Original Article

# Establishment Of A Novel RT- Qpcr Assay For The Detection Of Mutations In Myeloproliferative Neoplasms

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## Abstract

**Introduction:** Myeloproliferative Neoplasms (MPNs) are rare heterogeneous hematological disorders usually characterized by one or more lineages of myeloid cells in bone marrow and increase number of normal and abnormal cells. Janus kinase 2 valine to phenylalanine (JAK2-V617F) is usually present in Philadelphia-negative MPNs. Pathogenic mutation in JAK2-V617F cause's valine to phenylalanine substitution in JAK2 gene on exon-14. Different techniques such as Amplification refractory mutation system (ARMS-PCR), Allele-specific PCR (AS-PCR), High resolution melting (HRM) analysis and Molecular beacon probe-based RT-PCR are already available to diagnose JAK2-V617F mutation.

**Method:** Current study aimed to develop and optimize real-time PCR assay which will be available locally and be feasible, less expensive and less labor extensive. The DNA was extracted from 128 patients and analyzed on our optimized method using newly designed primers and probe. Standards were generated using in-vitro synthesized sequence (Kinco Biological) and Standard curve was obtained.

**Result:** Predicted sensitivity of the method is at least5% for allele burden of the mutation. The total of 128 MPN patients were included in the present study and 54 (42.1%) were JAK2-V617Fpositive according to the optimized protocols.

**Conclusion:** The study concluded that TaqMan Real time qPCR is efficient, sensitive and less expensive for the detection of JAK2-V617F mutation.

**Keywords:** MPNs, JAK2-V617F, PV, ET and PMF

## 1. INTRODUCTION

The MPNs are rare and heterogeneous class of hematological disorders with same characteristics [1]. MPNs involve conditions characterized by expansion of single or multiple myeloid cell lines in the blood formation site and a rise in the normal and abnormal peripheral blood cells. Instances include PV, ET and PMF. These disorders are stated to have a yearly occurrence of 2-3, 1.5-2, and 0.5-1.5 per 100,000 populations respectively [2-6].
The mutation contributes to a valine-phenylalanine (V617F) substitution in the JH2 region of the protein. The area of this gene was suggested to perform a direct role throughout the negative regulation for JAK2 signaling [7]. Patients great than a half are asymptomatic at the stage of diagnosis , while some from fatigue, weight loss, pruritus, exhaustion, fever and the early satiety. Bleeding and thrombosis may also be one of the early symptoms that ultimately contribute

to the identification of MPNs [8]. MPN Patients has a risk of vascular events (bleeding and thrombosis) and transformation to hematological malignancies and studies in Europe represent lower relative survival of MPN patients than controls [9].

In later years of the 20th century, the observation of Philadelphia (Ph) chromosome in individuals with CML have shown that the gained chromosomal aberrations could be the cause of cancer [10, 11]. MPNs continue over a longer period in patients, showing that JAK2-V617F mutated sequence is present within cells with long term capacity of self-renewal. In fact, JAK2-V617F is capable of being detected in early progenitor/ hematopoietic stem cells [12, 13].

Mutation like JAK2 that drive the most MPNs, it has set to the main focus for researchers on MPNs as germ-line predispositions. Although all mutations found in JAK2-V617F in undiagnosed MPNs are not germ-line variants, they may reflect the classical concept of indeterminate potential clonal hematopoiesis (CHIP). The 3 Copenhagen authors have observed 0.1-0.2% JAK2 mutation in somatic cells in population [14].In 2005, treatment and diagnosis of MPNs having no Ph chromosome were revolutionized by discovery of mutation in JAK2-V617F gene [15]. The JAK2-V617F mutation is having more importance in the diagnosis of ph-negative MPNs, especially in PV [2, 3]. Mutation in JAK2 gene is valuable diagnostic markers for the MPNs especially in PV. A single mutation (V617F) is observed in almost 95% of cases. Among the PV cases that are V617F-negative (almost 5%), many are linked with mutations in some other regions of the JAK2 gene within exon 12 [9, 16, 17].JAK2 is one of the member from Janus kinase group of cytoplasmic tyrosine kinases (consisting JAK1, JAK2, JAK3 and TYK2) associated with cytokine receptors domain which is present intracellular to mediate signals downstream in a response of ligand binding. Activation of JAK2 is essential for response to broad range of cytokines, like thrombopoietin (Tpo), granulocytes colony stimulating factors (G-CSF), erythropoietin (Epo), interleukin [3 and 5], and also interferon's[18].

