Concordance of assays designed for the quantification of *JAK2*V617F: a multicenter study

Eric Lippert,[1] François Girodon,[2] Emma Hammond,[3] Jaroslav Jelinek,[4] N. Scott Reading,[5] Boris Fehse,[6] Katy Hanlon,[7] Mirjam Hermans,[8] Céline Richard,[9] Sabina Swierczek,[10] Valérie Ugo,[11] Serge Carillo,[12] Véronique Harrivel,[13] Christophe Marzac,[13] Daniela Pietra,[14] Marta Sobas,[15] Morgane Mounier,[2] Marina Migeon,[1] Sian Ellard,[7] Nicolaus Kröger,[6] Richard Herrmann,[3] Josef T. Prchal,[5,10] Radek C. Skoda,[15] and Sylvie Hermouet[16]

[1]Laboratoire d'Hématologie, CHU, Bordeaux, France; [2]Laboratoire d'Hématologie, CHU, Dijon, France; [3]Murdoch University, Perth, Australia; [4]University of Texas MD Anderson Cancer Center, Houston, Texas, USA; [5]ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, Utah, USA; [6]Uniklinik Eppendorf, Hamburg, Germany; [7]Molecular Genetics, Royal Devon and Exeter Hospital, Exeter, UK; [8]Molecular Diagnostics, Jeroen Bosch Hospital, s-Hertogenbosch, Netherlands; [9]Laboratoire d'Hématologie, CHU, Clermont-Ferrand, France; [10]Hematology Division, University of Utah School of Medicine, Salt Lake City, Utah, USA; [11]Laboratoire d'Hématologie, CHU, Brest, France; [12]Laboratoire de Cytologie Clinique et Cytogénétique, CHU, Nîmes, France; [13]Laboratoire d'Hématologie, Hôtel-Dieu, Paris, France; [14]Department of Hematology, University of Pavia and Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; [15]Experimental Hematology, Department of Biomedicine, Basel University Hospital, Basel, Switzerland, and [16]Laboratoire d'Hématologie, CHU, Nantes, France

ABSTRACT

Background
Many different techniques have been designed for the quantification of *JAK2*V617F allelic burden, sometimes producing discrepant results.

Design and Methods
*JAK2*V617F quantification techniques were compared among 16 centers using 11 assays based on quantitative polymerase chain reaction (with mutation-specific primers or probes, or fluorescent resonance energy transfer/melting curve analysis), allele-specific polymerase chain reaction, conventional sequencing or pyrosequencing.

Results
A first series of blinded samples (granulocyte DNA, n=29) was analyzed. Seven assays (12 centers) reported values inside the mean±2SD; the mean coefficient of variation was 31%. Sequencing techniques lacked sensitivity, and strong discrepancies were observed with four techniques, which could be attributed to inadequate standards or to different modes of expression of results. Indeed, quantification of *JAK2*V617F in relation to another control gene produced higher than expected values, suggesting the possibility of more than two *JAK2* copies/cell. After calibration of assays with common 1% to 100% *JAK2*V617F standards (dilutions of UKE-1 cells in normal leukocytes), 14 centers tested ten new samples. *JAK2*V617F allelic burdens greater or equal than 1% were then reliably quantified by five techniques – one allele specific-polymerase chain reaction and four TaqMan allele-specific quantitative polymerase chain reaction assays, including one previously giving results outside the mean±2SD – with a lower mean coefficient of variation (21%). Of these, only the two TaqMan allele-specific quantitative polymerase chain reaction assays with primer-based specificity could detect 0.2% *JAK2*V617F.

Conclusions
Techniques expressing the allelic burden as *JAK2*V617F/total *JAK2* and using a common set of standards produced similar quantification results but with variable sensitivity. Calibration to a reference standard improved reproducibility.

Key words: *JAK2*V617F, quantification, standardization, allele-specific PCR, myeloproliferative diseases, multicenter study.

Citation: Lippert E, Girodon F, Hammond E, Jelinek J, Reading NS, Fehse B, Hanlon K, Hermans M, Richard C, Swierczek S, Ugo V, Carillo S, Harrivel V, Marzac C, Pietra D, Sobas M, Mounier M, Migeon M, Ellard S, Kröger N, Herrmann R, Prchal JT, Skoda RC, and Hermouet S. Concordance of assays designed for the quantification of JAK2V617F: a multicenter study. Haematologica 2009; 94:38-45. doi: 10.3324/haematol.13486

©2009 Ferrata Storti Foundation. This is an open-access paper.

Acknowledgments: the authors wish to thank Elizabeth Young, Katie Thomas, Lydie Lecucq, Sophie Salzes and Francis Belloc for expert technical help and the tumor bank of the CHU de Bordeaux for providing samples.

Funding: this study was supported by a grant from the Comité Grand Ouest and Comité Aquitaine Charente of the Ligue contre le Cancer to SH and EL, respectively, and by contributions from Exeter Leukaemia Fund to KH.

Manuscript received June 11, 2008. Revised version arrived August 14, 2008. Manuscript accepted August 25, 2008.

Correspondence: Sylvie Hermouet, Laboratoire d'Hématologie, Centre Hospitalier Universitaire, Institut de Biologie, 9 quai Moncousu, 44093 Nantes, France. E-mail: sylvie.hermouet@chu-nantes.fr

The online version of this article contains a supplementary appendix.

©Ferrata Storti Foundation

Introduction

The diagnosis of Philadelphia-negative myeloproliferative neoplasms has changed dramatically with the discovery of a somatic point mutation in the *JAK2* gene (*JAK2* 1849G>T or *JAK2*V617F).[1] Detection of this mutation is now a major criterion for the diagnosis of Philadelphia-negative myeloproliferative neoplasms.[2] Whether *JAK2*V617F is the initial genetic event responsible for *JAK2*V617F-positive myeloproliferative neoplasms is still a matter of debate.[3,4] Not all myeloid cells carry the mutation and, because of mitotic recombination, the mutation can be found on both *JAK2* alleles.[5,6] The variation in mutation burden thus reflects both the size of the myeloproliferative clone and the homozygous or heterozygous status of clonal cells. Homo/heterozygosity cannot be differentiated from *JAK2*V617F allelic burdens (%JAK2V617F) representing lower than 50% of total *JAK2*, but homozygosity can be inferred when %JAK2V617F is greater than or equal to 50%.

