

Template amplification and detection of PCR products on AmpliGrid AG480E using microarray technology

Combining PCR and Microarray Analysis on the Advalytix AmpliGrid.

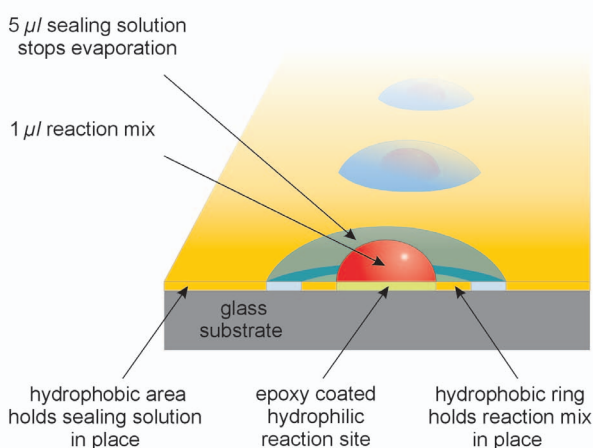
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Multiplex PCR is an easy standard method for generating a high number of different specific PCR products in parallel out of one template. The analysis of PCR products however usually is a time consuming process and the analysis of PCR product mixtures is difficult with gel electrophoresis if the length of different PCR products is similar and the number of PCR products is high. Specific hybridization of PCR products to arrayed capture probes that are immobilized on the reaction sites of AmpliGrid E slides is a reliable, fast and easy method for analyzing PCR product mixtures in multiplex PCR reactions in a seamless workflow that is also applicable in automated assays.

Introduction

This application report shows the array based specific detection of PCR products from multiplex PCR reactions using low amounts of extracted DNA. The AmpliGrid AG480E slide is a platform for ultra low volume applications in the 1 µL range, based on a chemically structured microscope slide. The 48 reaction sites of the AmpliGrid AG480E have an epoxy-coated surface for binding of oligonucleotides (figure 1).

1 Figure 1: Cross section of the AmpliGrid AG480E slide



PCR is performed in the presence of DNA arrays that are spotted onto the reaction sites of AmpliGrid AG480E slides. In the last step of the PCR the fluorescently marked PCR products are hybridized to the arrays and analyzed with a laser scanner.

Protocol

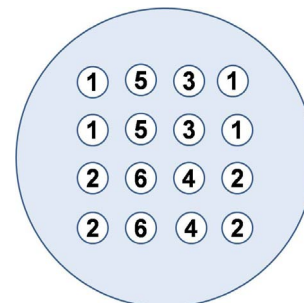
Capture probes and primers are obtained as 100 µM solution in water. Prior to spotting dilute probes to a 50 µM solution using AdvaSpot AT100 (Advalytix). Concentrations are measured using the Nanophotometer™ with Labelguard™ for ultravolume spectrophotometric analysis.

Perform spotting on AmpliGrid AG480E slides using an OmniGrid® Accent Spotter from GenomicsSolutions® Inc. and a SMP3 pin (Telechem International Inc.). Cool sample plates to 12°C and set humidity to 60% during the spotting run.

After spotting cure the slides at 42°C over saturated NaCl overnight, subsequently wash 5 minutes with 0.2% SDS and thoroughly rinse with nuclease free water. Afterwards wash the slides with nuclease free water at 50°C for 20 minutes and dry with compressed nitrogen.

All reaction sites contain identical arrays (4 x 4) according to the following spotting scheme in figure 2 and table A and B:

2 Figure 2: Array-Design (4 x 4 array)



A Table A: Probe sequences

1	5'-AAT ATT CAG GCT ATT TTG CTG CTT AGT GTC ACA TGT TTC TGG CAA -C7-aminolink-3'
2	5'-GGG CAC ACT ATG AAA ACT AAT ACC AGA AAC CAG ATC AAG ACA CCC C7-aminolink-3'
3	5'- GAG AGA AAA TCA GAG CAT GCG TAC TCT GAA CTT AAA GTA GCG AAT-C7-aminolink-3'
4	5'-GTC AAG ATT ATC AAA GGC ATC TTA TCG ACA CAA AAA GCA CTA TAA-C7-aminolink-3'
5	5'-ATT ACT CCC ACG TGA AAG GAA CAT TTA GAG TGC AAA TTG GGG CTA-C7-aminolink-3'
6	5'-AAA TAC TTA GGG TCA CCC CAA AAC TTT GAG GAT TAT AGT CAA GAT-C7-aminolink-3'

B Table B: Primer sequences (2pmol/µL each forward and reverse primer)

1	Forward: 5'-ATA CTA ACC ATG CCG GTT GC-3' Reverse: 5'-Cy3-AGA GGG ACA ACA AAC GTG CT-3'
2	Forward: 5'-GTG AGG ATT CTG GGC ACA CT-3' Reverse: 5'-Cy3-TGT TTA TTC TGG CAC TCC AAT G-3'
3	Forward: 5'-GAT AGC AAA TGC ACC ACG G-3' Reverse: 5'-Cy3-TTT TCC CGC CTA AAG CAT C-3'
4	Forward: 5'-AGG CAT TGT GGA GAT AAC GC-3' Reverse: 5'-Cy3-AAA CAT CAA AAT AGT CCA AGA TTC G-3'
5	Forward: 5'-TGG CCC CTG TGT TCA AGT-3' Reverse: 5'-Cy3-AGA ATT GCT GAA GTG TGT TAG CC-3'
6	Forward: 5'-GGT GGA TGC TTC TGC CTA AA-3' Reverse: 5'-Cy3-TTG GTT ATG GGT GCC AAG AT-3'

Pipette 1 μL of human male DNA (200 $\text{pg}/\mu\text{L}$; Promega #9948) to each reaction site and let air-dry.

