Standardization of RQ-PCR for BCR-ABL

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IRIS trial: variation in the three reference centres
IRIS trial: results following normalisation to 30 shared baseline samples
Realising the international scale for
BCR-ABL RQ-PCR

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

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International standardisation of BCR-ABL measurements

- BCR-ABL/ABL
- BCR-ABL/BCR
- BCR-ABL/GUS
- (other control genes?)
- Different primers/probes
- TaqMan
- LightCycler
- Corbett
- Others

Reference samples: lab-specific conversion factor

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

cf. International Normalized Ratio (INR) for Prothrombin time
The Adelaide reference lab has maintained consistency of data since the MMR value was established for the IRIS trial in 2001 - BCR-ABL/BCR 0.08%

Yearly quality control mean values

<table>
<thead>
<tr>
<th>Control</th>
<th>Target mean</th>
<th>2001 (from July)</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006 (to March)</th>
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<tbody>
<tr>
<td>Low b3a2</td>
<td>0.07</td>
<td>0.07</td>
<td>0.06</td>
<td>0.08</td>
<td>0.06</td>
<td>0.08</td>
<td>0.09</td>
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<tr>
<td>High b3a2</td>
<td>85</td>
<td>82</td>
<td>77</td>
<td>93</td>
<td>69</td>
<td>94</td>
<td>93</td>
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<tr>
<td>Low b2a2</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.09</td>
<td>0.08</td>
<td>0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>High b2a2</td>
<td>56</td>
<td>48</td>
<td>53</td>
<td>69</td>
<td>50</td>
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<td>111</td>
<td>147</td>
<td>156</td>
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<td>34</td>
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</table>
International standardisation of BCR-ABL measurements

BCR-ABL level vs CML cell dilution graph.
International standardisation of BCR-ABL measurements

BCR-ABL level

CML cell dilution
International standardisation of BCR-ABL measurements

BCR-ABL level vs. CML cell dilution

- Log scale for both axes
Conversion factors: current status

International reference labs
Adelaide, Mannheim

EQA schemes
**Derivation of conversion factors: current status**

- **Sample exchange:**
  - Either sends 20+ samples to each test lab; K562 or primary CML cells in normal leucocytes
  - Or receives 20+ patient samples from test lab

- **Derivation of conversion factor:**
  - Samples analysed in both centres; different operators, different days
  - conversion factor calculated from Bland-Altman bias plots

- **Validation:**
  - Samples (20-30) from test lab covering minimum 3 log range sent back to reference lab for analysis and results compared.
Lab 5 BCR-ABL/ABL\% \times 0.23 \text{ to convert to the international scale} \newline

The CF is the antilog of the Bias = 0.23 

Branford & Hughes
Validation of Conversion Factor

Identity line $X=Y$

$n=16$

$n=33$

Converted data

Branford & Hughes
### Concordance between Ref Lab and Lab 5

<table>
<thead>
<tr>
<th></th>
<th>Average fold bias</th>
<th>2-fold</th>
<th>5-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before conversion</strong></td>
<td>-4.3</td>
<td>15%</td>
<td>52%</td>
</tr>
<tr>
<td><strong>After conversion</strong></td>
<td>+1.2</td>
<td>61%</td>
<td>97%</td>
</tr>
</tbody>
</table>
What is achievable?

MMR: 83% concordance

Branford & Hughes
Conversion factors: current status

• Works well (for many labs), but very labour intensive
• Open to a limited number of labs at any time

• EUTOS programme: Europe including UK & Ireland
• Adelaide: Australasia/Asia
• USA: enthusiasm but not much action
Conversion factors: future status

International reference labs
↓
National or regional reference labs
↓
Primary reference standards
↓
Secondary reference standards

Lab 1  Lab 2  Lab 3  Lab 4  Lab 5  Lab 6  ..................  Lab n

EQA schemes
Primary reference standards

- Ideally be as close as possible to real samples.
- Must be stable over several years (=freeze dried) and batches prepared that last several years.
- Must cover all or most existing methods (including RNA extraction).
- Prepared centrally and WHO accredited by NIBSC (but other routes possible).

