Design and validation of a robust diagnostic assay (prv-1 gene) based on real-time RT-PCR

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Potential conflict of interest:

world-wide exclusive license of the prv-1 test
Basic requirements for assays in clinical routine

1. refundable
2. robust
3. legal considerations:
   harmonised standards of in vitro diagnostics need to be observed (e.g. ISO standards)
Clinical background
Polycythemia vera rubra

- Uncontrolled proliferation: precursors of erythrocytes, (thrombocytes, leucocytes)

- Classified as myeloproliferative syndrome (MPS, leukemia like disorder)
Primary diagnosis

Increased hematocrit

increased red blood cell counts,
blood hyperviscosity,
abnormal platelets
Reasons for increased hematocrit

- Relative polycythemia (e.g. dehydration)
- Secondary polycythemia (e.g. as a consequence of liver carcinoma)
- Inherited Polycythemia (mutation in the EPO receptor)
- Polycythemia vera
Diagnosis of PV

Until 2003: exclusion of other reasons

Since 2004: overexpression of the prv-1 gene in granulocytes

Since 2005: JAK2 mutation in granulocytes
Diagnostic qPCR assay for the prv-1 gene

1. Blood sampling
   - anticoagulant: EDTA
   - minimum: 20 ml
   - storage temperature: RT

2. Transportation
   - maximum time?
   - temperature: ??????

3. diagnostic Assay
   - Specific, sensitive, etc.
   - Whole procedure: Cheap and robust
Key features of the RT-qPCR assay

• Relative quantification
• Standard curve method
Step 1: set up of qPCR

- Target amplicon (prv-1)
  - Avoid known SNPS
  - Take care of potential splice variants
Step 2: choose appropriate reference gene

<table>
<thead>
<tr>
<th>Name</th>
<th>Function</th>
<th>Normfinder**</th>
<th>Genom***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ranking</td>
<td>Stability v.</td>
</tr>
<tr>
<td>b-actin</td>
<td>structure</td>
<td>1</td>
<td>0,078</td>
</tr>
<tr>
<td>Glucose-6-phosphate 1-dehydrogenase</td>
<td>metabolism</td>
<td>2</td>
<td>0,109</td>
</tr>
<tr>
<td>b-2-microglobulin</td>
<td>histocompatibility</td>
<td>3</td>
<td>0,153</td>
</tr>
<tr>
<td>b-Glucuronidase</td>
<td>metabolism</td>
<td>4</td>
<td>0,173</td>
</tr>
<tr>
<td>Glyceraldehyde dehydrogenase</td>
<td>metabolism</td>
<td>5</td>
<td>0,217</td>
</tr>
<tr>
<td>ABL</td>
<td>cell signalling</td>
<td>6</td>
<td>0,244</td>
</tr>
</tbody>
</table>

* Beillard et al., Leukemia 203; 17, 2474-2486
** Andersen et al., Cancer Res. 2004; 64, 5245-5220
*** Vandesompele et al., Genome Biology 2002; 3(7), research0034.1 - 11

reference gene panel of “Europe Against Cancer” *
Exon spanning, pseudogen-free amplicon of beta actin:

Kreutzer et al., Clin. Chem. 45(2), 297
Step 3: effect of blood storage

How stable is the relative value at different ambient temperature?

Delta Ct's of Blood sample #2

EDTA blood

4 °C  22 °C  30 °C

hours of blood storage

Delta Ct's of Blood sample #2

ABL
GUS
G6PD
B2M
AC
18S

EDTA blood aliquots
### blood storage: ranking
e.g. donor #2

<table>
<thead>
<tr>
<th>Gene</th>
<th>Var(DCt) $t_0-t_{48}$</th>
<th>rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL</td>
<td>0,26</td>
<td>3</td>
</tr>
<tr>
<td>GUS</td>
<td>0,74</td>
<td>5</td>
</tr>
<tr>
<td>G6PD</td>
<td>0,81</td>
<td>6</td>
</tr>
<tr>
<td>B2M</td>
<td>0,20</td>
<td>2</td>
</tr>
<tr>
<td>AC</td>
<td>0,20</td>
<td>1</td>
</tr>
<tr>
<td>18S</td>
<td>0,50</td>
<td>4</td>
</tr>
</tbody>
</table>

#### Delta Ct's of Blood sample #2

- ABL
- GUS
- G6PD
- B2M
- AC
- 18S

![Graph showing Delta Ct's of Blood sample #2](image)
# blood storage: results

mean of 3 donors

<table>
<thead>
<tr>
<th>Gene</th>
<th>t=0-24h</th>
<th>t=0-36h</th>
<th>t=0-48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD</td>
<td>1,3</td>
<td>2,7</td>
<td>3,7</td>
</tr>
<tr>
<td>AC</td>
<td>1,7</td>
<td>1,3</td>
<td>2,0</td>
</tr>
<tr>
<td>ABL</td>
<td>3,0</td>
<td>2,7</td>
<td>2,3</td>
</tr>
<tr>
<td>B2M</td>
<td>4,0</td>
<td>3,3</td>
<td>2,7</td>
</tr>
<tr>
<td>18S</td>
<td>5,3</td>
<td>5,0</td>
<td>4,7</td>
</tr>
<tr>
<td>GUS</td>
<td>5,7</td>
<td>6,0</td>
<td>5,7</td>
</tr>
</tbody>
</table>
Summary reference gene

- 100 patients, $t = 1$ day $T = RT$: $b\text{Actin} + G6PD$

- 3 patients, $t = \text{variable}$, $T = \text{variable}$: $b\text{Actin} (+ G6PD)$

- Economic considerations: $b\text{Actin}$
Step 4: produce a life time supply of plasmid standards

- QC*1: Sequencing
- QC2: qPCR
  - plasmid marker
  - bacterial RNA marker (23S RT-PCR)
  - bacterial DNA marker (adenylate cyclase)
- Photometry (whole spectrum)
- Dilute standard in “protective buffer”
- QC3: qPCR of plasmid marker
- Make aliquots (one or two freeze-thaw cycles)

* QC = quality control
Step 5: determine dynamic range of:

- cDNA synthesis
  (RNA concentration, RT primers, enzyme etc)

- qPCR
  (constant reference gene – vary target gene)
Step 6: diagnostic threshold values, assay sensitivity and specificity

- logarhytm of the prv-1 expression

- polycythemia vera
- grey area
- healthy

false negative
Step 7: clinical validation
(not only PV patients!!)

- 133 patients: PV plus other forms of MPS
- 40 have MPS and are prv-1 positive
- 0 have NO MPS and are prv-1 positive
- 31 have MPS and are prv-1 negative

100% specificity
56% sensitivity
Step 8: check constantly for uniform performance

• data collection from positive and negative controls
Finally

- Never change reagents
- Never change thermocycler

In case you need to revalidate your assay

- ca. 100 specimens measured in parallel
  -> correction factor
- Validate correction factor with ca. 100 specimens
Acknowledgements:

Kooperationsgemeinschaft molekulare Labordiagnostik:
  • Melanie Schneider
  • Elisabeth Böhm

University of Freiburg, Center for Clinical Research:
  • Prof. Dr. Heike Pahl
  • Dr. med. Snezana Temerinac

Klinikum Chemnitz gGmbH, Klinik für Innere Medizin III
  • Dr. med. Regina Herbst