We previously reported that *JAK2*V617F burdens are higher in polycythemia vera than in essential thrombocythemia.[7,8] In addition, a high %JAK2V617F has been associated with increased risk of thrombotic events in essential thrombocythemia; increased risk of cardiovascular events, larger spleen and greater treatment requirement in polycythemia vera; and higher leukocytosis and risk of acute transformation in primary myelofibrosis.[9-11] Some of these findings, however, are controversial: Pemmaraju *et al*.[12] found no correlation between the %JAK2V617F in neutrophils and occurrence of thrombotic events. Thus reproducible quantification of the *JAK2*V617F burden is of major interest in the evaluation of myeloproliferative neoplasms. Quantification of *JAK2*V617F may also be important in the follow-up of patients who have received a bone marrow transplant or treatment with interferon α-2a and the soon-to-come JAK2 inhibitors.[13,14]

Understanding the discrepancies between the results of the studies reported here is partly dependent on the comparison of the techniques used for quantification. We, therefore, undertook a study comparing 11 different techniques, carried out in 16 laboratories using various instruments. After a first round of analyses, we distributed dilutions of *JAK2*V617F-positive DNA to standardize the assays. With adjustment for the mode of expression of the results and the use of common standards, five techniques were similarly reliable for the quantification of *JAK2*V617F loads greater than or equal to 1% of total *JAK2*; two of these techniques could detect 0.2% *JAK2*V617F. The advantages and disadvantages of the five techniques are presented.

Design and Methods

Design of the study

From April to June 2007, laboratories with published assays designed for the quantification of *JAK2*V617F were identified via a PubMed search and contacted. Sixteen centers using 11 different techniques responded, agreeing to participate in a comparative study of *JAK2*V617F assays. Initially, a series of 29 blinded DNA samples from purified blood granulocytes of patients referred to three participating laboratories (centers 4, 5 and 7) were distributed to the 16 laboratories. Three of the 29 samples had been found negative; the *JAK2*V617F burden was lower than 10% in ten samples and greater than 10% in 16 samples. Each center was asked to process the sample according to their standard procedures and report the %JAK2V617F [(*JAK2*V617F/total *JAK2*) × 100].

The standards used by eight participating centers were analyzed in center 7 using an allele-specific quantitative polymerase chain reaction (AS-qPCR).[7,15] Subsequently, a set of standards based on a *JAK2*V617F-positive, diploid cell line (UKE-1) was defined and distributed to all centers. Following the calibration of local standards, a second series of test samples, comprising ten samples from patients and a series of DNA dilutions of two cell lines (HEL/Karpas 299, prepared in center 9), were assessed blindly. The HEL/Karpas series included four samples with a *JAK2*V617F allelic burden lower than or equal to 2%, to provide further information on the sensitivity of the various techniques. Technical issues prevented centers 8 and 13 from participating in this part of the study.

Patients

The diagnosis of myeloproliferative neoplasm was established according to WHO guidelines for all *JAK2*V617F-positive patients. Diagnoses were, for the two series of samples, respectively, secondary erythrocytosis (1; 1), atopic hypereosinophilia (1; 0), essential thrombocythemia (16; 3), polycythemia vera (10; 5) and primary myelofibrosis (1; 1).

Cell line characterization and DNA preparations

UKE-1 cells, kindly provided by Dr. Walter Fiedler (Hamburg, Germany), were grown as described elsewhere.[16] DNA content was assessed by flow cytometry after treatment of ethanol-permeabilized cells with RNAse and staining with propidium iodide. The karyotype was established using standard cytogenetic techniques, synchronization with synchroset (Amplitech, France), heat denaturation and Giemsa staining of R bands. For preparation of *JAK2*V617F standards, UKE-1 cells were counted with a hemocytometer and diluted with leukocytes (buffy coat white blood cells, WBC) obtained from normal healthy individuals. Pellets of each mix of UKE-1 and WBC (100% UKE-1, 75% UKE-1 + 25% WBC, 50% UKE-1 + 50% WBC, ...) were kept frozen at -20°C. Genomic DNA was extracted using the QiaAmp kit. An additional cell line dilution series was obtained by diluting HEL into Karpas-299 cells. A mixture of 20% HEL + 80% Karpas 299 was found to have 50% *JAK2*V617F. Further dilutions were performed, taking this percentage into account.

Plasmids

Three sets of plasmids were used for quantification. Plasmids A and B were prepared in center 4 as described elsewhere.[7] Briefly, a PCR fragment obtained by reverse transcriptase PCR of U937 or HEL (respectively wild type

©Ferrata Storti Foundation

and mutant) with primers 3'-TTATGGACAACAGT-CAAACAACA ATTCT-5' and 3'-AACTCCTGTTAAAT-TATAGTTTACTGACACC-5', was cloned into a TopoTA vector (Invitrogen, Carlsbad, CA, USA). Plasmids C and D were generated in center 8 by amplification of an 881 bp fragment with primers 3'-GATTCACTAATCATAC-CCAG-5' and 3'-TTGAACCTGCCATAATCTCT-5', cloned into pGEM T Easy (Promega,WI, USA). Plasmid E (mutant only) was prepared in center 15 as described by Hammond *et al.*[20] The fragment obtained with primers 5'-TATCCCAAAGGACAGAAGTATTCATT-3' and 5'-TTGTGTTTCCCTCCCT GTTGGA-3' was cloned into Zero Blunt Topo (Invitrogen).

