Achieving Higher Call Rates With the SNPlex™ Genotyping System Using Modified OLA Conditions

Introduction

The SNPlex™ Genotyping System allows researchers to design custom assays for SNPs, MNPs, and indels of up to 6 bases at multiplex levels of 12–48 per reaction. Although the SNPlex™ design pipeline uses algorithms to minimize unwanted probe–probe interactions, and most assays yield call rates of >90%, some assays do not perform at this level, for reasons including nonspecific oligonucleotide interactions, the presence of neighboring polymorphisms, high GC content, and hindered accessibility of the target locus. Here we describe modified thermal cycling conditions for the OLA (oligonucleotide ligation assay) reaction that increase both signal intensity and specificity of hybridization, resulting in higher genotyping call rates.

Overview

The SNPlex™ Genotyping System OLA reaction is based upon allele-specific hybridization of two out of three oligonucleotides per target on genomic DNA in the presence of up to 141 other assay oligonucleotides (three per polymorphism) and approximately 100 universal linker oligonucleotides in each probe pool. The thermal cycling conditions for the OLA reaction are set so that each of the 25 ligation cycles takes approximately 7 minutes (Figure 1). This results in an overall OLA reaction time of approximately 4.5 hours, which is sufficient for most assays. However, experiments show that increasing the time for each ligation cycle to 12 minutes (about 6.5 hours for the entire OLA reaction) improves assay performance by increasing both ligation product yield and specificity of hybridization, resulting in higher genotyping call rates. Some assays that fail to yield genotype clusters under standard OLA conditions are able to produce clusters under the extended cycle conditions.

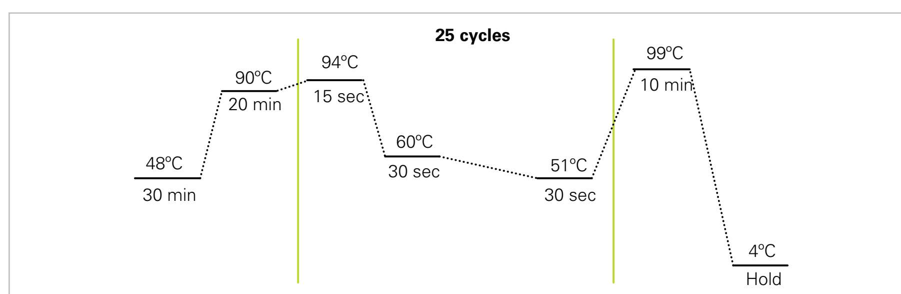

Figure 1. Thermal Cycling Conditions. Thermal cycling conditions for the SNPlex™ Genotyping System OLA reaction include 25 cycles of ligation (between the vertical bars), each of which takes about 7 minutes. During each cycle, annealing of oligonucleotides to genomic DNA target loci takes place by slow cooling from 60°C to 51°C.

Experimental Workflow

Incorporating the longer OLA protocol into the experimental workflow may be accomplished without increasing the number of days required to complete the assay, if only a small number of plates are processed. Instead of the standard two-day procedure, during which the OLA reaction is performed at the beginning of the first day, the OLA reaction is set up at the end of the first day and allowed to proceed overnight. The remaining steps—up to starting the capillary electrophoresis run—can be completed the second day in about 8 hours. If a large number of plates are processed and it is not possible to complete the procedure on the remaining day, it is advised to complete the PCR reaction setup on the second day and proceed with the post-PCR steps the following day.

The extended thermal cycling conditions (Table 1) can be programmed in 11 steps, where after a brief heat denaturation at 94°C for 15 seconds, the annealing occurs by decreasing the temperature one degree per 1 minute 20 seconds from 60°C to 51°C.* For the Applied Biosystems 9600 Thermal Cycler and non–Applied Biosystems thermal cyclers, adjust the 1 minute 20 seconds so that a single ligation cycle (indicated in orange) is completed in approximately 12 minutes.

It is important to prevent evaporation during the reaction by using durable plate seals. For this application it is recommended to use the MicroAmp® Optical Adhesive Film (P/N 4311971 or 4360954) directly in contact with the plate covered with a second seal of greater thickness, such as the MicroAmp™ Clear Adhesive Film (P/N 4306311). Place a MicroAmp™ Optical Film Compression Pad (P/N 4312639) over both seals. This configuration is suitable for both 384-well and 96-well plates on Applied Biosystems thermal cyclers. For 96-well plates, the MicroAmp™ 96-Well Full Plate Cover (P/N N8010550) may also be used.

* Note: The single-plate Aluminum 96-Well GeneAmp® PCR System 9700 (P/N 4314879) and Aluminum 96-Well GeneAmp® PCR System 9700 with Sample Block Module (P/N 4314445) are not recommended for use with the SNPlex™ Genotyping System. However, all other GeneAmp® PCR System 9700 configurations can be used, including both types of aluminum dual-plate block modules. The 9800 Fast Thermal Cycler and all Veriti™ Thermal Cyclers may also be used with this protocol.

