Validation of KASPar™ Dual FRET Based SNP Genotyping Assay

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Overview

KASPar™ SNP Genotyping Assay from KBioscience was validated in this work with Thermo Scientific thermal cyclers and microplate fluorometers. Both basic PCR instrument, Thermo Scientific Arktik, and real-time PCR instrument, Thermo Scientific PikoReal, were used for the DNA amplification and end point fluorescence signal was measured with filter based Thermo Scientific Fluoroskan Ascent and with Thermo Scientific Varioskan Flash spectral scanning multimode reader.

The key findings of the validation were:
• KASPar assay could well be run in both 96- and 384-well formats with identical performance.
• Fluorescence reading can be performed from the top or through the bottom of the PCR plate without any influence to the result quality, and sealed microplate can be used as well in the fluorometric reading.
• Generally, monochromator based Varioskan Flash produced results with slightly better resolution of the two fluorescent signals. This is based on the more precise wavelength optimization that is possible with monochromator based spectrofluorometer.
• DNA amplification can be run with any of the tested thermal cycler instruments and assay results have always full 100% correlation with the reference data when known DNA samples are tested.
• Arktik thermal cycler combined with either Varioskan Flash or Fluoroskan Ascent microplate fluorometer offer an efficient and reliable system for running KBioscience KASPar SNP genotyping assays.

Introduction

KASPar™ Genotyping System from KBioscience is a novel competitive allele specific dual FRET based assay for SNP genotyping. It uses two FRET cassettes where fluorometric dye, HEX or FAM, is conjugated to primer but quenched via resonance energy transfer. Sample DNA is amplified with thermal cycler using allele specific primers, leading to the separation of fluorometric dye and quencher when FRET cassette primer is hybridized with DNA. The principle of KASPar Genotyping System is presented in the animation at http://www.kbioscience.co.uk/reagents/KASP/KASP.html

In this work, fluorescence detection of the KASPar system was optimized with the Varioskan Flash spectral scanning fluorometer and with the Fluoroskan Ascent filter fluorometer. They key optimization parameters were excitation and emission wavelengths for both FRET pairs used in the assay. One of the dyes, HEX, was especially interesting for optimization because it has quite narrow Stoke’s shift, about 20 nm, that is somewhat challenging for most of the fluorometers. With common filter based fluorometers one cannot normally use excitation and emission filters that would have less than 40 nm difference. When quadruple monochromator based fluorometer is used, one can set excitation and emission much closer than with filter readers, in this case it should remarkably increase the resolution of fluorescent signal from the background. In addition, optimal measurement wavelengths are affected by the possible other dyes that exist in the sample, therefore normally recommended settings are not always optimal for the dual assays.

Methods

Microplate reader validation was performed with KBioscience KASPar Validation kit that contains three fluorescent dye solutions, one with FAM dye, one with HEX dye and one with FAM/HEX combination. These dye solutions were added into 96-well PCR plates (ABgene SuperPlate 96, cat. no. AB-2800) as 8 μl volume and in 384-PCR plates (Applied Biosystems® MicroAmp® Optical 384-Well Reaction Plate, cat. no. 4343370) using 4 μl volume. PBS buffer was used as a blank sample and 24 replicate wells were used in all validation assays.

The validation plates were measured with Varioskan Flash and Fluoroskan Ascent fluorometers using 100 ms measurement time. Excitation and emission peak wavelengths for FAM are reported to be 485 nm and 520 nm and for HEX the peaks are at 535 nm and 556 nm. Several wavelength pairs around these peak values were tested for both labels with spectrofluorometer and few filters within these areas were tested with filter fluorometer. Test plates were always measured using both top and bottom reading and the plates were first measured without sealing and then with the plastic seal (Thermo Scientific ABgene Clear Seal Diamond, prod. no. 0812).

System validation was performed with the known DNA samples supplied in the validation kit. Samples include 45 DNA samples and three negative controls. DNA amplification was run according to the protocol from the validation kit using either normal 96-well PCR plate or special Piko PCR plate that has 96 sample wells with 384-well PCR plate format, to mimic 384-well plate PCR assay. The plates were measured after the thermal cycling using the best wavelengths or filters based on the reader validation.
Results

Reader Validation

Varioskan Flash scanning spectrofluorometer and Fluoroskan Ascent filter based fluorometer were used in this KASPar assay validation. Result plots measured with the best excitation / emission wavelengths or filters using 96-well PCR plate are shown in Figures 1 and using 384-well PCR plate in Figure 2. The best measurement settings were selected based on the highest FAM/HEX signal ratio and the highest signal to blank and signal to noise ratios.

FIGURE 1. KASPar reader validation results with Varioskan Flash and Fluoroskan Ascent using 96 well PCR plates. The assay can be read equally well with or without seal and from top or from bottom. Bottom reading and using the seal reduces signal levels slightly, both about 20-25%. These signal reductions do not anyhow effect on the resolution of the different sample groups. The KASPar assay plate can be read using any of the options. When the filter reader is used, FAM emission is commonly measured with about 535 – 540 nm filter. In this case such filter should not be used because of the notable HEX emission such filter will collect in addition to the FAM signal.

FIGURE 2. KASPar reader validation results with Varioskan Flash and Fluoroskan Ascent measured with 384 well PCR plates. The 384-well PCR plate assay can as well be performed with top or bottom reading. Recommended measurement settings are, as expected, identical to 96-well plate and performing 384-well assays with lower volume (4 μl instead of 8 μl) does not cause any remarkable effects on the results.
System Validation (Thermal Cycler + Reader)

The complete KASPar assay system was validated with normal 96-well PCR plates using Arktik Thermal Cycler and with PikoReal real-time PCR instruments. After the DNA amplification the assay plates were measured and final results were analyzed based on the FAM / HEX signal ratio. Based on the ratio the DNA samples were classified into three groups: FAM homozygotes that produce mainly FAM signal, heterozygotes that produce both FAM and HEX signal in about equal intensity and HEX homozygotes that produce mainly HEX signal. The limit values between the groups need to be determined separately for each instrument and wavelength or filter combinations used in the assay. Third fluorometric dye, 6-ROX, was used as a normalization reference in these assays and results were analyzed both with and without the ROX normalization. Results of the 96-well plate assays are shown in Figure 3 and results of 96-well Piko PCR plate simulating 384-well assay are shown in Figure 4.

**FIGURE 3.** KASPar system validation results with Varioskan Flash and Fluoroskan Ascent measured with 96 well PCR plates. Both system combinations perform the assay with 100% accuracy, all reference samples are easily classified into correct category. When the measurement wavelengths and filters are correctly selected it is also possible to use the same classification limit values with both instruments. Using ROX normalization reduces the result variability somewhat but it is not necessary for the correct result analysis. These three sample groups are separated with large margins between them based on FAM / HEX ratio. The highest FAM / HEX ratio of HEX homozygote samples were 0.31 with Varioskan Flash and 0.43 with Fluoroskan Ascent when the lowest value of heterozygote sample were 0.81 and 1.07 respectively. Similarly, difference between heterozygote and FAM homozygote groups was clear; the highest ratio obtained from heterozygotes was 1.08 or 1.36 when the lowest values from FAM homozygotes were 5.99 and 5.18. These large gaps between the groups show the reliability of this assay.
FIGURE 4. KASPar system validation results with Varioskan Flash and Fluoroskan Ascent measured with 96 well Piko PCR plates that simulates 384-well format. The full 100% accuracy was obtained also with this plate format, but classification limits needed to be adjusted for each instrument. The effect of the ROX normalization was similar to normal 96-well plate, improves data but is not necessary.

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