Currently numbers of diagnostic techniques are available internationally for JAK2-V617F mutation detection including Melting curve analysis using the Light Cycler System, High resolution melting (HRM), Amplification refractory mutation sequencing (ARMS) PCR and Allele-specific PCR but these methods have many loopholes such as expensiveness, time consuming, costly and labor intensive in comparison to the current optimized assay[19- 23].

## 2. MATERIAL AND METHODS

### 2.1. Ethical statement

All the patients were properly informed about the research of study and written informed consent was obtained from all the patients. Most human researches are conducted according to ethical standard declaration of Helsinki [24]. The study was conducted after the approval of synopsis from Board of study and Advance study research board (ASRB), Department of MLT, The University of Haripur.

### 2.2. Sample collection

According to WHO criteria, whole blood samples were collected from conform MPN patients at Rehman Medical Institute (RMI) and Institute of Basic Medical Science, Khyber Medical University (IBMS-KMU) Peshawar KP. Proper informed consent was taken from all participants. A total of 4 ml of blood were collected from all participants in aseptic condition and DNA was extracted from blood samples.

### 2.3. DNA extraction

QIAamp Blood mini kit (Qiagen) was used for the extraction of DNA as per manufacturer guidelines. The Principle of DNA extraction is based on silica membrane column and performed as described previously [25].This method gave higher concentration of DNA yield and easy to extract DNA as per manufacturer instructions.

### 2.4. Primers and probe design

Newly synthesized primers were used in the current study. The JAK2 gene exon 14 regions was used to generate primers.Wild type forward primer CATTTGGTTTTAAATTATGGAGTATGTG,mutation V617F specific forward primer CATTTGGTTTTAAATTATGGAGTATGTT, common reverse primer CTGACACCTAGCTGTGATCCTG

and common probe $^{FAM}$TGCCTTTCTCAGAGCATCTG$^{BHQ1}$. The primers and probe were manufactured by Molecular Biology Products (Eurofins-USA). For internal control, the following forward (TGCTGAAAGTAGGAGAAAGTGC) and reverse (CCTACAGTGTTTTCAGTTTCAAAAA) were used.

## 2.5. Optimization of the Real Time PCR amplification conditions

The Real-time PCR was done using ABI-7500 thermal cycler. The current method uses a single pair of primers and probe used in optimization. First, in the optimization phase, DNA from positive cases was used. Reaction mixture for PCR had 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.6 µl of each primer, 0.6 µl of probe, 2 µl of DNA and 6.2 µl PCR water giving final volume of 20 µl reaction mixtures. The reactions were added to PCR plate and sealed with sealing film. The ABI 7500 Real-Time PCR systems was used for qPCR tests using 40 CT cycles each reaction. Analysis of the result was done by RQ manager software (Applied Biosystems). The following conditions was used to performthe PCR reactions; initially holding stage at 50ºC for 2 minutes, 2nd holding stage at 95ºC for 10 minutes, cycling stage at 95ºC for 15 second and at 60ºC for 1 minute and cycling stage repeat for 40 cycles. Fluorescent signals were recorded, and were analyzed after PCR performed upon ABI-7500.

## 2.6. Assay validation

TaqMan q-PCR probe was tested for inter and intra-assay reliability using 1 mutant JAK2-V617F and 1 wild-type samples, these samples were run in 2 and 10 replicates respectively, tested in multiple experiments. Thermal cycler ABI-7500 was used for real time PCR throughout the study.