JAK2V617F quantification

The characteristics of the 11 techniques, used either for diagnosis/follow-up of patients with myeloproliferative neoplasms or for research, are presented in Table 1, while sequences of primers and probes can be found in the *Online Supplementary Table S1*. Most (13/16) centers had developed a quantitative real time PCR assay, predominantly (11/13) involving hydrolysis probe-based quantification. The same Taqman assay was used in five centers (centers 4, 5, 6, 7, and 8) with different real-time PCR machines; another assay was used in centers 9 and 10 on different qPCR platforms. Center 2 used fluorescent resonance energy transfer (FRET) probes, center 13 used SYBRgreen chemistry. Five assays based the specificity of detection on a primer, which at centers 9 and 10 was modified by inclusion of a mismatch and a locked nucleic acid. Center 12 quantified the products of a final point allele-specific PCR. Centers 3 and 16 performed DNA sequencing, with pyro-sequencing and conventional Big Dye terminator chemistry, respectively. The assay used in center 1 was based on the TaqMan allelic discrimination assay principle. Results were expressed as percentages of mutated allele (%JAK2V617F) by all laboratories but center 15, which determined the ratio of *JAK2*V617F *versus* a control gene, human growth hormone (*HGH*). The different standards used for calibration included plasmids, cell lines or genomic DNA from patients.

Statistics

The mean and standard deviation (SD) of results of the 16 centers (mean16 and SD16, respectively) were established for each sample tested. Results were then labeled as within 2 SD from the mean (mean16±2SD16) or as exceeding 2 SD from the mean. Centers with more than three results outside the mean16±2SD16 interval were excluded, and new mean and SD of values were then calculated. Similar analyses were conducted for the second series of samples, also using boundaries of 2 SD. Coefficients of variation (CV) were used to analyze the variability in results reported for each sample. Results from the different centers were also compared using a two factor analysis of variance (ANOVA).

Results

Assessment of %JAK2V617F in the first series of samples

A first series of 29 blinded DNA samples was proposed to the 16 participating centers (Table 2). There was one false positive (declared weakly positive at 0.1%) and six false negative results in two centers that used sequencing-based techniques. The mean and SD of results of the 16 centers were established for each sample tested. Centers 13, 14 and 15 had more than three results outside of the mean 16±2 SD16. In addition, centers 1 and 13 had results systematically lower than the mean16. A new, narrower mean 12±2 SD12 range was calculated from results gener-

Table 1. Overview of *JAK2*V617F assays employed by the participating centers.

Center	Type of assay	Specificity	Standards	Instrument
1	Taqman qPCR	Double Dye probe	Cell line (HEL)	ABI 7300
2	FRET	Melting curve	Patient's DNA	Light Cycler 1
3	Pyro-sequencing	DNA sequence	N/A	PSQ HS96-Biotage
4	Taqman AS-qPCR	Fwd primer	Plasmids A-B	Rotorgene 3000
5	Taqman AS-qPCR	Fwd primer	Plasmids A-B	Light Cycler 480
6	Taqman AS-qPCR	Fwd primer	Plasmids A-B	Light Cycler 1
7	Taqman AS-qPCR	Fwd primer	Plasmids A-B	Light Cycler 1
8	Taqman AS-qPCR	Fwd primer	Plasmids C-D	Light Cycler 2
9	Taqman AS-qPCR	Rev primer, 1 LNA,1 MM	Cell line (HEL/Karpas 299)	Light Cycler 480
10	Taqman AS-qPCR	Rev primer, 1 LNA, 1 MM	Patient's DNA	ABI 7000
11	Taqman qPCR	Hydrolysis probes	Patient's DNA	ABI 7500
12	AS-PCR	Rev primer	Cell line (DAMI), DNA from heterozygous NK clone	ABI 3100
13	AS-PCR	Rev primer, 1 MM	Patient's DNA	ABI Prism 5700
14	Taqman AS-qPCR	Rev primer	UKE-1 dilution	ABI Prism7700 / PTC-200
15	Taqman AS-qPCR	Fwd primer, 1 MM	Plasmid E	ABI 7700
16	DNA sequencing and peak height analysis	N/A	Plasmids C-D	ABI Prism 3730

AS: allele-specific, Fwd: forward, Rev: reverse, LNA: locked nucleic acid, MM: mismatch; N/A: not applicable.

©Ferrata Storti Foundation

Table 2. **%JAK2V617F of total *JAK2* in DNA of the first series of 29 clinical samples as determined by the 16 participating centers.**

