction

By Alexander Dobrovic, Kevin J. Trainor, and Alexander A. Morley

Blood, Vol 72, No 6 (December), 1988; pp 2003-2005

Molecular relapse in chronic myelogenous leukemia patients after bone marrow transplantation detected by polymerase chain reaction
(BCR-ABL/Philadelphia chromosome)

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*Department of Medicine, Division of Hematology-Oncology, University of California at Los Angeles Center for the Health Sciences, and the Jonsson Comprehensive Cancer Center, Los Angeles, CA 90024; ‡Howard Hughes Medical Institute, Department of Microbiology and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024; and †Department of Molecular Biology, Cetus Corporation, Emeryville, CA 94608
Detection of BCR-ABL mRNA by nested RT-PCR

[Diagram showing the steps of nested RT-PCR for BCR and ABL genes, with bands for b3a2, b2a2, BCR, ABL, and ABL control]
Qualitative nested RT-PCR results after BMT

• Seattle:
  – BCR-ABL positivity 6-12 months post BMT is an independent predictor of subsequent relapse.
  – BCR-ABL positivity >12 months less predictive of relapse

• Tokyo/Nagoya:
  – BCR-ABL positivity is frequent and does not correlate with relapse.

• Hammersmith:
  – BCR-ABL positivity common up to 6-9 months post BMT and does not correlate with subsequent relapse.
  – BCR-ABL positivity uncommon >12 months but weakly correlated with subsequent relapse.
Competitive RT-PCR to quantify BCR-ABL mRNA

Competitive RT-PCR to quantify BCR-ABL mRNA

Patterns of remission post BMT

- **Cytogenetic Relapse**
- **Haematologic Relapse**
- **PCR positive**
- **PCR negative**

**BCR-ABL/ABL ratio (%)**

Months post BMT:
- 0
- 6
- 12
- 18
- 24
Patterns of relapse post BMT

**Haematologic Relapse**

**Cytogenetic Relapse**

**PCR positive**

**PCR negative**

<table>
<thead>
<tr>
<th>Months post BMT</th>
<th>PCR negative</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td>6</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td>12</td>
<td>28/30</td>
<td>0/30</td>
</tr>
<tr>
<td>18</td>
<td>5/30</td>
<td>0/30</td>
</tr>
<tr>
<td>24</td>
<td>12/30</td>
<td></td>
</tr>
</tbody>
</table>

**BCR-ABL/ABL ratio (%)**

- 0.0001
- 0.001
- 0.01
- 0.1

*Patterns of relapse post BMT*
Sequential quantitative PCR and subsequent relapse post BMT

High or rising BCR-ABL levels (n = 33)
≥ 0.02% BCR-ABL/ABL
(≥ 100 BCR-ABL/μg RNA)

Low or falling BCR-ABL levels (n = 80)
< 0.02% BCR-ABL/ABL
(≥ 100 BCR-ABL/μg RNA)

n = 113
p < 0.0001
Quantitative PCR results according to cytogenetic response status

Complete cytogenetic responders on IFN-therapy: Relapse free survival according to residual BCR-ABL transcripts

![Graph showing relapse free survival over time for two groups with different BCR-ABL/ABL ratios.](image)

- Ratio BCR-ABL/ABL > 0.045%, n=27
- Ratio BCR-ABL/ABL ≤ 0.045%, n=27

**p < 0.0001**
Relapse following withdrawal of IFN during CCR

Patient JS

BCR-ABL/ABL (%)

Years after CCR

Off IFN

Cy relapse

median CCR

0.001

0.01

0.1

0

0.01

0.1

1

10

100

0

1

2

3

4

5

6
Sustained remission following withdrawal of IFN during CCR

Patient AP

BCR-ABL/ABL (%) vs Years after CCR

Off IFN

Cy relapse

median CCR
Detection of BCR-ABL transcripts by RT-PCR

- Diagnosis / Relapse
- Partial remission
- Conventional
  - RT-PCR positive
  - RT-PCR negative
- IFN
  - Healthy subjects
  - 'Optimized'
    - RT-PCR positive
    - RT-PCR negative
- Conventional
  - RT-PCR positive
  - RT-PCR negative

Sensitivity of PCR:
- $10^{-2}$
- $10^{-6}$
- $10^{-8}$
1996: imatinib and RQ-PCR

**ARTICLES**

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

Brian J. Druker, Shiu Tamura, Elisabeth Buchdunger, Sayuri Ohno, Gerald M. Segal, Shane Fanning, Jürg Zimmermann & Nicholas B. Lydon

Nat Med. 1996;2:561-6

**GENOME METHODS**

**Real Time Quantitative PCR**

Christian A. Heid, Junko Stevens, Kenneth J. Livak, and P. Mickey Williams

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

Genome Res. 1996;6: 986-994
Peripheral blood

Extract RNA from total leukocytes

Reverse transcribe to cDNA

RQ-PCR for BCR-ABL

RQ-PCR for control gene

Positive specimens: Ratio of BCR-ABL to control gene

Negative specimens: No. of control gene transcripts as indicator of sensitivity

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 regardless of their proliferative status
  – unaffected by therapeutic regimens
  – invariant between individuals
  – expressed a level similar to BCR-ABL mRNA
  – Subject to degradation at the same rate as BCR-ABL mRNA
What is the best internal control gene?

