

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

Freising
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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

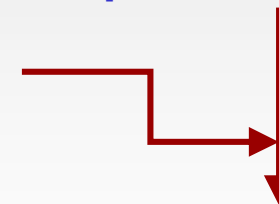


maximum time?
temperature: ?????

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

Name	Function	Normfinder**		Genorm***	
		Ranking	Stability v.	Ranking	M value
b-actin	structure	1	0,078	1	0,72
Glucose-6-phosphate 1-dehydrogenase	metabolism	2	0,109	2	0,76
b-2-microglobulin	histocompatibility	3	0,153	5	0,99
b-Glucuronidase	metabolism	4	0,173	4	0,97
glycerine aldehyde dehydrogenase	metabolism	5	0,217	3	0,94
ABL	cell signalling	6	0,244	6	1,12

Best

Worst

b-2-microglobulin etc. reference gene panel of "Europe Against Cancer" *

* Beillard et al., Leukemia 2003; 17, 2474-2486

** Andersen et al., Cancer Res. 2004; 64, 5245-5220

*** Vandesompele et al., Genome Biology 2002; 3(7), research0034.1 - 11

Exon spanning, pseudogen-free amplicon of beta actin:

Kreutzer et al., Clin. Chem. 45(2), 297

Step 3: effect of blood storage

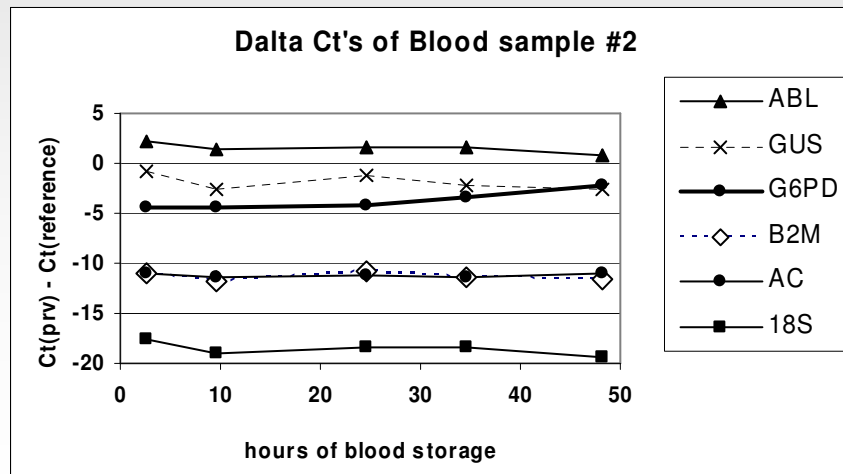
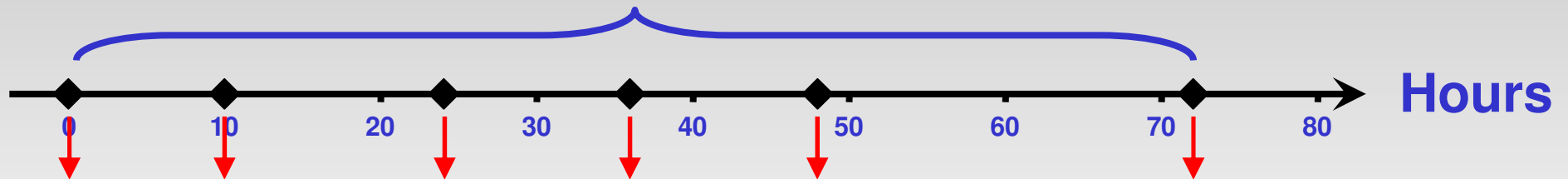
How stable is the relative value at different ambient temperature?



