

## Development and implantation of PCR-SSP for the genotyping of *JAK2 V617F* mutation

### Desenvolvimento e implantação de metodologia molecular baseada em PCR-SSP para genotipagem da mutação V617F de *JAK2*

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**ABSTRACT**

The JAK2 protein promotes cells growth and proliferation, and mutations in the *JAK2* gene can result in increase of the number of blood cells and in development of myeloproliferative neoplasms. This study aimed to develop and implement the PCR-SSP (Sequence-Specific Primer - Polymerase Chain Reaction) to detect the *JAK2V617F* mutation. Therefore, a literature review about genotyping methodologies for this mutation was conducted and primers were based on the model proposed by Xavier (2009), tested and adjusted to the best amplification condition. The PCR-SSP technique was standardized and was effective for the detection of the *JAK2V617F* mutation. As it is not expensive, the technique reduces costs and can be implanted even in small molecular biology laboratories, helping the diagnosis of patients with myeloproliferative diseases and favoring research related to this gene.

**Keywords:** Genotyping techniques, Myelodysplastic-Myeloproliferative Diseases, Janus Kinase 2.

**RESUMO**

A proteína JAK2 participa do crescimento e da proliferação celular e as mutações no gene *JAK2* podem resultar em aumento do número de células sanguíneas e no desenvolvimento de neoplasias mieloproliferativas. Desta forma, este estudo teve o objetivo de desenvolver e implantar a técnica de PCR-SSP (*Sequence-Specific Primer-Polymerase Chain Reaction*) para a detecção da mutação V617F de *JAK2*. Para tanto, foi realizada uma revisão de literatura sobre metodologias de genotipagem da mutação em questão e os *primers* foram confeccionados baseados no modelo proposto por Xavier (2009), testados e ajustados às melhores condições de amplificação. A técnica de PCR-SSP foi padronizada e mostrou-se eficaz para a detecção da mutação *JAK2V617F*. Por ser pouco dispendiosa, este método diminui custos e pode ser implantado inclusive em laboratórios de biologia molecular de pequeno porte, auxiliando o diagnóstico de pacientes com doenças mieloproliferativas e favorecendo as pesquisas relacionadas a este gene.

**Palavras-Chave:** Técnicas de genotipagem, Doenças Mieloproliferativas-Mielodisplásicas, Janus Quinase 2.

**1 INTRODUCTION**

In 1951, William Dameshek introduced the term myeloproliferative disorders (MPD) to clonal hematopoietic stem cell diseases related to the overproduction and abnormal proliferation of one or more myeloid lineages in the bone marrow and the

increase of mature cells in the peripheral blood. Clinically, this is associated with risks of hemorrhagic and thrombotic complications and fibrotic or leukemic evolution (CAMPBELL; GREEN, 2006; PEDRAZZINI et al., 2016; LEVINE et al., 2005; BENCH et al., 2013). Also according to Dameshek (1951), this group of diseases contained, among other pathologies, four classic representatives: Polycythemia Vera (PV), Essential Thrombocythemia (ET), Primary Myelofibrosis (PMF) and Chronic Myeloid Leukemia (CML). So far, there is no cure for these diseases (GOULDING et al., 2008; ZHAO et al., 2011).

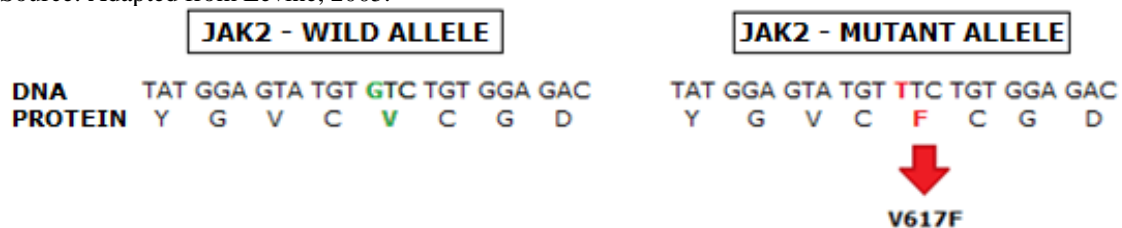
With the discovery of the Philadelphia chromosome (Ph) in 1960, CML was classified as a Philadelphia chromosome-positive (Ph+) myeloproliferative disease, since the translocation between chromosomes 9 and 22 was found in individuals with the disease (NOWELL; HUNGERFORD, 1960). As a result, a BCR-ABL chimeric protein with tyrosine kinase activity was formed (MARCHIANI, 2015; MEIRELES, 2011; SPIVAK, 2002). PV, ET and PMF were classified as myeloproliferative diseases with a negative Philadelphia chromosome (Ph-), due to the absence of 9; 22 translocation. In this group of diseases, mutations in the *JAK2* gene (Janus kinase 2) are usually involved, and its appearance was found in 98% of patients with PV and in 50-60% of patients with ET and PMF (PEDRAZZANI, 2016; MARCHIANI, 2015; LEVINE et al., 2005; BENCH, 2013). In 2008, the WHO (World Health Organization) changed the term myeloproliferative diseases (MPDs) to myeloproliferative neoplasms (MPNs) due to the neoplastic character of cell proliferation (MARCHIANI, 2015).