- EAC: recommended ABL, GUSB or β2M

- CML expert group: recommended ABL, GUSB and BCR
IRIS Trial: Standardization by normalisation of results to 30 Shared Baseline 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 Keeda, 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., Iris C. van Hoomissen, B.Sc., N., John M. Goldman, D.M., and Gerald P. Radich, M.D. for the International Randomised Study of Interferon versus STIC71 (IRIS) Study Group


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**Before normalization**

**After normalization**
The International Scale (IS) for BCR-ABL

**LOCAL ASSAY**

- BCR-ABL/ABL
- BCR-ABL/BCR
- BCR-ABL/GUS
  (other control genes?)

Different primers/probes

- TaqMan
- LightCycler
- Corbett
- Others

**Conversion factor/Reference samples**

**International Scale**

- 100% [IRIS baseline]
- 10%
- 1%
- 0.1% [IRIS MMR; 3 log reduction]
- 0.01%
- 0.001%

Standardisation of molecular monitoring in CML: Background

- Historical (IRIS trial; 2000): The mean BCR-ABL levels of 30 CML patients was defined as 100% in each of the three participating laboratories using BCR as a control gene\(^1\)

- The value corresponding to MMR in each laboratory was defined as 0.1% (reduction of 3 log from IRIS baseline)

- International Scale (IS) fixed to these key points

Realising the International Scale for BCR-ABL

- Derivation of laboratory specific conversion factors
  - Methodology developed by Adelaide laboratory
  - Expanded in Europe by European LeukemiaNet / EUTOS

- Development of accredited reference reagents

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?

The International Scale for BCR-ABL:
Before standardisation

Hypothetical schematic.
The International Scale for BCR-ABL: After standardisation

BCR-ABL level

CML cell dilution

Hypothetical schematic.
RQ-PCR results at low levels are inevitably more variable than those at high levels.

Conversion factors: current status

- Works well (for many labs), but very labour intensive
- Open to a limited number of labs at any time
- 300-500 labs testing for BCR-ABL by RQ-PCR worldwide?
- Unclear how often conversion factors need to be calculated
- Process has highlighted problem of assay instability in some testing labs.
Internal Quality Assurance: High / low BCR-ABL standards – Adelaide model

Quality Control Chart

Low control
Mean: 0.072%
1st: 0.018
Range: 0.036-0.108

High control
Mean: 89%
1st: 10
Range: 65-105

Value = 0.19
>3sd
reject run

Control Result

>2SD
No

Yes

>3SD

No

2 results
>2SD

Yes

Reject Run

Accept Run


Improved comparability using a common calibration plasmid

- Problem: no universally accepted reference standards / calibrators

White and Cross, unpublished
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
Formulation for primary reagents

- [CML cells (primary or K562) diluted in normal leucocytes]
- Cell line mixtures

- K562 is fine for BCR-ABL (b3a2)
- Non BCR-ABL: 30 lines tested: KG1 and HL60 had ratios of ABL:BCR:GUSB closest to that seen in normal leukocytes
  - ??500 labs worldwide
  - Median 3 runs/week = 78,000 runs pa
  - Four dilutions, each containing $10^6$ cells
  - Would need $3 \times 10^{11}$ cells (>600 litres culture volume) per annum to satisfy worldwide demand for reference reagents to be included in each run
Likely use of primary reference reagents

Available to routine testing laboratories
Reference Reagents: full scale production

- Both freeze dried cells combinations performed well in international field trials
- Large scale grow ups of HL60 (40L)
- Mixtures made with K562 to yield approx 10%, 1%, 0.1%, 0.01% BCR-ABL\textsuperscript{IS}.
- 3000 vials at each dilution freeze dried by NIBSC
- Initial in house evaluations successful
- International field trial
- Assignment of IS values (\textit{ABL, BCR, GUSB})

Primary reference materials: stability studies

- Reagents available from [www.nibsc.ac.uk](http://www.nibsc.ac.uk) but limited to companies/labs that want to produce and calibrate secondary reference reagents for distribution or sale

What is Armored RNA Quant® (ARQ)?

- A patented and proven technology to manufacture and stabilize RNA for use as:
  - Positive/Negative control
  - Process control (e.g., spike in non-target RNA)
  - Assay calibrator (e.g., standard curves)
  - External calibrator (e.g., proficiency panel)
- Nuclease resistant and stable in biological matrices*
- Precisely quantified using a NIST-traceable reference standard
- Performance well established:
  - In clinical molecular virology applications (HIV, HCV…)
  - And now in clinical hematology applications
- Manufactured under cGMP to ensure lot-to-lot consistency critical for reagents used in clinical molecular analyses