### 2.6.1. Generation of standard

In-vitro synthesized target sequence (**Kinco Biological**) was cloned in bacterial plasmid for mutant DNA sequence having 161-bp lengths. The control containing 4µg of transformed plasmid was delivered. The control containing 100% of plasmid DNA was used for development of standard curve and positive control. For negative control, pooled DNA from 20 healthy individuals was used.

### 2.6.2. Standard curve development

The standard curve was developed by using transformed-plasmid. The 100% transformed-plasmid containing mutant sequence was serially diluted. For internal control, the following forward (TGCTGAAAGTAGGAGAAAGTGC) and reverse (CCTACAGTGTTTTCAGTTTCAAAAA) were used.

### 2.6.3 Analytical sensitivity and specificity

Serial dilution of DNA was made to test the sensitivity of the assay. Dilutions of 1:1, 1:2, 2:4, 1:8, 1:16 and 1:32 were used for JAK2-V617F DNA having wild-type DNA with a reproducible limit of detection (LOD) of 5% mutation positive DNA. Our optimized assay proved 99% specificity and 98% accuracy.

## 2.7. Statistical analysis

Data were analyzed by the help of SPSS software (Statistical Package for the Social Sciences Inc. Chicago, Illinois USA, version22).Quantitative data analysis was performed on GraphPad Prism 6.0 (GraphPad software, Inc., La Jolla, CA, USA). Cross tabulation were applied to the study variables.

# 3. RESULTS

The current research was conducted on Real time PCR assay for JAK2-V617F mutation using 6-carboxyfluorescein reporter and BHQ-1-labeled probes at 5 prime and 3 prime sites respectivel. Common probe was used both for wild type and mutant sequence.

## 3.1 Assay validation

For inter-assay reliability of TaqMan q-PCR probe we have chosen 1 mutant JAK2-V617F samples and 1 wild-type samples, each sample was run in 2 replicates on 5 days. The same protocol was used for intra-assay reliability,

choosing 1 mutants and 1 wild-type in 10 replicates. The procedure was performed upon ABI-7500 thermal cycler (Applied Biosystems).
After inter-/ intra-assay reliability testing, a desirable amplification plot was generated for both JAK-V617F and wild-type chosen samples (Figure 1). The Ct values were used to determine the CV for determining imprecision of our procedure. For intra-assay, the CV values of 10 replicates for mutant and wild-type were 1.61% and 1.73%, these values reveal that almost 99% intra-assay reliability exist. For inter-assay, CV values of 3 replicates for mutant and wild-type were 1.9 and 2.02; these values reveal that almost 98% inter-assay reliability (Table 1). Overall, less than 3%, indicating desired reproducibility for the current procedure. The assay imprecision was determined using CV value.