In humans, the *JAK2* gene is located on the short arm of chromosome 9 (9p24.1) and has an essential role in myeloid differentiation, especially in the erythroid lineage. Once activated, the *JAK2* gene triggers the activation of mediators, which stimulate the activation and transcription of genes that promote erythropoiesis (ZAGO; CALADO, 2013). The Janus kinase 2 (JAK2), encoded by the *JAK2* gene, is a member of the Janus kinase family (Janus kinases - JAKs), which also include JAK1, JAK3 and TYK2 (tyrosine kinase 2) proteins. JAKs have seven homologous domains (JH1-JH7) located in four regions: JH1 (JAK homology 1 - kinase domain); JH2 (JAK homology 2 - pseudokinase domain); FERM (band four-point-one, ezrin, radixin, moesin homology domains - JH5, JH6, JH7 and part of JH4 domains); and SH2-like (SRC homology 2-like - JH3 domain and another part of JH4) (XAVIER, 2009; SCHINDLER et al., 2007). Moreover, JAKs proteins are influenced by growth factors and cytokine-mediated

signaling, and also participate in changes observed in intracellular signaling molecules, such as constitutive activation of STAT3 (Signal transducer and activator of transcription 3) and overexpression of Bcl-xl.

The main genetic variation present in Philadelphia chromosome-negative myeloproliferative neoplasms (Ph-MPNs) is the acquired mutation in the *JAK2* gene, c.1849G>T (rs77375493), which results in the encoding of an altered protein with the substitution of the amino acid valine for phenylalanine in codon 617, abbreviated as V617F, in the Janus 2 kinase, *JAK2V617F* (FANTASIA et al., 2014). This variation is schematically represented in Figure 1.

Figure 1. DNA sequence and protein translation for wild-type (represented in green) and mutant (represented in red) alleles of *JAK2*. The substitution of guanine for thymine results in a substitution of valine (V) for phenylalanine (F) at codon 617, characterizing the V617F *JAK2* mutation (red arrow). Source: Adapted from Levine, 2005.



As the mutation in the *JAK2* gene, essentially *JAK2V617F*, appears in most of people affected by the myeloproliferative neoplasms mentioned, the detection of this mutation is a criterion for the diagnosis of these pathologies according to the World Health Organization (LEVINE et al., 2005; BENCH, 2013; ARBER et al., 2016).

Several molecular techniques have been developed to detect the *JAK2V617F* mutation, such as allele-specific polymerase chain reaction (AS-PCR), real-time polymerase chain reaction (RT-PCR), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing techniques (BENCH, 2013; ZHAO et al., 2011). Among these molecular methods, there are variations in sensitivity, in the cost of reagents, in the execution time and in the equipment needed to carry them out: sequencing techniques, for example, demand expensive and complex equipment; PCR-RFLP uses restriction enzymes, a costly reagent. At the same time, the polymerase chain reaction sequence-specific primers (PCR-SSP) technique, which uses specific primers to detect the nucleotide sequence of polymorphic alleles, was chosen as the method of this study mainly because it has low cost and does not require complex equipment for its development, which means that even small molecular biology laboratories would be able

to employ this diagnostic method. Moreover, this molecular technique has an allelic load detection sensitivity ranging from 0.1 to 5%, being able to diagnose most patients with myeloproliferative neoplasms (BENCH, 2013; ZHAO et al., 2011; QUIRINO et al., 2019).

Therefore, the objective of this study was to develop and implant the PCR-SSP technique for the detection of the V617F mutation of the *JAK2* gene. This standardization may help in the diagnosis of patients with myeloproliferative neoplasms.

## 2 MATERIALS AND METHODS

The biological material used was whole blood, collected in ethylenediamine tetraacetic acid (EDTA), from positive and negative individuals for the *JAK2* mutation, previously genotyped by Sanger sequencing and stored at the Immunogenetics Laboratory of the Maringá State University (LIG-UEM). DNA was extracted with the commercial QIAprep Spin Miniprep Kit (QIAGEN®, Germany), according to the manufacturer's recommendations. The use of these biological samples was approved by the Ethics Committee on Human Research of the Maringá State University (UEM) (CAAE 14508313.2.0000.0104).

To standardize the PCR-SSP technique, which is based on the specificity of the amplification of mutated DNA sequences using a mutation-specific primer, three primers were designed based on the model proposed by Xavier (2009). The *JAK2*-F1 forward primer was constructed to amplify the conserved region of the *JAK2* gene, which is present in all DNA samples and is used to verify the integrity of the PCR reaction (internal control); therefore, regardless of whether the fragment to be analyzed contains the mutated allele or not, an amplification product (amplicon) of 364 bp (base pairs) will be generated. The *JAK2*-F2 forward primer was constructed to anneal the nucleotide sequence containing the SNV (Single Nucleotide Variation) with rs77375493, which would generate a 203 bp product. Finally, the reverse primer *JAK2*-R1 was constructed, which is complementary to the final sequence to be amplified and, consequently, does not reach the SNV region (Table 1).