Sample	Diag.	Ctr 1	Ctr 2	Ctr 3	Ctr 4	Ctr 5	Ctr 6	Ctr 7	Ctr 8	Ctr 9	Ctr 10	Ctr 11	Ctr 12	Ctr 16	Ctr 13	Ctr 14	Ctr 15	Mean 16	SD16	Mean 12	SD12
B11	SE	nd	nd	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0
D584	Eosino.	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.1**	0	0	0.0	0.0	0.0	0.0
N580	ET	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0	0.0
B93	ET	nd	nd	0.2	0.7	2	1	1	nd	1.1	**0**	1.5	2	**0**	0.1	1.1	2	1.0	0.8	1.0	0.8
N342	ET	< 0.5	2	0.5	3	2	1	2	nd	1.1	2.1	1.6	3	**0**	0.3	1.4	3	1.7	1.0	1.7	1.0
N508	ET	< 0.5	3	2.3	3	1	2	2	nd	1.2	2.7	2.7	3	**0**	0.3	0.7	3	1.9	1.1	2.0	1.0
N295	ET	0.5	3	1.2	2	2	nd	nd	nd	1.9	4	3.5	2	2	0.4	1	3	2.0	1.1	2.4	0.9
N585	ET	0.5-1	3	1.7	3.5	1	3	2	12	1.9	2.6	2.4	2	**0**	0.3	0.5	3	2.6	2.8	2.9	3.0
N396	ET	2	5	2.5	4	6	6	5	nd	4	7.8	4.5	7	5	1.5	7	8	5.0	2.0	5.1	1.5
N474	ET	1	5	4.1	4	4	4	6	8	4.6	6.3	6	7	5	0.4	7	11	5.2	2.6	5.3	1.4
D345	ET	2	10	nd	2	3	14	8	nd	7.6	9.3	6	10	7	1.7	6	nd	6.6	3.7	7.7	3.5
B17	ET	nd	nd	15.4	12	15	13	5	15	14.6	11	7	26	17	5	45	29	16.4	10.7	13.7	5.5
N525	ET	5	15	14.3	11	14	15	15	13	12.3	14.8	15	21	14	3	7	19	13.0	4.7	14.5	2.4
D571	ET	5	20	17.4	9	8	25	28	39	18	25.7	16	26	19	4	14	27	18.8	9.5	20.9	8.6
B85	ET	nd	nd	33.5	27	32	25	31	32	30	39	27	42	35	8	29	48	31.2	9.3	32.0	5.2
D329	ET	15	40	36.2	24	19	32	46	51	38.6	39.4	34	48	42	21	24	58	35.5	12.4	37.5	9.5
N530	ET	15	40	40.3	34	32	41	38	32	39.7	46.9	35	50	44	16	31	33	35.5	9.5	39.3	5.7
N583	ET	20	50	51	50	37	54	46	62	47.9	50.7	46	59	52	18	90	88	51.3	19.0	50.4	6.5
B87	IMF	nd	nd	91.2	96	96	98	97	99	98.2	98.4	99	92	100	87	100	207	104.1	29.9	96.7	2.9
N450	PV	0.5	2	1.1	1	2	2	2	6	1.1	1.8	1.3	2	**0**	0.3	1.3	2	1.6	1.3	1.8	1.5
N393	PV	0,5	3	0.5	2	4	4	4	nd	2.8	4.5	4.1	5	4	0.6	1.7	4	2.9	1.5	3.4	1.3
D510	PV	5	20	15.4	9	3	22	19	20	16.7	22.1	14	22	18	nd	24	13	16.2	6.4	16.7	5.9
N578	PV	10	30	29.1	26	24	36	22	30	29.2	33.6	31	36	27	8	7	68	27.9	14.1	29.4	4.4
D77	PV	20	55	52	37	28	50	50	54	54.1	51.3	52	63	61	31	32	56	46.6	12.9	50.5	9.7
B46	PV	nd	nd	60	57	62	61	63	56	61.2	66.7	57	69	62	20	82	142	65.6	25.6	61.4	4.0
N564	PV	30	70	65.4	66	50	66	69	83	67.2	72.6	63	54	72	37	14	74	59.5	18.3	66.4	8.6
T54	PV	40	80	73	68	52	78	79	84	79.3	82.6	74	80	80	45	26	110	70.7	20.5	75.8	8.7
N581	PV	70	95	89.4	91	84	93	91	96	93	95.4	93	92	94	79	100	128	92.7	11.9	92.2	3.4
D586	PV	100	100	92	97	96	98	98	98	97.6	97.9	99	92	95	93	36	67	90.9	16.6	96.6	2.5

Diag.: diagnosis; nd: not done (not tested). Mean16/SD16: mean/standard deviation of %JAK2V617F for all 16 laboratories. Mean12/SD12: mean/SD of %JAK2V617F for 12 laboratories remaining after those giving outlying results (centers 1, 13, 14, 15) were withdrawn. Bold characters indicate the false positive/false negative results. Heavy shading indicates values outside the mean16±2SD16. Light shading indicates values outside the mean12±2SD12.

©Ferrata Storti Foundation

ated by the remaining 12 centers. The mean CV in %JAK2V617F assessment by these 12 centers was 31%. Concordance improved with increasing mutational load: the mean CV was 63.4% for samples containing lower than 5% *JAK2*V617F, and 19.8% for samples with greater or equal than 5% *JAK2*V617F (p<0.001). Because the results of three centers were systematically low (centers 1 and 13) or high (center 15), we suspected under- and over-estimation of %JAK2V617F due to inaccurate calibration of standards. In contrast to other centers, center 15 expressed results as a percentage of number of copies of *JAK2*V617F compared to the number of copies of a control gene (*HGH*), instead of %JAK2V617F DNA of total *JAK2* DNA. The 100% allelic ratio was defined as an equal number of *JAK2*V617F and *HGH* copies, while a ratio greater than 100% implied more *JAK2*V617F than *HGH* copies. Center 14, whose results were within the mean16±2SD16 for samples with lower than 10% *JAK2*V617F, but outside for *JAK2*V617F loads greater than 10%, also determined the %JAK2V617F by comparison with a control gene other than *JAK2* (hemopoietic cell kinase, *HCK*). Results were then plotted against those of a cell line, UKE-1, with known *JAK2*V617F status (*see below*). These additional steps, although not hampering detection of low levels of mutant – the primary goal of this technique – were likely to be responsible for the variations in ratios observed in samples with high mutational loads.

Comparison and optimization of standards

To confirm that part of the discrepancies could be attributed to the use of different references, standards were compared on the same apparatus with a single technique. Although there was a slight difference in crossing points obtained with linear (A,B) *versus* circular (C,D,E) plasmids (Figure 1), ratios of numbers of *JAK2*V617F/total *JAK2* copies determined in unknown samples using both sets of plasmids yielded similar results. %JAK2V617F in standards from center 1 (HEL cells diluted in normal peripheral blood lymphocytes (PBL), or HEL/PBL standard) and center 11 (patient's DNA), were quantified against sets of plasmids A-B and

C-D, with similar results. However the observed %JAK2V617F in the HEL/PBL standard was consistently higher than that reported by center 1 (*Online Supplementary Table S2*). This was consistent with the fact that HEL cells have more than two copies of *JAK2*, and explained the systematic underestimation of the *JAK2* mutational load by center 1.