*Pasloske et al., J Clin Microbiol 1998
ARQ IS Calibrator Panel™ Design

- Two panels, one for e13a2 and one for e14a2, designed to be compatible with most of the common RT-qPCR methods using ABL1 or BCR as endogenous controls
- Each panel consists of 4-level Calibrators (like the WHO primary standard) and one negative control
- Each Calibrator has an assigned nominal IS % ratio determined relatively to the reference values of the WHO primary standard as described in White et al., Blood 2010
- Testing with local RT-qPCR methods and comparison to the nominal IS values can be performed either directly (heat-lysis) or after RNA extraction*

*For research use only. Not for use in diagnostic procedures.
Example of Results – Linearity

- Similar slopes and R² with or without RNA extraction*
- Good linearity over 3-log dilution for both control genes*

<table>
<thead>
<tr>
<th></th>
<th>ABL1</th>
<th>BCR</th>
<th>Avg</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heat-lysis</td>
<td>QIAamp</td>
<td>TRlzol</td>
<td>Heat-lysis</td>
</tr>
<tr>
<td>e13a2-1</td>
<td>6.6</td>
<td>7.1</td>
<td>5.9</td>
<td>9.4</td>
</tr>
<tr>
<td>e13a2-2</td>
<td>0.62</td>
<td>0.66</td>
<td>0.63</td>
<td>0.98</td>
</tr>
<tr>
<td>e13a2-3</td>
<td>0.066</td>
<td>0.079</td>
<td>0.054</td>
<td>0.115</td>
</tr>
<tr>
<td>e13a2-4</td>
<td>0.0081</td>
<td>0.0093</td>
<td>0.0077</td>
<td>0.0135</td>
</tr>
</tbody>
</table>

*Preliminary research data. The performance characteristics of these reagents have not been established.
Evaluation of ARQ IS Calibrator Panel™

- Third international field trial ongoing in 10 IS-standardized labs
- The goal is to compare the ARQ IS Calibrator Panel nominal IS % ratios (anchored to the mean reference values of the WHO primary standard) to IS % ratios measured by local RT-qPCR methods
- To date, slopes and $R^2$ are similar and all results are within ±5-fold confirming alignment of the ARQ IS Calibrator Panels to IS-standardized methods with similar scale (measure of log change) and classification accuracy (MMR)

Dash lines represent ±5 fold from nominal values
Results at WNGRL

**e13a2 Panel**

- Calibrator lot 1
- February 2011

- Dash lines represent ±2.5 fold from nominal values

**e14a2 Panel**

- Calibrator lot 2
- August 2011

- Dash lines represent ±2.5 fold from nominal values
Potential uses of calibrated secondary reference reagents

• Direct calibration of results to the International Scale

• Quality Control: definition of assay variation / drift

• Troubleshooting

• Standardization of ‘Complete Molecular Response’
Should Complete Molecular Response be considered as Optimal Molecular Response?

- CMR generally understood to mean undetectable disease
  - Depends how hard you look
  - Variability of sample quality within centres
  - Variability of assay sensitivity between centres

- Need a robust definition of CMR that is reproducible between laboratories, takes into account intrinsic variability and is able to accommodate future technological improvements
Definitions of Complete Molecular Response

CMR\(^{4.0}\) (≥4 log reduction; ≤0.01%\(^{IS}\))

CMR\(^{4.5}\) (≥4.5 log reduction; ≤0.0032%\(^{IS}\))

CMR\(^{5.0}\) (≥5 log reduction; ≤0.001%\(^{IS}\))

log reduction = reduction from IRIS baseline, not individual pretreatment levels
Definitions of Molecular Response

- **MR^{4.0} (≥4 log reduction; ≤0.01\%^{IS})**
- **MR^{4.5} (≥4.5 log reduction; ≤0.0032\%^{IS})**
- **MR^{5.0} (≥5 log reduction; ≤0.001\%^{IS})**

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

**International Scale**

BCR-ABL undetectable

- 100% [IRIS baseline]
- 10%
- 1%
- 0.1% [IRIS MMR]
- 0.01%
- 0.001%
**Working definition of MR**

- **MR\(^4.0\)** = either (i) detectable disease ≤0.01% BCR-ABL\(^{IS}\) or (ii) undetectable disease in cDNA with ≥10,000 ABL transcripts*

- **MR\(^4.5\)** = either (i) detectable disease ≤0.0032% BCR-ABL\(^{IS}\) or (ii) undetectable disease with in cDNA with ≥32,000 ABL transcripts

- Need to define equivalent transcript numbers for other controls genes, e.g. *GUSB, BCR*

* i.e. no of ABL transcripts in the same volume of cDNA used to detect BCR-ABL
Can the sensitivity of PCR for BCR-ABL be pushed further?

- PCR from genomic DNA enables more sensitive detection of MRD, e.g. MR\textsuperscript{5.0}
- Expensive: breakpoints need to be identified; patient-specific primer/probe pairs need to be designed and tested
Can the sensitivity of PCR for BCR-ABL be pushed further?

Can the sensitivity of PCR for BCR-ABL be pushed further?

Patient 1: stopped imatinib, remained in stable CMR for 2 years but persistently DNA PCR positive

Patient 2: stopped imatinib, rapidly became DNA PCR positive, then RT-PCR positive

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International BCR-ABL Standardization Group