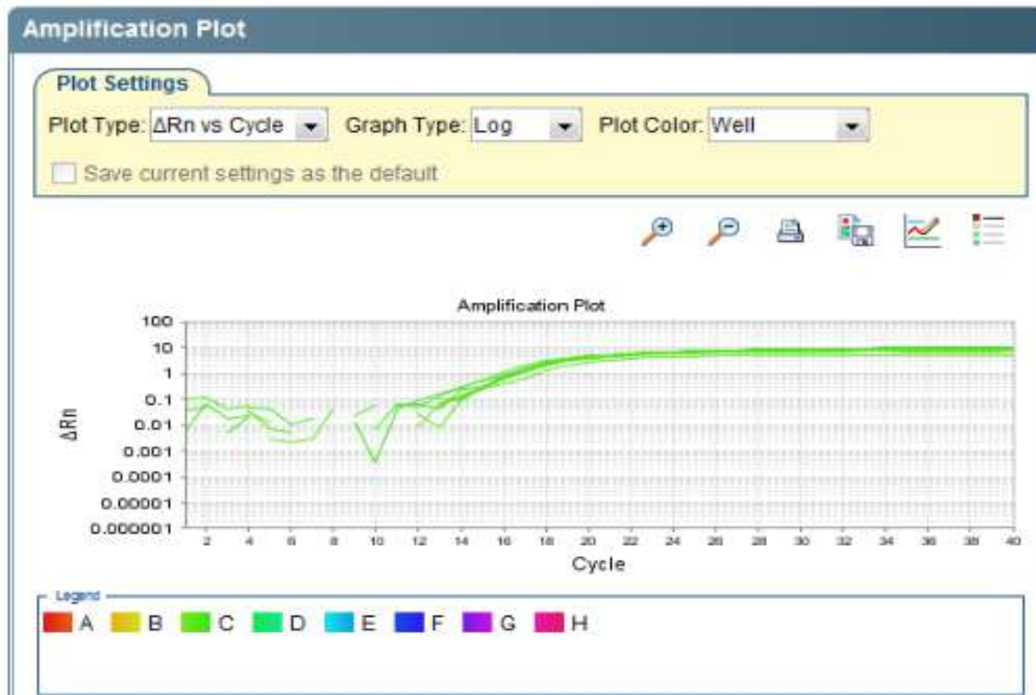


**Figure 1: Amplification plot for Intra-assay reliability**

**Table 1: Inter and intra-assay precision of TaqMan Q-PCR.**

| Specimen | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|
| | Mean Ct | SD | CV% | Mean Ct | SD | CV% |
| *JAK2* wild-type | 15.551 | 0.269 | 1.61 | 16.879 | 0.3408 | 2.02 |
| *JAK2* mutant (*V617F*) | 25.409 | 0.4096 | 1.73 | 26.321 | 0.4999 | 1.9 |

## 3.2.1 Standard curve development

In-vitro synthesized target sequence (Kinco Biological) was cloned in bacterial plasmid for mutant DNA sequence having 161-bp lengths. Serial dilutions were used from cloned fragments for both wild type and mutant sequences. Cloned fragment was used in the range of 1 to $10^6$ copies. Then, serial dilution was performed upon each mutated plasmid allele, from 0.1%-100%, in a solution of DNA from a normal individual as in heterogeneous DNA samples which was for utilized for diagnostic purposes. Figure 2 shows performance of the assay for JAK2-V617F allele,

which allowed for the detection of 0.5% mutant allele in the wild-type DNA background. Using these dilutions, standard curve was formed.