Prepare six PCR master mixes using the Qiagen Multiplex PCR Kit primer mixes 1 to 6 as described in table C:

C Table C: Master mix composition for primer mixes 1 - 6

Component	20x
2x Multiplex PCR Master Mix	10 μL
5x Q-Solution	1.2 μL
Primer mix singleplex 1 to 6 (2 μM each)	2 μL
Nuclease free water	6.8 μL
Total volume	20 μL

Prepare PCR master mix 7 containing primer mixes (forward and reverse) 1, 3 and 5 as triplex mix; prepare PCR master mix 8 containing primer mixes (forward and reverse) 2, 4 and 6 as triplex mix according to table D:

D Table D: Master mix composition for primer mixes 7 and 8

Component	20x
2x Multiplex PCR Master Mix	10 μL
5x Q-Solution	1.2 μL
Primer mix triplex (1,3 & 5 or 2, 4 & 6)	3 x 2 μL
Nuclease free water	2.8 μL
Total volume	20 μL

Pipette 1 μL of each master mix to the corresponding reaction sites on the AmpliGrid and immediately cover with 5 μL of sealing solution.

Transfer the AmpliGrid to the AmpliSpeed slide cyclor and run the program described in table E:

E Table E: Amplification program

Temperature	Time	Cycle
95°C	10 min	
94°C	30 sec	
64°C	75 sec	35 cycles
72°C	75 sec	
95°C	30 sec	
64°C	20 min	hybridization

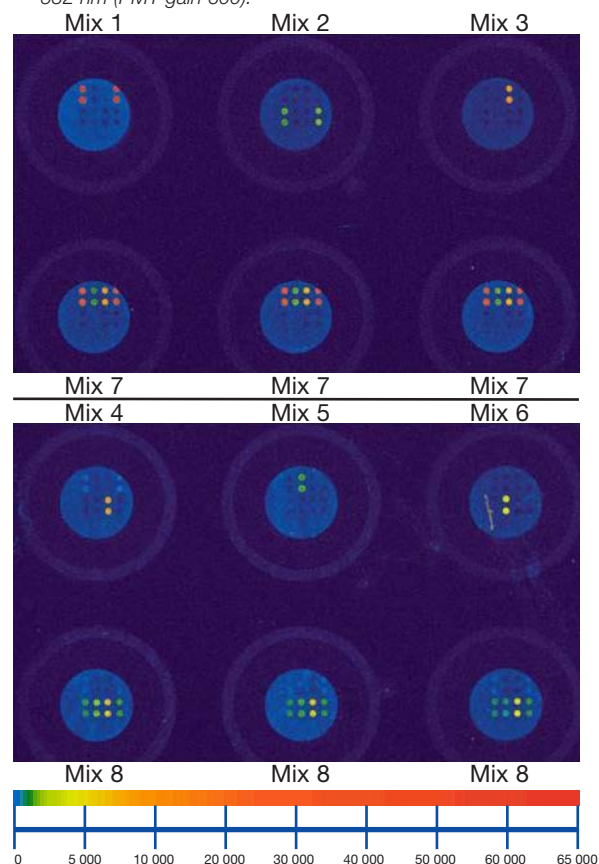
Wash the slide with washing buffer 1 (2x SSC, 0.2% Triton X 100) and incubate for 5 min. Afterwards incubate the slide 5 min in washing buffer 2 (2x SSC). Finally incubate the slide 5 min in washing buffer 3 (0.2x SSC). The washing procedure can be automated using the AdvaWash slide washing station. Immediately after taking the slide out of washing buffer 3, dry the surface of the slide with compressed nitrogen and scan the slide at 532 nm (PMT gain 600).

Results

Figure 3 shows the scan picture of the AmpliGrid AG480E PCR and hybridization assay. The singleplex PCR reactions

of mix 1 to 6 show that first and foremost the expected spots show specific binding. Signal / noise ratios up to 200 are obtained (see colour intensity correlation in figure 3). Mix 4 shows minimal cross reaction, but the intensity of the correct spots is a factor of about 40 higher than the intensity of the false spots which is close to the background. The very low background fluorescence shows the high quality of the AmpliGrid surface. The capture probes on each reaction site have been spotted with either four (mix 1 and 2) or two (mix 3-6) replicates. Furthermore for multiplex PCR reactions the expected fluorescence spots are obtained. Additionally the multiplex assay shows the same intensity of fluorescence in the different spots and very low background fluorescence proving that there is no cross reaction between the different PCR products.

3 Figure 3: Scan of the AmpliGrid AG480E PCR and hybridization assay at 532 nm (PMT gain 600).



Discussion

Hybridization of PCR products on AmpliGrid AG480E is a very specific process. Highly multiplexed assays can be done in a fast and sensitive way that PCR with fluorescently labelled primers creates a high amount of fluorescently labelled PCR products allowing hybridization analysis within several minutes. Highly automation as well as high throughput is possible by using the described system. Because of the low reaction volume this assay is also the method of choice for all users who are taking care of affordability.

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