A. Primer and Eprobe design without Edesign function (values shown were re-calculated by Edesign.)

<table>
<thead>
<tr>
<th>OLIGO</th>
<th>start</th>
<th>len</th>
<th>tm</th>
<th>tm (VAR)</th>
<th>gc%</th>
<th>any</th>
<th>3' hairpin</th>
<th>seq</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEFT Primer</td>
<td>158</td>
<td>23</td>
<td>57.02</td>
<td>57.02</td>
<td>43.48</td>
<td>2.80</td>
<td>2.40</td>
<td>27.58</td>
</tr>
<tr>
<td>RIGHT Primer</td>
<td>350</td>
<td>20</td>
<td>58.91</td>
<td>58.91</td>
<td>55.00</td>
<td>3.00</td>
<td>2.40</td>
<td>0.00</td>
</tr>
<tr>
<td>Eprobe (Reverse)</td>
<td>215</td>
<td>17</td>
<td>58.39</td>
<td>55.92</td>
<td>41.18</td>
<td>5.40</td>
<td>3.40</td>
<td>54.32</td>
</tr>
</tbody>
</table>

Product size: 193,
Primer pair compl any: 4.40, 3': 3.20,
LEFT Primer-Probe compl any: 4.20, 3': 1.60,
RIGHT Primer-Probe compl any: 4.00, 3': 2.00

Location of designed primers and Eprobe

B. Primer and Eprobe design by Edesign

<table>
<thead>
<tr>
<th>OLIGO</th>
<th>start</th>
<th>len</th>
<th>tm</th>
<th>tm (VAR)</th>
<th>gc%</th>
<th>any</th>
<th>3' hairpin</th>
<th>seq</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEFT Primer</td>
<td>156</td>
<td>25</td>
<td>58.89</td>
<td>58.89</td>
<td>40.00</td>
<td>2.80</td>
<td>2.40</td>
<td>27.58</td>
</tr>
<tr>
<td>RIGHT Primer</td>
<td>366</td>
<td>21</td>
<td>58.79</td>
<td>58.79</td>
<td>52.38</td>
<td>2.40</td>
<td>0.00</td>
<td>1.81</td>
</tr>
<tr>
<td>Eprobe (Reverse)</td>
<td>204</td>
<td>21</td>
<td>61.72</td>
<td>56.41</td>
<td>38.10</td>
<td>7.00</td>
<td>0.60</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Product size: 211,
Primer pair compl any: 3.80, 3': 1.20,
LEFT Primer-Probe compl any: 3.60, 3': 0.20,
RIGHT Primer-Probe compl any: 3.60, 3': 2.40

Location of designed primers and Eprobe

S7 Fig. Designed Eprobes and primers for evaluation in mutation detection.
Design-A was conducted without any upgrade implemented in Edesign other than $T_m$ calculation of Eprobe/DNA duplex. Design-B was conducted by Edesign. Design-A has higher predicted hairpin $T_m$ of Eprobe and higher pair complementarity than Design-B.
Target sequence: pandemic 2009 H1N1 influenza virus genome segment 6 (neuraminidase) H275Y mutation

ACAACGGCATAATAACAGACACTATCAAGAGTTGGGAAGAACATATATTTGAAGACACAAAGAGTCTGAATGTCATG
TGTAATGCTGTCTTTACTGTAATGCGATGGGACCAAGTGTGATGGGACACGCCGTCAACAGATCTTCAGAATA
GAAAAGGGAAAGATAGTCAAATACGTCGAAATGGAATGCCCTAATTAT[CT]ACTATGAGGAATGCTCTGCTTTATCC
TGATTCTAGTGAATACATGTGTGCGAGGATAACTGGCATGGCTCGAATCGAC
CGTGGGTGTCTTTCAACCA
GAATCTGGAATATCAGATAGGATACATATGCAGTGGGATTTTCGGAGACAATCCACGCCCTAATGATAAGACAGGC
AGTTGTGGTCCAGTATCGTCTAAT

Edesign setting:

Positions below were set as excluded positions (non-target mutations) for primers and internal Eprobe:

Genotyping by Internal Probe: ON

PRIMER_PRODUCT_SIZE_RANGE: 150-250 100-300 301-400 401-500 501-600 601-700 701-850 851-1000

Prime Size Min: 18 Opt: 20 Max: 25

TH: Primer Max Hairpin: 47 (No primers found with the default value 24)

Internal Probe Size Min: 16 Opt: 18 Max: 21

Internal Probe Tm Min: 57.0, Opt: 62.0, Max: 67.0

Weight of Internal Probe Tm Difference by Target Variant: 1.0

Weight of OLD: Pair 3’ Complementarity: 1.0

Other parameter values were the same as the default settings of Edesign version 2.0.

Experimental Protocol:

PCR reactions were setup using 5 µl of 5×Light-Cycler 480 Genotyping Master (with 5% Formamide), 5 µl template DNA, 0.2 µM Eprobe, 0.9 µM primer in opposite strand of Eprobe and 0.3 µM primer in the same strand of Eprobe, in a total volume of 25 µl. Real-time PCR experiments were run on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) after activation of the hot-start enzyme for 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 72°C.

Amplification signals were detected during the annealing step of each cycle at 57°C, using a SYBR Green I (483 nm–533 nm) filter for thiazole orange (D514). For melting curve analysis, the PCR was followed by heating the reaction mixture to 95°C for 15 s, cooling to 37°C, holding at 37°C for 7 min, and then slowly heating again to 95°C at a ramp rate 2.2°C/s and continuous fluorescence acquisition at the indicated wave length. All PCR reactions and melting curve experiments were always performed in triplicate, and each experiment included a negative control where 1×TE Buffer (Promega Japan, Tokyo, Japan) was added instead of a template DNA.