To avoid variation of results due to differences in standards, we searched for a standard that could be used by all laboratories. Plasmid DNA standards allow reproducible and reliable quantification; however, not all diagnostic laboratories are able to produce plasmids. We reasoned that DNA from diploid cells characterized as heterozygous for the *JAK2*V617F mutation would be the best standard. DNA extracted from heterozygous erythroid colonies represents the ideal 50% standard. Unfortunately, single colonies not contaminated by non-mutated cells are difficult to obtain and provide limited amounts of DNA. As a substitute, we used the DNA of the UKE-1 cell line, generated from a transformed myeloproliferative neoplasm. This cell line bears a bi-allelic *JAK2* mutation in the context of a roughly diploid genome, as demonstrated by cytogenetic and DNA content analyses (*Online Supplementary Figure S1*). Standard dilutions were generated by diluting UKE-1 cells with white blood cells (WBC) obtained from normal healthy volunteers. DNA was then extracted and the allelic ratio for each dilution (100%, 75%, 50%, 25%, 10%, 5% and 1% *JAK2*V617F) was found to be similar to the theoretical value using A-B and C-D plasmid dilutions as standards (*Online Supplementary Table S2*). To validate the UKE-1 standards definitively, the results from a 50% dilution (50% UKE-1 cells/50% normal WBC) were compared to results obtained with DNA from single colonies of *JAK2*V617F-positive patients. Although not always exactly identical, the %JAK2V617F observed in DNA from heterozygous colonies and in the UKE-1 50% sample were very similar (*Online Supplementary Table S2*).

Assessment of %JAK2V617F using common UKE-1 standards

Fourteen laboratories, using ten assays, participated in this second part of the comparisons. The UKE-1 standards were distributed to all centers, with the instruction to calibrate local standards if necessary. A new series of samples from ten patients plus a series of DNA dilutions of HEL/Karpas 299 cells including four samples with lower or equal than 2% *JAK2*V617F, were then blindly assessed.

Only one false positive result was reported (DNA pool of healthy individuals declared to contain 0.15% *JAK2*V617F) (Table 3). The sample with the lowest theoretical mutation load (0.15% *JAK2*V617F) was found to be positive by only four of the ten techniques. Three other samples with low mutation burden (0.2-1.8%) were declared negative by centers 3 and 16. Center 16 also reported two samples with 1.6% and 2.1% *JAK2*V617F (mean values) as negative.

Three centers reported three or more values outside the mean 14±2 SD14. These were centers 14 and 15, for the reasons mentioned above, as well as center 2. Means and SD (mean11, SD11) were re-calculated without these three centers. For these 11 remaining centers, the use of common UKE-1 standards reduced the variability of *JAK2*V617F quantification: no center reported systematically high or low values (Figure 2) and all values from center 1 were now within the mean11±2 SD11.

Hence, as summarized in Table 4, when using common standards, 11 centers using seven assays consistently reported values inside the mean±2 SD for samples with 5% or more *JAK2*V617F. The %JAK2V617F reported by these 11 centers for the UKE-1 and HEL/Karpas 299 DNA dilutions were found to be not significantly different from the theoretical %JAK2V617F. These seven assays detected *JAK2*V617F with different levels of sensitivity, ranging from 5% for conventional DNA sequencing to 0.15-0.20% for TaqMan assays with specificity based on primers. The mean CV in assessment of %JAK2V617F using the five assays with a sensitivity of 1% or less was reduced to 21%.

Discussion

This study highlights the importance of using defined, accurate standards to calibrate *JAK2*V617F quantitative assays. The semi-quantitative assay of center 1, which reported values outside the mean±2 SD in the first part of

©Ferrata Storti Foundation

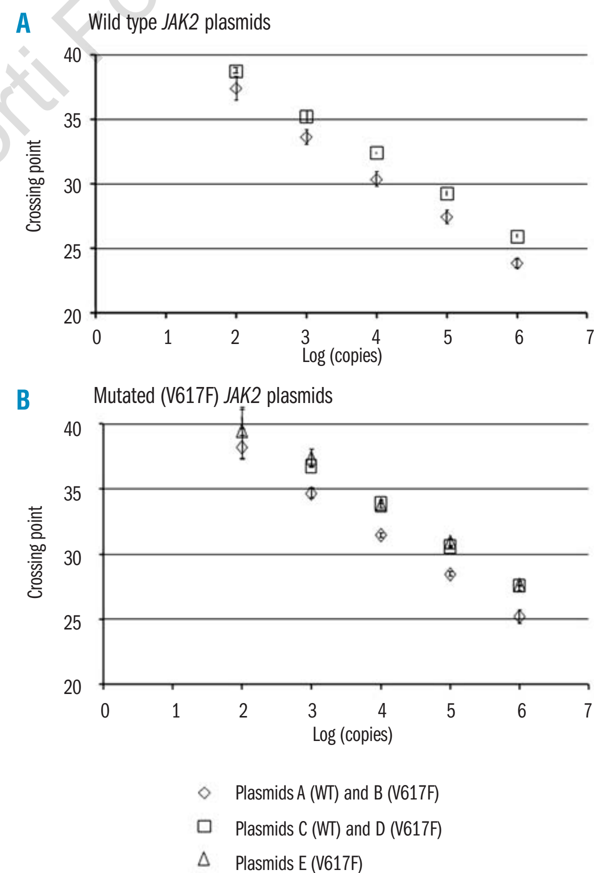

Figure 1. Comparison of *JAK2* plasmids. TaqMan AS-qPCR were performed in center 7 to determine the crossing points for five concentrations of plasmids A, C [wild type (WT) A], and B,D, and E (*JAK2*V617F, B).

Table 3. Quantification of *JAK2*V617F using common UKE-1 standards.