Table 1. Acquired primers for standardization according to Xavier, 2009. Note the intentional mismatch in the JAK2-F2 primer sequence (highlighted in red) and the genotype-specific nucleotide for the *JAK2V617F* mutation underlined.

Source: Author, 2021.

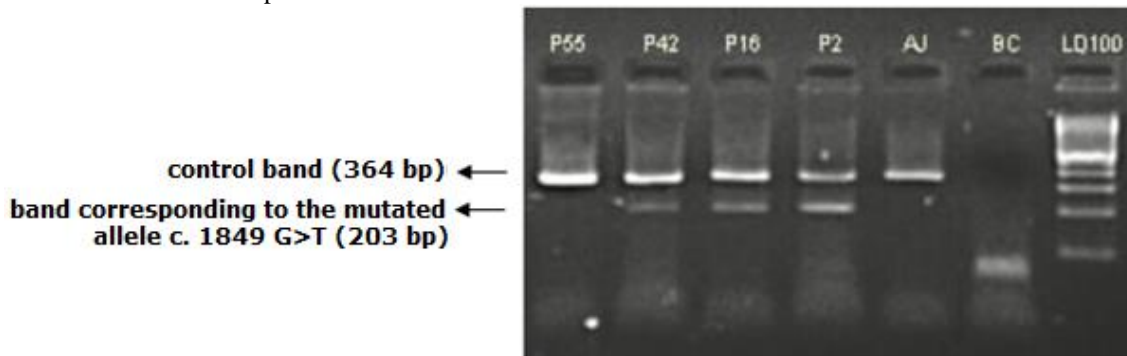
Primers	Sequence	Synthesis scale
JAK2-F1	5'-ATCTATAGTCATGCTGAAAGTAGGAGAAAG	25N
JAK2-F2	5'-AGCATTTGGTTTTAAATTATGGAGTATATT	25N
JAK2-R1	5'-CTGAATAGTCCTACAGTGTTCAGTTTCA	25N

The PCR-SSP reaction took place with a final volume of 10  $\mu$ L, containing 1X buffer; 0.2 mM dNTP; 1.5 mM MgCl<sub>2</sub>; 0.5  $\mu$ M of each of the forward primers (JAK2-F1 and JAK2-F2) (Life Technologies, Brazil) and 1  $\mu$ M of the reverse primer JAK2-R1; 1 U/ $\mu$ L of Taq DNA Polymerase (Invitrogen™, USA) and 100 ng/ $\mu$ L of DNA. The PCR thermocycling conditions, performed in a Veriti thermocycler (Applied Biosystems), were: 1 initial incubation cycle at 94°C for 10 minutes; 36 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds; and 1 cycle of final extension at 72°C for 6 minutes. The amplified products were evaluated by electrophoresis using 3% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen Life Technologies, Grand Island, NY), it runned at 150W, 300mA, 150V for 15 minutes and visualized under ultraviolet light.

### 3 RESULTS

After performing the PCR-SSP technique using the built primers (JAK2-F1, JAK2-F2 and JAK2-R1) for the detection of *JAK2V617F* mutation, two scenarios were expected depending on the genotype of the analyzed individual: (1) the samples that were negative for the *JAK2V617F* mutation (wild sequence) would generate a 364 bp amplicon, result from the amplification between the JAK2-F1 and JAK2-R1 primers; (2) positive samples for the *JAK2V617F* mutation (sequence containing the mutated allele) would generate the same 364 bp amplicon, amplification result between JAK2-F1 and JAK2-R1 primers, and a second 203 bp amplicon (containing the SNP), generated by the amplification between JAK2-F2 and JAK2-R1 primers. Given the success of the results, the validation of the technique was confirmed and its standardization established (Figure 2).

Figure 2. Electrophoresis using 3% agarose gel of PCR-SSP for detection of the V617F mutation of the *JAK2* gene. P42, P16 and P2: Positive controls for the V617F mutation of the *JAK2* gene; P55 and AJ: Negative controls; BC: Negative reaction control - no DNA present; LD100: 100 base pairs (bp) molecular weight marker. Note that samples P55 and AJ contain only the control band, result of amplification between *JAK2*-F1 and *JAK2*-R1 primers. Samples P42, P16 and P2 contain, besides the control band, the band indicative of the sequence containing the mutated allele (*JAK2*V617F), result of amplification between *JAK2*-F2 and *JAK2*-R1 primers.



Source: Author, 2021.

#### 4 CONCLUSION

The technique developed, besides being effective for the mutation in question, facilitates genotyping and aids in the diagnosis of patients suspected of having myeloproliferative neoplasms. As this entire process offers adequate sensitivity at a low cost, even small molecular biology laboratories can perform this methodology, which drives current and future research into the *JAK2* gene and the consequences of its mutations.

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