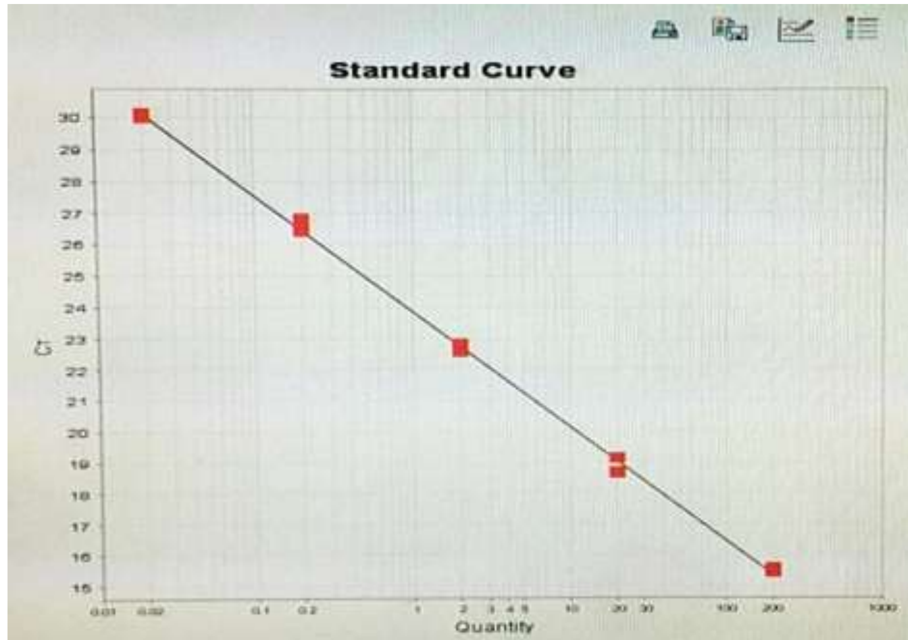


**Figure 2: Standard curve for JAK2-V617F Slope = -3.188, Y-Inter = 42.497, $R^2$0.994 and Eff% = 105.886**

### 3.2.2 Analytical sensitivity and specificity

Sensitivity analysis was performed using JAK2-V617F positive samples, described in Material and Methods. Assay sensitivity was determined using 100% mutated DNA and 100% wild type DNA. Serial dilutions were used for JAK2-V617F DNA with wild-type DNA shown a reproducible LOD of 5% mutation positive DNA (Figure 3). All samples were interpreted positive according to the dilution when the mutant allele presents at least 5% in a positive sample.

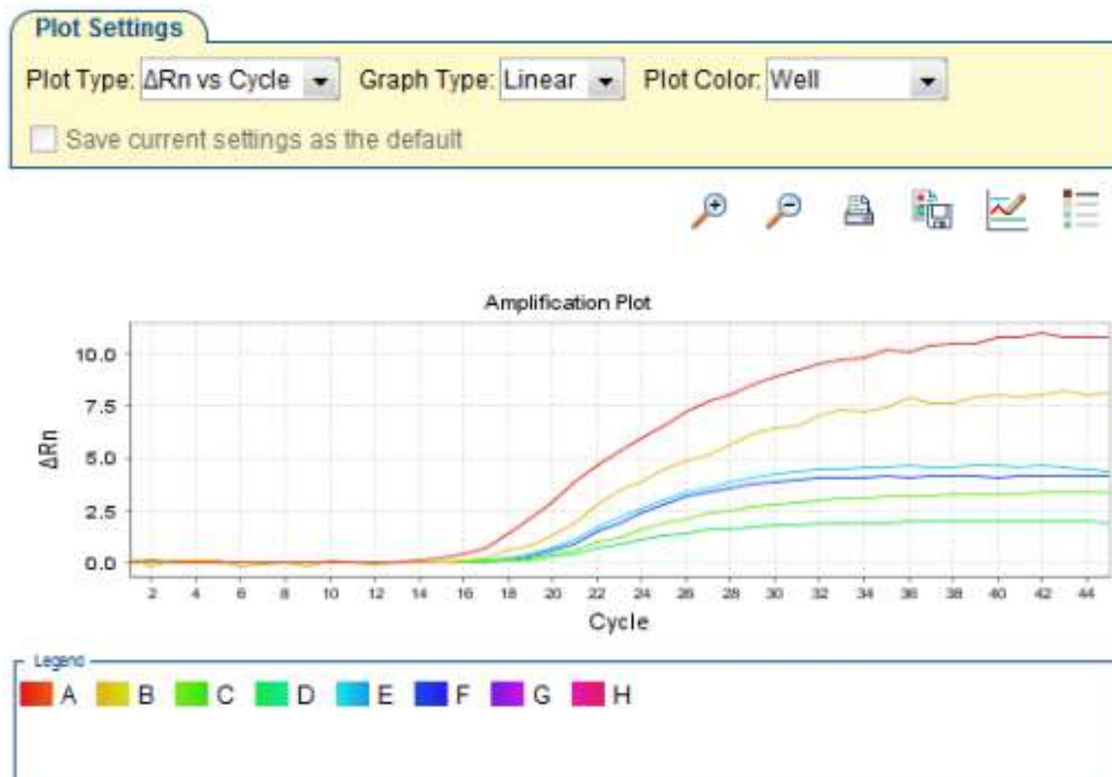

**Figure 3: LOD for JAK2-V617F positive DNA**

## 4. DISCUSSIONS

The current research performed to optimize assay for the detection of JAK-2 mutation in MPNs. Advancement and development of newly optimized can be more reliable for the mutation detections. The current study evaluated different techniques to detect the JAK2-V617F mutation in MPNs, in comparison to that techniques here in this study we developed a novel TaqMan qPCR diagnostic assay that could be used for patients and would be enough sensitive to detect and identify mutation in a sample containing low level of JAK2-V617F mutant allele burden in MPNs.