Sample	Ctr 1	Ctr 3	Ctr 4	Ctr 5	Ctr 6	Ctr 7	Ctr 9	Ctr 10	Ctr 11	Ctr 12	Ctr 16	Ctr 2	Ctr 14	Ctr 15	Mean 14	SD14	Mean 11	SD 11
0	NA	0	0	0	0	0	0	**0.15**	0	0	0	0	0	0	**0.0**	0.0	**0.0**	0.1
1	NA	**0**	0.9	1.3	1.1	1.3	0.6	0.95	0.9	1	**0**	1	0.9	1	**0.8**	0.4	**0.8**	0.5
5	NA	5.1	3.3	6.3	6	4.8	4.4	4.4	5.3	5	5.8	3	5.3	3	**4.7**	1.1	**5.0**	0.9
10	NA	8.4	8.0	10.5	13	10	9.9	10	7.7	10	13	8	9.2	8	**9.6**	1.8	**10.0**	1.9
25	NA	26.8	21	23	25	28.2	25	25	19	25	29	40	24	36	**26.8**	6.0	**24.6**	3.3
50	NA	50.1	41	nd	52	55.3	47	50	51	50	54	60	51	42	**50.3**	5.5	**50.1**	4.5
75	NA	66.7	68	74	77	61	75	78	73	75	79	70	74	67	**71.9**	5.4	**72.4**	6.0
100	NA	94	100	100	100	100	100	100	101	100	100	100	110	100	**100.4**	3.5	**99.4**	2.1
100	100	93	100	100	100	100	100	100	101	99	100	100	88	100	**98.6**	3.6	**99.4**	2.2
80	76	78.3	72	83	81	80	81	80	77	79	83	80	51	100	**78.7**	10.1	**79.1**	3.2
60	65	60.1	58	60	58	57	63	61	61	60	65	70	38	100	**62.6**	12.9	**60.7**	2.7
50	53	nd	failed	53	26	38	nd	48	50	18	failed	60	>100	100	**49.6**	23.4	**40.9**	14.0
20	20	19	16	24	14	20	22	21	24	19	22	30	11	32	**21.0**	5.6	**20.1**	3.1
18	16	15	18	18	12	16	17	15	21	15	19	25	12	38	**18.4**	6.6	**16.5**	2.4
2.5	3	0.4	2.2	2.5	2	2.5	2.5	2.4	3	2	**0**	1	1.8	5	**2.2**	1.2	**2.0**	1.0
1.7	3	0.3	2.1	2.3	1.6	1	1.7	1.9	2.5	2	**0**	5	1.7	3	**2.0**	1.2	**1.7**	0.9
0.9	1	**0**	1	1.1	1.1	1	3.1	0.8	1	1	**0**	3	0.6	2	**1.2**	0.9	**1.0**	0.8
0.15	**0**	**0**	0.4	0.25	0.1	**0**	0.15	0.2	**0**	**0**	**0**	**0**	0.2	0.4	**0.1**	0.1	**0.1**	0.1
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.0**	0.0	**0.0**	0.0
ET	25	22	17	23	17	20	23	22	10	22	29	20	7.7	ND	**19.8**	5.8	**20.9**	4.9
PV	34	30	20	29	24	31	31	27	19	27	33	30	8.2	37	**27.2**	7.4	**27.7**	5.0
PV	57	nd	34	54	41	62	47	48	48	47	54	60	26	100	**52.2**	17.6	**49.2**	8.0
ET	7	6	5.7	8	5	12	5.3	6.6	3.8	6	8	5	4.1	10	**6.6**	2.3	**6.7**	2.2
PV	32	30	28	16	30	37	32	30	26	28	35	50	12	68	**32.4**	13.6	**29.5**	5.5
PV (HU)	19	16	15	9	11	25	22	17	6	15	21	30	5.2	38	**17.8**	9.1	**16.0**	5.7
ET	2	**0**	1.3	1	1	0.9	0.6	1	0	2	**0**	5	0.7	2	**1.3**	1.3	**0.9**	0.7
PMF	93	93	92	96	96	96	97	96	97	98	100	100	46	100	**92.9**	13.7	**95.8**	2.4
PV	70	66	60	51	65	75	74	68	65	67	72	70	31	100	**66.7**	14.8	**66.6**	6.8
SE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.0**	0.0	**0.0**	0.0

NA: not applicable; nd: not determined; HU: hydroxyurea treatment. Mean14/SD14: Mean/SD of %JAK2V617F for all 14 laboratories. Mean11/SD11: Mean/SD of %JAK2V617F for 11 laboratories remaining after those giving outlying results (centers 2, 14 ,15) were withdrawn. Bold characters indicate the false positive/false negative results. Heavy shading indicates values outside the mean14±2SD14. Light shading indicates values outside the mean11±2SD11.

©Ferrata Storti Foundation

the study, reported values inside in the second part of the study thanks to the application of common standards. In addition, use of common standards reduced the variability of results. Although the choice of standards remains the prerogative of each center, we suggest combining plasmid DNA dilutions, which allow precise quantification of the number of copies of *JAK2*, with at least one well-calibrated genomic DNA sample, as an internal control. Dilutions of the diploid UKE-1 cell line, with two copies of mutated *JAK2* per cell, represent useful internal genomic DNA standards.

The study also underlined the necessity to use a single mode of expression of results, the percentage of total *JAK2* represented by *JAK2*V617F (%JAK2V617F). Centers that expressed *JAK2*V617F as percentages of genes other than *JAK2* consistently reported values outside the mean±2 SD. However, the %JAK2V617F values reported by center 15 that were more than two-fold the average values were exclusively found in samples from patients with polycythemia vera and primay myelofibrosis. Cells with more than two copies of wild type or mutated *JAK2* may be found in cases of myeloproliferative neoplasms with chromosome 9 trisomy, which occurs in approximately one third of cases of polycythemia vera.[23] Future studies with techniques evaluating the absolute number of copies of *JAK2* per cell by quantifying against one or several control genes[20,22] may bring useful information on the status and evolution of patients with myeloproliferative neoplasms.

Remarkably, using common UKE-1 standards, seven assays – conventional sequencing, pyro-sequencing, one AS-PCR assay and four TaqMan assays – were found to assess *JAK2*V617F similarly. The mean CV in assessing 1% or more *JAK2*V617F in granulocyte DNA using the TaqMan and AS-PCR assays was relatively low (21%). Hence, the choice of technique depends on the desired sensitivity and equipment available. Not surprisingly, the two techniques with the poorest sensitivity were sequencing techniques. Both techniques allow rapid processing of large series of samples; pyro-sequencing also enables the detection of potential additional mutations present less than six to eight bases downstream of

1849G>T (V617F). Less expected was the finding of three very different techniques providing values inside the mean±2 SD with 1% sensitivity: one was quantitative (TaqMan assay with sensitivity based on probes, center 11) but two were semi-quantitative assays (TaqMan with double dye probe, center 1; AS-PCR, center 12).