- Depending on formulation may be very limited in supply
- Used by reference labs or companies to calibrate secondary reference standards
Secondary reference standards

• Easily available: used by testing labs on every run? Every week?

• Prepared locally/nationally? Or by companies?
  – Asuragen, Molecular MD, (Ipsogen)

• Calibrated to primary reference reagents

• Similar to real samples: function to monitor efficiency of RNA extraction and/or RT and assay drift but may also provide conversion factor
Formulation for primary and/or secondary reagents

- [CML cells (primary or K562) diluted in normal leucocytes]
- Cell line mixtures
- Armored RNAs
Cell lines: current status

Aim to find cell line or cell line mixture for which the ratio of BCR:ABL:GUS in the same as median of normal leucocytes

If successful, dilution standards will define points on IS independent of control gene used (BCR, GUS or ABL)

10%

1%

0.1% [MMR; 3 log reduction]

0.01%

0.001%

International scale
What happens if we cannot find a line in which \textbf{BCR:ABL:GUS} is spot on?

Eg. if BCR:ABL:GUS in normal leucocytes is 1:2:4
cell line has a ratio 1:0.5:2

\begin{itemize}
  \item 2\% (BCR)
  \item 8\% (ABL)
  \item 4\% (GUS)
  \item 0.45\% (BCR)
  \item 1.8\% (ABL)
  \item 0.9\% (GUS)
  \item 0.0015\% (BCR)
  \item 0.006\% (ABL)
  \item 0.003\% (GUS)
\end{itemize}

International scale
Evaluation of cell lines

- K562 is fine for BCR-ABL
- Non BCR-ABL: Control genes (BCR, ABL and GUS) need to be expressed at levels comparable to normal leukocytes: KG1 and HL60 (at least the subclones we have tested).

- Pilot batch of freeze dried samples:
  - Cells grown and mixtures made in Salisbury
  - 4 dilutions of K562 in both KG1 and HL60; 10%-0.01%; 3x10^6 cells/vial (10^9 cells total)
  - Freeze dried at NIBSC (April 2007)
  - Initial tests at Salisbury, Mannheim and Marseilles
  - Full performance evaluation involving 14 labs worldwide July-October 2007
**Field Trial Protocol**

- Freeze Dried Cells sent to 14 labs (4 control genes; 7 protocols; 9 platforms)
- Each lab sent 24 vials packaged into 3 batches
- Each batch contained 8 vials: HL60/K562 Levels 1-4, KG1/K562 Levels 1-4
- Cells resuspended directly into 1ml Trizol (Invitrogen) or 600µl RLT Buffer (QIAGEN)
- Usual lab protocol for cDNA synthesis and RQ-PCR:
Freeze dried cells: yields of RNA

Median 30ug RNA/vial

Stability studies ongoing
Stability of freeze dried cells

**HL60/K562**

**KG1/K562**
**Trial summary**

- Majority of labs obtained linear results with expected slope
- CVs were comparable to those described in other trials with patient samples
- Both HL60 and KG1 performed well
Next steps

• Current trial: report to be circulated to participants for comments, then published/circulated more widely

• Selection of cell line combination and levels (HL60/K562?)
• Large scale grow ups and freeze drying
• Performance evaluation and certification

• $1.5 \times 10^6$ cells/vial
• 4 levels: Spanning range 10% - 0.01%
• Values assigned to dilutions by reference labs
How will vials be used?

• Vials should not be simply be available for any testing lab on demand: risk of depleting stock too quickly

• Essential to promote production and widespread availability of secondary reference reagents
Armored RNAs

- Easily available in large quantities
- Stable
- Good track record for calibration of RNA virus detection assays
- Easy to adjust BCR:ABL:GUS ratio
- Flexible: can use directly for reverse transcription after heat lysis, put through RNA extraction or negative sample spike-ins.
Armored RNAs: current status

- Survey of primer sets performed July 2006
- Plasmids made (BCR, ABL, GUS, b2a2, b3a2) that cover the regions targeted by all members of the international group
- Sequence verified; sent (essentially gifted) to Asuragen Nov 2006
- Armored RNAs arrived in Salisbury May 2007
- Copy numbers estimated by NIST-traceable phosphate assay (except ABL)
  - Due to lack of sufficient ABL product yield, target was quantified through a standard OD260 conversion used for estimating copy numbers for Armored RNA non-quantitative products.
- Preliminary in house evaluation successful
- International evaluation round
  October – November 2007
Initial stability tests
Armored RNA Field Trial