EDTA blood

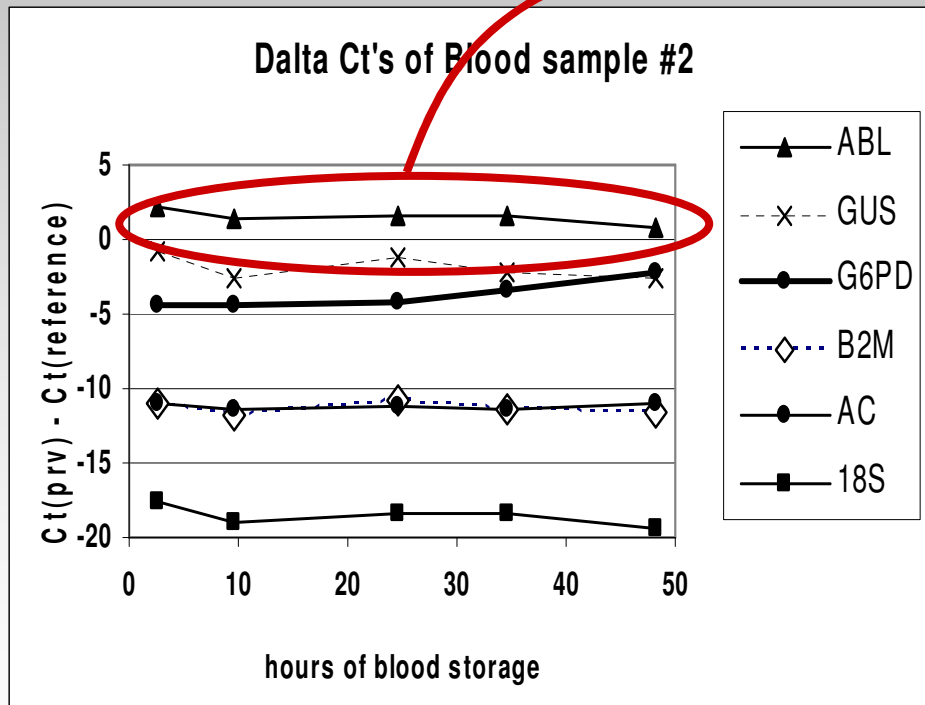
aliquots

4 °C 22 °C 30 °C



blood storage: ranking

e.g. donor #2



Gene	Var(DCt) t_0-t_{48}	rank
ABL	0,26	3
GUS	0,74	5
G6PD	0,81	6
B2M	0,20	2
AC	0,20	1
18S	0,50	4

blood storage: results

mean of 3 donors

Gene	Ranking		
	t=0-24h	t=0-36h	t=0-48h
G6PD	1,3	2,7	3,7
AC	1,7	1,3	2,0
ABL	3,0	2,7	2,3
B2M	4,0	3,3	2,7
18S	5,3	5,0	4,7
GUS	5,7	6,0	5,7

Summary reference gene

- 100 patients, t = 1 day T = RT: **bActin + G6PD**
- 3 patients, t = variable, T = variable : **bActin (+ G6PD)**
- economic considerations : **bActin**

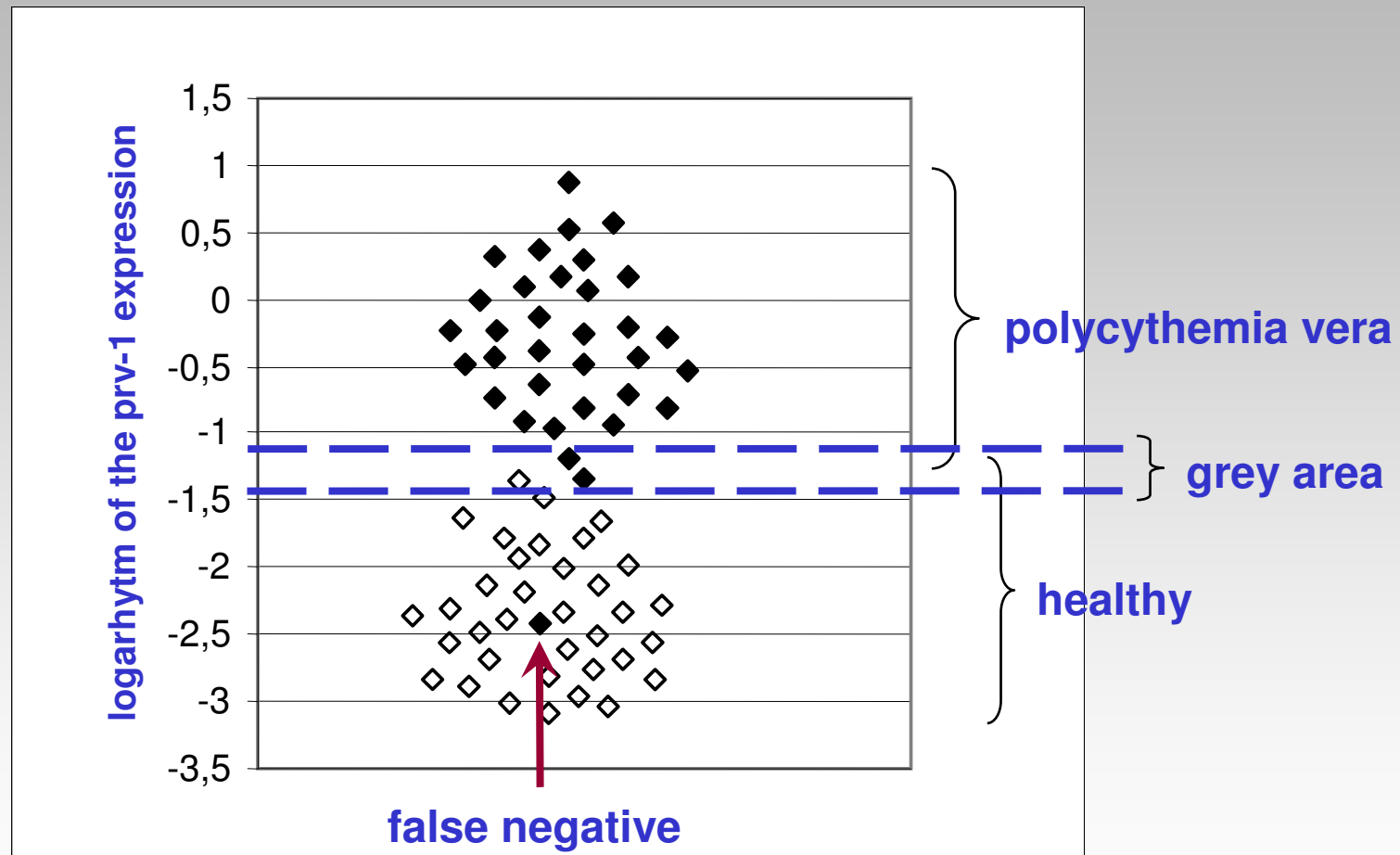
Step 4: produce a life time supply **of plasmid standards**

- 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)

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



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

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