A study conducted on MPNs patients [26] who reported 63.7% (n=644) out 1010 registered patients in different hospitals. Another recent study in Pakistan conducted on 137 MPNs patients reported 37.2% (n=51) are same as the current study [27].The current optimized method was able to achieve sensitivity level of 5% mutated allele in the wild-type background of sample, value that is higher as compared to bidirectional sequencing, which detect 10 to 15% mutation burden [28] and melting curve technique with 5% sensitivity but it is too costly compared to our study [29]. In the current study, a novel method is developed for the detection of JAK2-V617F mutation. If we compare current protocol with sequencing, the current assay has advantages of being less expensive, being less time consuming and require less personnel.

Previously published detection techniques included; allele-specific PCR which is sensitive enough to detect 2.5% and another study has 0.01% of sensitivity for mutant DNA[30-32], droplet digital PCR have sensitivity limit of 96%[33], allele-specific competitive blocker PCR (ACB-PCR) have 1%[34], an ARMS [35], pyro-sequencing have 5-10%[6, 36], sequencing 10-40%[2, 4, 15], restriction fragment length polymorphism (RFLP) has 5% [32], high resolution melting curve analysis (HRM) having sensitivity of 0.5-1%[37]. Although these approaches are enough sensitive but are not quantitative. The current study reported 99% intra-assay reliability and 98% inter-assay reliability which is almost same as a most recent published works [25]. Multiple studies also performed specificity analysis, claiming 100% specificity and 98.5% accuracy for detection of JAK2-V617F [33, 35, 38]. The current optimized assay proved 99% specificity and 98% accuracy which are almost same as previously mentioned studies.

## 5. CONCLUSION

This studyconclude that the TaqMan qPCR assay is efficient, sensitive, less expensive and less labor intensive techniquehaving ability to detectJAK2-V617F mutation in MPNs patients containing PV, ET and PMF. Additionally, the assay might beperformed inroutine laboratoryfor the diagnosis of Ph-negative MPNs. However, this technique may also useful for monitoring change in mutated allele burden in patient under treatment.

## 6. RECOMMENDATIONS

- ✓ The practical applicability of this assay on other real time instrument still need further testing. Same type of assay can also be expended to detect other JAK2-V617F and other mutations.
- ✓ Detection of rare mutation other than JAK2V617F needs study on large scale sample.
- ✓ A multiplex q-PCR can be optimized for detection of both exon 12 and 14, which will greatly decrease cost.
- ✓ The local availability of the current assay will become choice of method for the detection of JAK2-V617F.

## 7. STUDY LIMITATIONS

i. The study is not applicable to detect mutant allele burden of MPNs patients.
ii. The assay is confined to ABI-7500, other tools may not be applied to the current assay.

## 8. STATEMENTS AND DECLARATIONS

**Ethical approval**

The study was approved by the ethical committee and Advanced research board committee of the University of Haripur department of Medical Laboratory Technology.

**Consent to participate**
Informed consent was obtained from all the individual participants included in the study.

**Consent to publish**
The authors affirm that human research participants provided informed consent for publication.

**Availability of data**
All data generated or analyzed during this study are included in this published article.

**Competing interest**
The authors have no relevant financial or non-financial interest to disclose.

**Author contributions**
Muhammad Umair, Dr. Yasar Mehmood Yousafzai and Dr. Aamir Ali Khan conceive the idea, Muhammad Umair and NaeemUllah perform the experiments. Jamshid Ullah, Nasir Ahmad, Waqar Saeed, Iftikhar Ahmad and Dr. Saima Qayum assisted the experimental work and help in the supply of required materials. Dr. Abdul Jabbar, Dr. Sadiq Noor Khan, Dr. Aamir Ali Khan and Muhammad Umair write the initial draft of the manuscript. Dr. Yasar Mehmood Yousafzai and Dr. Aamir Ali Khan validate the data and provides the overall supervision.

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