The best sensitivity – 0.2% *JAK2*V617F – was achieved by three assays. One was reliable only for mutation loads of less than 10% *JAK2*V617F. Thus only two TaqMan AS-qPCR assays with primer-based specificity, using plasmid dilutions for wild type and mutated *JAK2* quantification, consistently provided values inside the mean±2 SD with a sensitivity estimated at 0.2%. Both assays require two PCR reactions. The assay using LNA-modified primers gave the only false positive result (less than 0.2%). One might question the necessity to detect less than 1% *JAK2*V617F. However, highly sensitive techniques are required to study residual disease, notably for those patients who have received allografts or treatment with interferon α-2a.[13,14] Moreover, we and others also recently demonstrated that hydroxyurea treatment can reduce *JAK2*V617F burden to very low or undetectable

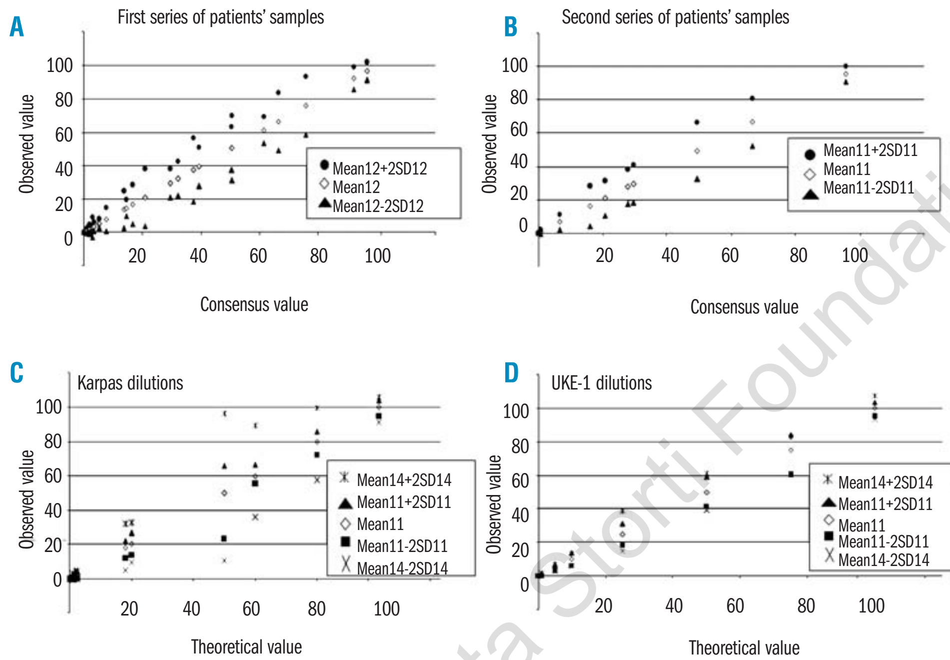

Figure 2. Observed %JAK2V617F values compared to average and theoretical %JAK2V617F values. A and B. Results obtained by the various techniques giving values inside the mean±2 SD were compared to mean values (open diamonds) for samples from patients in the first (A) and second (B) series. Mean plus 2 SD (closed circles) and mean minus 2 SD (closed triangles) are indicated. C and D: results obtained by the various techniques giving values inside the mean±2 SD were compared to theoretical values (open diamonds) for dilutions of the Karpas (C) and UKE-1 (D) cell lines. Mean plus 2 SD (asterisk) and mean minus 2 SD (cross) obtained by all 14 laboratories or by the 11 laboratories producing values inside the mean±2 SD (closed triangles and closed squares) are represented.

©Ferrata Storti Foundation

Table 4. Advantages and disadvantages of the seven *JAK2*V617F assays providing results inside the mean±2 SD.

Center	Type of assay	Specificity	Ref.	Sensitivity	N. of false negatives	N. of false positives	Advantages	Disadvantages
4,5,6,7,8	Taqman AS-qPCR	Forward primer	7, 15	0.15%	1	0	Sensitivity Quantification of *JAK2* copies against plasmid dilutions	2 PCR reactions
9,10	Taqman AS-qPCR	Reverse primer, 1 mismatch, 1 LNA	4	0.2%	1	1	Sensitivity Quantification of *JAK2* copies against plasmid dilutions	2 PCR reactions False positive
1	Taqman qPCR	Double dye probe	18	1%	1	0	1 PCR reaction	
11	Taqman qPCR	Hydrolysis probes	19	1%	2	0	1 PCR reaction	
12	AS-PCR	Reverse primer	3	1%	1	0	1 PCR reaction	*JAK2* copy number not assessed
3	Pyrosequencing	N/A	21	2%	4	0	1 PCR reaction Detection of other mutation(s) Potential for high throughput screening of large cohorts	Limited sensitivity *JAK2* copy number not assessed
16	DNA sequencing with peak height analysis	Big dye terminator chemistry	N/A	5%	11	0	1 PCR reaction Straightforward analysis Detection of other mutation(s) Potential for high throughput screening of large cohorts	Limited sensitivity *JAK2* copy number not assessed

Ref.: reference of publication; N/A: not applicable; AS: allele-specific; qPCR: quantitative polymerase chain reaction; LNA: locked nucleic acid.

levels.[24,25] Obviously, for a less than 1% sensitivity to be meaningful, *JAK2*V617F assays should be performed on purified cells, not on the highly variable mix of cells of whole blood.[26]

Finally, none of the assays tested can guarantee accurate quantification of *JAK2*V617F for all patients. Any unsuspected additional mutation occurring within the sequence of primers can potentially reduce or prevent amplification of the concerned allele, resulting in inaccurate quantification of *JAK2*V617F and possible false negative results. In these instances, a second assay based on primers or probes with non-overlapping sequences will confirm the initial assessment of %JAK2V617F.

Authorship and Disclosures

EL: performed analyses, provided samples, analysed the data and wrote the paper; FG: performed analyses and provided samples; EH: performed analyses, provided samples and plasmids, NSR: performed analyses and provided samples; JJ, BF, KH, MH, CR, SS, VU, SC, VH, CM, DP, MS, MM, SE, NK, RH, JTP, RCS: designed assays and performed analyses; MM: performed statistical analyses; SH: designed the study, performed analyses, provided plasmids, and wrote the paper.

The authors reported no potential conflicts of interest.