Evaluation round

- aRNA mixes prepared in Salisbury

- 4 levels of BCR-ABL (in background of BCR+ ABL+ GUS) armored RNA mixtures tested before and after ‘RNA’ extraction protocol

- Samples sent at ambient temperature

- data return by 30th Nov 2007

aRNA sent to 29 labs (19 returned data so far: 18 ABL; 4 BCR; 11 GUS)

22 European: UK (5), France (4), Italy (3), Czech Republic (2), Germany (2), Spain (2), Austria (1), Finland (1), Greece (1), Sweden (1)

USA (3), Australia (1), Korea (1), Singapore (1), Japan (1)
aRNA Field Trial Protocol

**Batch 1**
- aRNA Samples (n=5 or 9)
  - Extract aRNA from Trizol (n=5 or 9)
  - No extraction (Heat 80°C 3min) (n=5 or 9)
- cDNA (1) Perform cDNA reactions at same time with same reagents and analyse in same RQ PCR run
- cDNA (2) Perform cDNA reactions at same time with same reagents and analyse in same RQ PCR run
- RQ PCR

**Batch 2**
- aRNA Samples (n=5 or 9)
  - Extract aRNA from Trizol (n=5 or 9)
  - No extraction (Heat 80°C 3min) (n=5 or 9)
- cDNA (1)
- cDNA (2) Perform cDNA reactions at same time with same reagents and analyse in same RQ PCR run
- RQ PCR

**Batch 3**
- aRNA Samples (n=5 or 9)
  - Extract aRNA from Trizol (n=5 or 9)
  - No extraction (Heat 80°C 3min) (n=5 or 9)
- cDNA (1)
- cDNA (2) Perform cDNA reactions at same time with same reagents and analyse in same RQ PCR run
- RQ PCR
**aRNA results: BCR & GUS absolute copy numbers**

<table>
<thead>
<tr>
<th>Expected</th>
<th>1</th>
<th>8</th>
<th>13</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR Unextracted (Heat lysed)</td>
<td>1.E+00</td>
<td>1.E+00</td>
<td>1.E+00</td>
<td>1.E+00</td>
</tr>
<tr>
<td>BCR Extracted</td>
<td>1.E-01</td>
<td>1.E+00</td>
<td>1.E+00</td>
<td>1.E+00</td>
</tr>
</tbody>
</table>

- Expected = $3 \times 10^4$
- Median unextracted = $1.6 \times 10^4$
- Median extracted = $4.5 \times 10^2$
- n=4

<table>
<thead>
<tr>
<th>Expected</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>12</th>
<th>13</th>
<th>15</th>
<th>16</th>
<th>19</th>
</tr>
</thead>
</table>

- Expected = $3 \times 10^4$
- Median unextracted = $7.8 \times 10^3$
- Median extracted = $3.4 \times 10^2$
- n=10
**aRNA results: ABL absolute copy number (corrected for protocol differences)**

Expected = 3x10^4; Median unextracted = 1.60x10^5 ; median extracted = 8.5x10^3 ; n=18

Median ABL target values as formulated based on OD260 conversion and not NIST-traceable phosphate assay show an approximately 1 log difference from the expected value.
aRNA losses during extraction: trizol vs Qiagen
Summary

- aRNAs survived ambient temperature transportation
- aRNAs worked very well when heat lysed and directly converted to cDNA
- Variable losses on RNA extraction (particularly Trizol)
- Comparability between normalised values was good

Next steps
- Need to explore addition of carrier to improve Trizol extractions
- Need to send out more concentrated aRNAs
- Further trial?
  - Adjustment of BCR/GUS/ABL ratios to those seen in leucocytes
  - Provide test samples (and plasmid dilutions)
  - Test ability of aRNAs to provide comparable results for test samples
  - Test utility of spiking BCR-ABL RNAs into normal leucocytes
Acknowledgements

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  - Tim Hughes, Sue Branford
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- NIBSC
- Asuragen
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