Real-Time monitoring of the JAK2V617F mutation by ultra-rapid, high sensitive Fluorescent AS-LAMP

Giulia Minnuci1, Giulia Amicarelli1, Elena D’Agostini1, Riccardo Mesturini1, Silvia Salmoiraghi2, Orietta Spinelli2, Fabrizio Bonelli1,
Francesco Colotta1, Alessandro Rambaldi2
1DiaSorin SpA, Gerenzano (VA), Italy; 2Ospedali Rumliri di Bergamo, Bergamo, Italy

INTRODUCTION

The V617F mutation in the JAK2 gene causes constitutive activation of the Janus 2 kinase, leading to uncontrolled cell proliferation and resistance to apoptosis (1). JAK2V617F is frequent in Myeloproliferative Neoplasms (MPNs), being found in 80-95% of Polycythemia Vera (PV), 35-95% of Idiopathic Myelofibrosis (IMF), and 23-57% of Essential Thrombocythemia (ET) (2). Therefore the molecular detection of the JAK2V617F mutation is mandatory in the diagnostic work-up of MPNs, as recommended by the 2008 WHO classification criteria (3).

Several molecular diagnostic techniques are currently used (4) but with some important limitations such as a low sensitivity, low specificity, long reaction time, the requirement of labor intensive procedures performed by specialized and high cost equipments.

We recently described an innovative, non-PCR method called A
ty Specific Loop-mediated isothermal Amplification (AS-LAMP) (5). In this study we present an improvement of the AS-LAMP assay, introducing an internal control reaction in a duplex format, to increase the overall reliability, the rapidity and to allow real-time signal monitoring.

METHOD

The Duplex Fluorescent AS-LAMP reaction consists in:

- a primer set for JAK2V617F alleles
- a primer set for ABL Internal Control gene

To establish the specificity, Fluorescent AS-LAMP assay was performed on DNA extracted from 11 cell lines negative for JAK2V617F mutation. The assay amplified exclusively the Internal Control ABL gene on all the samples tested (315 replicates), allowing to validate the JAK2V617F negative results.

The two primer sets incubated at constant temperature in presence of a DNA Polymerase with strand-displacement activity and an intercalating dye, efficiently amplify the JAK2V617F and ABL DNA. The amplified products are characterized by two distinct melting temperature, detectable by performing an annealing analysis. Fluo AS-LAMP generates an annealing peak for JAK2V617F mutation at 83.7°C±0.3 and a different peak at 91.2°C±0.7 for ABL internal control.

RESULTS

Level of Sensitivity of Duplex Fluorescent AS-LAMP

The Fluorescent Duplex AS-LAMP reaction amplifies and distinguishes JAK2V617F and Internal Control ABL genes thanks to the employment of two primer sets, each one consisting in a pair of external primers (F3, B3) and a pair of internal primers (FIP, BIP) that recognize 6 distinct regions on the target gene (6). The addition of a DNA probe and a self-annealed loop primer to block and amplify the JAK2 wild type and mutant sequences respectively, ensures a high level of selectivity. The amplification of ABL gene has been optimized in order to produce a signal in JAK2V617F negative and low positive samples, for validation of negative results.

The sensitivity of the duplex Fluorescent AS-LAMP assay was established on 25 ng DNA extracted from UKE1 cell line (JAK2V617F positive) serially diluted into wild type DNA from BJAB cell line.

The assay detects the JAK2V617F mutation down to 0.005% within the reaction time (30 min). Interestingly, a direct relationship between the amount of mutant target tested and the height of peaks obtained after annealing analysis has been consistently observed.

Comparison between ASO-PCR and Fluo AS-LAMP on clinical samples

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Total samples tested</th>
<th>Positive by ASO-PCR</th>
<th>Positive by Fluorescent AS-LAMP</th>
<th>Concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycythemia Vera</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>100%</td>
</tr>
<tr>
<td>Essential Thrombocythemia</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>100%</td>
</tr>
<tr>
<td>Other hematological malignancies</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>100%</td>
</tr>
<tr>
<td>Negative Controls*</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>100%</td>
</tr>
</tbody>
</table>

*negative controls are ET, 1.3 PPo-ET and 10/10 healthy donors

the high sensitivity level of the assay allowed to correctly detect the JAK2V617F mutation on 17 DNA samples extracted directly from peripheral blood, avoiding the step of granulocytes isolation.

CONCLUSION

We have developed a novel Fluorescent Duplex AS-LAMP assay for simultaneous amplification and discrimination of JAK2V617F and ABL sequences in clinical samples. This novel assay represents an improvement of the previously described AS-LAMP (5) by implementation of an Internal Control reaction that ensure reliability by excluding that negative results are due to poor DNA target quality, presence of inhibitors or suboptimal reaction conditions. This feature has been achieved by adding an intercalating dye to the reaction mix, and by performing an annealing analysis after isothermal incubation.

In conclusion Fluorescent AS-LAMP is a highly sensitive and specific assay for the rapid detection of JAK2V617F mutation in patients affected by Myeloproliferative Neoplasms.

References:
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(3) A Tefferi et al. Leukemia 2008; 22, 14
(5) G Minnucci et al. Haematologica 2012, E83
(6) T Notomi et al. NAR 2000; 15:63
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