APPLICATION NOTE

Detect SNPs with KASP genotyping technology on SpectraMax microplate readers

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Introduction
Genotyping is a process for analyzing genetic differences among individuals by examining their DNA sequences. The many methods available for genotyping enable researchers to investigate genomic diversity in humans, microorganisms, and plants. Single nucleotide polymorphisms (SNPs) are one of the most common types of genetic variation, consisting of a single nucleotide mutation at a specific locus. SNP genotyping has proven very useful in identifying disease-related mutations in various species, and as a result many techniques for SNP detection have been developed.

Kompetitive Allele Specific PCR (KASP) is one of the most widely used techniques for SNP genotyping as it is highly accurate, cost effective, and provides great flexibility in terms of assay design. KASP uses two allele-specific forward primers and one common reverse primer to amplify target DNA sequences via polymerase chain reaction (PCR) such that the reaction products are labeled with the fluorescent dye corresponding to their sequence. If a sample is homozygous, PCR products will be labeled with the fluorescent dye HEX or FAM only, but if it is heterozygous both HEX- and FAM-labeled products will be present. Unincorporated fluorescent dyes are quenched.

In this application note, we demonstrate how SpectraMax microplate readers can be used to read final KASP products using a KASP assay validation kit from LGC Genomics.

The first experiment was performed to validate that the readers generated distinctive signals for each fluorophore including FAM, HEX, and FAM/HEX. In the second experiment, the KASP assay was performed with known DNA samples provided by the vendor, and the PCR products were detected on the readers to verify correct genotyping of the samples.

Materials
- Standard ROX validation kit (LGC Genomics cat. KBS-1014-101)
- Thermal cycler (MJ Research cat. PTC-200)
- TempPlate semi-skirted 96-well PCR plate (USA Scientific cat. #1402-9700)
- 384-well low volume solid black microplate (Corning cat. #3676)
- Microseal B PCR plate sealing film (Bio-Rad cat. #MSB1001)
- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices cat. #i3x)
- SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices cat. #M5e)
Methods

Microplate reader compatibility test

The validation kit included individual tubes of diluted fluorophores: FAM, HEX, and FAM+HEX. These were dispensed directly into a microplate and did not require any thermal cycling. Each tube of fluorophore also contained ROX, a passive reference dye that enables normalization of HEX and FAM signal, eliminating the effects of variation due to pipetting. 5 µL of each fluorophore (HEX, FAM, and HEX+FAM) was dispensed to triplicate wells of a 384-well plate. The plate was sealed with a clear film and then centrifuged at 560 x g for one minute. After centrifugation, the plate was immediately read on the M5e and i3x readers using the instrument settings listed in Table 1.

Detection of KASP genotyping reactions

For the second test, the validation kit contained 36 known samples, including 33 DNA samples and 3 no-template controls (NTC). 5 µL of each sample was pipetted into a 96-well plate for PCR. The same volume of genotyping mixture (2x KASP Master mix plus KASP Assay mix) was added to each well. The passive reference dye ROX was included in the KASP Master mix. The plate was sealed with a clear film and centrifuged at 560 x g for one minute. A thermal cycling reaction was immediately initiated. Detailed thermal cycling conditions are listed in Table 2. After the reaction was complete, 5 µL of each sample was transferred into wells of a 384-well plate and detected on the microplate readers using the instrument settings listed in Table 1.

Results

For the first test, relative fluorescence units of each fluorophore, including FAM, HEX, and a mixture of both, were normalized to ROX and the normalized values were plotted in SoftMax® Pro Software. As shown in Figure 1, three distinct clusters were generated.

For the second test, the ROX-normalized fluorescence of amplified DNA samples were plotted in SoftMax Pro software (Figure 2). Three well-defined clusters were detected. FAM homozygotes were located near the X axis, whereas HEX homozygotes were located close to the Y axis, and the heterozygote samples

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles/step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Activation</td>
<td>94°C</td>
<td>15 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>20 sec</td>
<td>10 cycles</td>
</tr>
<tr>
<td></td>
<td>Annealing/Elongation</td>
<td>61-55°C</td>
<td>60 sec (drop 0.6°C per cycle)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Denaturation</td>
<td>94°C</td>
<td>20 sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td></td>
<td>Annealing/Elongation</td>
<td>55°C</td>
<td>60 sec</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Plate reader settings used for data acquisitions.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>M5e</th>
<th>i3x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Read mode</td>
<td>Fluorescence</td>
<td>Endpoint</td>
</tr>
<tr>
<td>Read type</td>
<td>Endpoint</td>
<td>Endpoint</td>
</tr>
<tr>
<td>Wavelength</td>
<td>Lm1 (FAM)</td>
<td>485 ex/515 cutoff/520 em</td>
</tr>
<tr>
<td></td>
<td>Lm2 (HEX)</td>
<td>535 ex/550 cutoff/556 em</td>
</tr>
<tr>
<td></td>
<td>Lm3 (ROX)</td>
<td>575 ex/610 cutoff/610 em</td>
</tr>
<tr>
<td>PMT and optics</td>
<td>Auto, 6 flashes/read</td>
<td>6 flashes/read</td>
</tr>
</tbody>
</table>

Table 2. Thermal cycle settings for KASP genotyping reactions.

![Figure 1. Reader validation using three fluorophores.](image-url) HEX/ROX and FAM/ROX values obtained from both M5e and i3x formed three distinctive clusters.
containing both FAM and HEX formed a cluster between the two homozygotes clusters. No-template controls formed a cluster near the origin as expected. The data closely matched the genotyping results provided by the vendor for the validation kit.

**Conclusion**

SpectraMax M5e and i3x plate readers have been fully validated for use with KASP genotyping assays. Monochromator-based optics easily enable setup of the three excitation/emission wavelength pairs required to detect fluorescent PCR products representing all three genotypes present in the reaction. Consistent results are obtained on both instruments. Data analysis and graphing of genotypic clusters by SoftMax Pro Software provides rapid visualization of the normalized results.

**References**


**Figure 2. KASP genotyping results.** PCR products read on the plate readers clustered into three different groups including FAM homozygotes (blue), HEX homozygotes (red), and FAM/HEX heterozygotes (green). No-template controls (NTC) clustered near the origin.
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9/17 2128A
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