Assay Results

Most assays show moderate improvement in both genotype cluster separation and signal intensity (compare columns 1 and 2 in Figure 2). The average sigma separation for three cluster SNPs increased from 13.02 using the 7-minute protocol to 23.10 for the 12-minute protocol. The average signal intensity increased from 630 RFUs to 1,200 RFUs for these assays. In addition, some assays that failed to yield genotype clusters using the 7-minute ligation protocol (column 3) were able to produce genotype clusters using the 12-minute protocol (column 4). Note that the no-template controls (NTCs, depicted with black boxes) also showed a lower signal in the 12-minute protocol (columns 2 and 4), demonstrating increased specificity of the ligation reaction. None of the tested assays displayed a decrease in average signal intensity or sigma separation under the extended OLA conditions. The size of this study was five pools of 230 assays.

Summary

Improved genotyping call rates can be obtained by slowing the rate of cooling during the annealing stage of each ligation cycle from 7 minutes per cycle to 12 minutes per cycle. This results in more ligation product as indicated by higher average signal, and increased specificity of ligation as indicated by a higher average of sigma separation values, resulting in higher genotyping call rates.

1 cycle	48°C, 30 min
1 cycle	90°C, 20 min
	94°C, 15 sec
	60°C, 1 min 20 sec
	59°C, 1 min 20 sec
	58°C, 1 min 20 sec
	57°C, 1 min 20 sec
25 cycles	56°C, 1 min 20 sec
	55°C, 1 min 20 sec
	54°C, 1 min 20 sec
	53°C, 1 min 20 sec
	52°C, 1 min 20 sec
	51°C, 1 min 20 sec
1 cycle	99°C, 10 min
Hold	4°C

Table 1. Step Cycle Parameters for the Extended OLA Reaction. The region highlighted in orange shows the steps for the 12-min ligation stage of the protocol, which lies between the phosphorylation/amplicon decontamination stage and the ligase inactivation stage shown in blue. These conditions can be used for all GeneAmp® PCR System 9700 thermal cyclers, the 9800 Fast Thermal Cycler, and all Veriti™ Thermal Cyclers. When using a GeneAmp® PCR System 9700, verify that the 9600 emulation mode is not selected, and check the length of the ligation cycle prior to performing the first run. When using a 9600 Thermal Cycler, substitute 1 min steps for the 1 min 20 sec steps shown above from 60°C to 51°C.

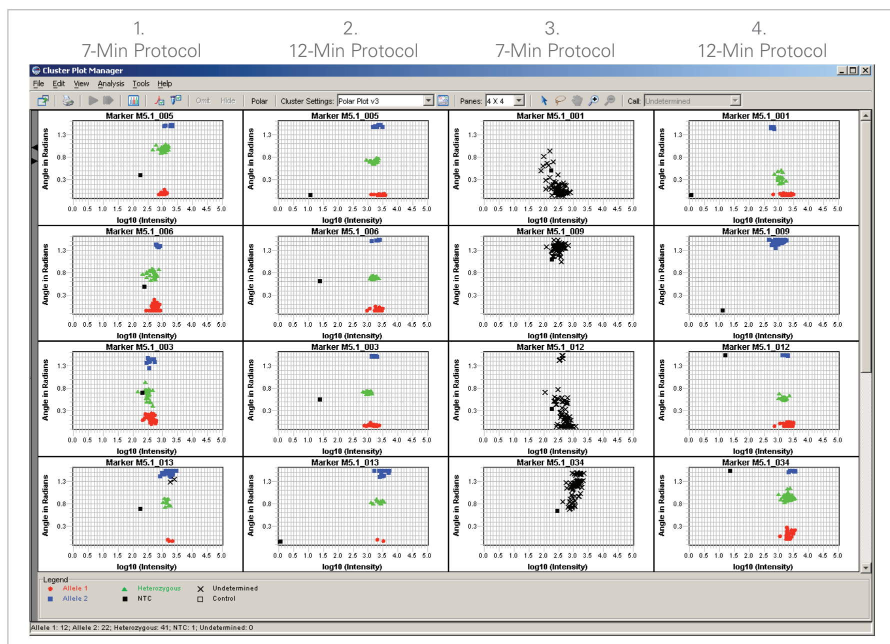

Figure 2. Genotyping Results From the 7-Min Ligation Protocol (Columns 1 and 3) and the 12-Min Ligation Protocol (Columns 2 and 4). In columns 1 and 2, the same set of four assays is shown (left to right). Another set of four assays is shown in columns 3 and 4. Note the increase in log10 (intensity) along the x-axis, showing the increase in signal intensity in columns 2 and 4 relative to columns 1 and 3, respectively. Also note the increase in sigma separation (decrease in cluster size) in columns 2 and 4 relative to columns 1 and 3, respectively. Note the substantial improvement in assay performance between columns 3 and 4. The results were obtained using a Dual 384-Well GeneAmp® PCR System 9700 for the OLA reaction and the plate seal configuration described in the experimental workflow.

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