References

1. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005;434: 1144-8.
2. Tefferi A, Vardiman JW. Classification and diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. Leukemia 2008;22:14-22.
3. Kralovics R, Teo S-S, Li S, Theocharides A, Buser AS, Tichelli A, Skoda RC. The V617F mutation of JAK2 is a late genetic event in a subset of patients. Blood 2006;108:1377-80.
4. Nussenzveig RH, Swierczek SI, Jelinek J, Gaikwad A, Liu E, Verstovsek S, et al. Polycythemia vera is not initiated by JAK2V617F mutation. Exp Hematol 2007;35:32-8.
5. Kralovics R, Guan Y, Prchal JT. Acquired uniparental disomy of chromosome 9p is a frequent stem cell defect in polycythemia vera. Exp Hematol 2002;30:229-36.
6. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R. Passweg JR, et al. A gain-of-function mutation of JAK-2 in myeloproliferative disorders. New Engl J Med 2005;352:1779-90.
7. Lippert E, Boissinot M, Kralovics R, Girodon F, Dobo I, Praloran V, et al. The JAK2-V617F mutation is frequently present at diagnosis in patients with essential thrombocythemia and polycythemia vera. Blood 2006;108:1865-7.
8. Passamonti F, Rumi E, Pietra D, Della Porta MG, Boveri E, Pascutto C, et al. Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. Blood 2006;107:3676-82.
9. Vannucchi AM, Antonioli E, Guglielmelli P, Rambaldi A, Barosi G, Marchioli R, et al. Clinical profile of homozygous JAK2 617V>F mutation in patients with polycythemia vera or essential thrombocythemia. Blood 2007;110:840-6.
10. Vannucchi AM, Antonioli E, Guglielmelli P, Longo G, Pancrazzi A, Ponziani V, et al. Prospective identification of high-risk polycythemia vera patients based on JAK2(V617F) allele burden. Leukemia 2007;21:1952-9.
11. Barosi G, Bergamaschi G, Marchetti M, Vannucchi AM, Guglielmelli P, Antonioli E, et al. JAK2 V617F mutational status predicts progression to large splenomegaly and leukemic transformation in primary myelofibrosis. Blood 2007;110:4030-6.
12. Pemmaraju N, Moliterno AR, Williams DM, Rogers O, Spivak JL. The quantitative JAK2 V617F neutrophil allele burden does not correlate with thrombotic risk in essential thrombocytosis. Leukemia 2007;21: 2210-2.
13. Kröger N, Badbaran A, Holler E, Hahn J, Kobbe G, Bornhäuser M, et al. Monitoring of the JAK2-V617F mutation by highly sensitive quantitative real-time PCR after allogeneic stem cell transplantation in patients with myelofibrosis. Blood 2007;109: 1316-21.
14. Kiladjian JJ, Cassinat B, Turlure P, Cambier N, Roussel M, Bellucci S, et al. High molecular response rate of polycythemia vera patients treated with pegylated interferon α-2a. Blood 2006;108:2037-40.
15. Boissinot M, Lippert E, Girodon F, Dobo I, Fouassier M, Masliah C, et al. Latent myeloproliferative disorder revealed by the JAK2-V617F mutation in patients with splanchnic vein thrombosis. Blood 2006;108:3223-4.
16. Fiedler W, Henke RP, Ergün S, Schumacher U, Gehling UM, Vohwinkel G, et al. Derivation of a new hematopoietic cell line with endothelial features from a patient with transformed myeloproliferative syndrome. Cancer 2000;88:344-51.
17. James C, Delhommeau F, Marzac C, Teyssandier I, Couedic JP, Giraudier S, et al. Detection of JAK2 V617F as a first intention diagnostic test for erythrocytosis. Leukemia 2006;20:350-3.
18. Ugo V, Le Gal G, Lecucq L, Mottier D, Oger E; EDITH Collaborative Study Group. Prevalence of the JAK2 V617F mutation is low among unselected patients with a first episode of unprovoked venous thromboembolism. J Thromb Haemost 2008;6: 203-5.
19. Poodt J, Fijnheer R, Walsh IB, Hermans MH. A sensitive and reliable semi-quantitative real-time PCR assay to detect JAK2 V617F in blood. Hematol Oncol 2006;4:227-33.
20. Hammond E, Shaw K, Carnley B, P'ng S, James I, Herrmann R. Quantitative determination of JAK2 V617F by TaqMan: an absolute measure of averaged copies per cell that may be associated with the different types of myeloproliferative disorders. J Mol Diagn 2007;2:242-8.
21. Jelinek J, Oki Y, Gharibyan V, Bueso-Ramos C, Prchal JT, Verstovsek S, et al. JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia. Blood 2005;106:3370-3.
22. Hammond E, Shaw K, Herrmann R. The JAK2 V617F mutation is detectable in granulocyte populations at greater than two copies per cell among individuals with myeloproliferative disorders. Leukemia 2007;21:815-6.
23. Najfeld V, Cozza A, Berkofsy-Fessler W, Prchal J, Scalise A. Numerical gain and structural rearrangements of JAK2, identified by FISH, characterize both JAK2 617V>F-positive and –negative patients with Ph-negative MPD, myelodysplasia, and B lymphoid neoplasms. Exp Hematol 2007; 35:1668-76.
24. Ricksten A, Palmqvist L, Johansson P, Andreasson B. Rapid decline of JAK2V617F levels during hydroxyurea treatment in patients with polycythemia vera and essential thrombocythemia. Haematologica 2008;93:1260-1.
25. Girodon F, Schaeffer C, Cleyrat C, Mounier M, Lafont I, Dos Santos F, et al. Frequent reduction or absence of detection of the JAK2-mutated clone in JAK2V617F-positive patients within the first years of hydroxyurea therapy. Haematologica 2008;93:1723-27.
26. Hermouet S, Dobo I, Lippert E, Boursier M-C, Ergand L, Perrault-Hu F, Pineau D. Comparison of whole blood vs purified blood granulocytes for the detection and quantification of JAK2V617F. Leukemia 2007;21: 1128-30.

©Ferrata Storti Foundation