

Standardization of PRV-1 mRNA Quantification

Deliverable WP 9.6

Background

PRV-1 mRNA quantification is a molecular marker which has been recommended by the German Society of Hematology and Oncology (DGHO) as well as the German Society for Internal Medicine (DGIM) for the diagnosis of Polycythemia vera (PV) (Cilloni, *et al.*, 2004, Griesshammer, *et al.*, 2002, Klippel, *et al.*, 2003, Kralovics, *et al.*, 2003). While many independent laboratories have reported overexpression of PRV-1 in > 90% of PV patients, some publications document lower proportions of PRV-1 overexpression in their cohorts (Liu, *et al.*, 2003). The main reason for this discrepancy are most likely technical differences.

The most frequently reported differences in the assays used include:

- 1) The cell fraction assayed. While we have demonstrated that purified granulocytes give the most accurate reading of PRV-1 expression (Palmqvist, *et al.*, 2003), several other groups use mononuclear cells or whole blood leukocytes for their assays (Ricksten, *et al.*, 2002, Spinelli, *et al.*, 2002).
- 2) The time elapsing between blood sampling and RNA extraction. Dr. Prchal has published (and we have identical unpublished data) that PRV-1 quantification in samples stored for very different amounts of time can obscure the results (Jelinek, *et al.*, 2004)
- 3) The housekeeping reference gene used. The various groups reporting PRV-1 assays have used GAPDH, 18S, GUS, b2-microglobulin, c-abl as well as RPL19 for comparison of the amount of RNA used. Not all of these genes are similarly well suited as housekeeping genes. Some bear no introns and are thus sensitive to DNA contamination, some are expressed at vastly higher concentrations than the target genes, some may be regulated differentially in PV patients versus controls.
- 4) PRV-1 Primers and Probes used. Because of the highly repetitive nature of the PRV-1 mRNA, it was initially very difficult to find PCR primer pairs

that would amplify well. Therefore, many of the published assays are conducted with primers that do not span an intron/exon junction, making them sensitive to DNA contamination.

- 5) The method of RNA isolation. Total cellular RNA preparations are frequently contaminated with genomic DNA. Depending on the isolation method used, this contamination may be more or less severe. Using primers for both PRV-1 and the housekeeping gene that do not span intron/exon junctions amplifies this problem.

Because of the myriad differences in PRV-1 protocols, and the increasing role of PRV-1 mRNA quantification for diagnosis it is essential that a standardized PRV-1 protocol be established and endorsed by the European Leukemia Network.

Standardization of PRV-1 mRNA Quantification

- 1) In a study published recently, we established conclusively that the use of whole blood or mononuclear cells in place of purified granulocytes for PRV-1 quantification, can yield false negative results (Palmqvist, et al., 2003).
 - Therefore, the use of purified granulocytes as the standard for PRV-1 quantification is recommended.
- 2) The PRV-1 primers and probe for quantitative RT-PCR have been redesigned, with the following advantages
 - The primers span an intron/exon border, so that contaminated DNA is not amplified
 - The 5' primer lies within exon 3, which is not present in the duplicated PRV-1 "pseudogene" so that only bona fide PRV-1 mRNA is detected.
 - The probe has been designed as a "MGB" probe, which allows the use of shorter probes and offers some technical advantages
- 3) The optimal housekeeping gene is being determined. Extensive experiments have been conducted to find a gene whose
 - Expression level is similar to PRV-1

- M-RNA stability is similar to that of PRV-1
 - Expression variability between individuals is minimal
- 4) The method of data analysis has been changed to a standard curve analysis, in which results are reported as copy number PRV-1 per copy number housekeeping gene

With these improvements, a standardized PRV-1 mRNA quantification assay will be available which should show minimal inter-laboratory variability making results comparable throughout the area of the European Leukemia Network.

Literature:

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