Efficient DNA amplification and cycle sequencing from single cells without template preparation

Based on AmpliGrid technology.

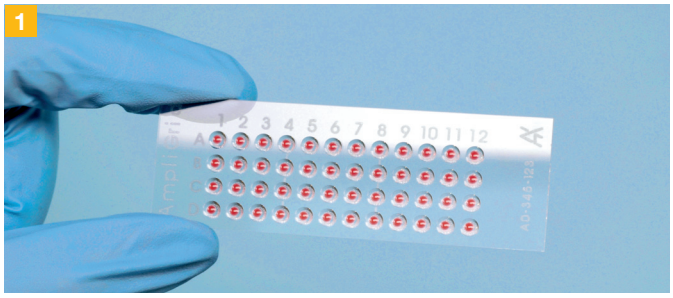
Based on new advanced AmpliGrid technology from Advalytix, DNA amplification and cycle sequencing are now possible in a low volume reaction format using single cells as the template source. Using Advalytix' AmpliGrid technology, DNA amplification doesn't require any template preparation. Due to the flexibility of the AmpliGrid technology, PCR and cycle sequencing can be performed on the same reaction site of the AmpliGrid AG480F.

Cell isolation

Lymphocytes were isolated from human peripheral blood by PANCOLL (PAN Biotech) density centrifugation and transferred in Phosphate buffered saline (PBS).

Cell sorting

Single cells were deposited onto each of the 48 AmpliGrid reaction sites (fig. 1) by MoFlo™[1] High Performance cell sorter (Dako Cytomation). Vital cells were sorted according to their side and forward scatter signals.



AmpliGrid AG480F slide

PCR

PCR amplification of mtDNA fragments from a single cell was performed using primers L29 and H3812 on the Advalytix AmpliSpeed slide cycler (fig. 2). The sequences of the primers are shown in table A.

A *Table A: Primer sequences*

Primer	Sequence
L29	5'- CTCACGGGAGCTCTCCATGC -3'
H381	5'- GCTGGTGTTAGGGTTCTTTG -3'

A PCR master mix was prepared as described in table B. After transferring 1 µL of master mix to each reaction site on the AmpliGrid slide, each PCR droplet was covered with 5 µL of sealing solution (fig. 3). The loaded AmpliGrid was placed on the AmpliSpeed slide cycler and the amplification programme (tab. C) started. After cycling the AmpliGrid was cooled down to room temperature.

B *Table B: Contents of master mix, PCR-Kit (Applied Biosystems)*

Component	1 µL PCR Mix
AmpliTaq Gold	0.1 µL
10x GeneAmp buffer I with 15mM $MgCl_2$	0.1 µL
Primer (5 pmol/µl each)	0.1 µL
dNTPs (2.5 µM each)	0.1 µL
ddH_2O (PCR clean)	ad 1 µL

Sequencing reaction

Hydrolysis of single stranded DNA and dephosphorylation of residual dNTPs was done by injection of 0.4 µL ExoSap-IT® (USB Corporation) through the covering solution and subsequent incubation for 60 min at 37°C. Enzymes were then inactivated by heating to 80°C for 15 min. After exonuclease digestion, 0.6 µL sequencing mix consisting of 0.5 µL BigDye™ Terminator Ready Reaction Mix (Applied Biosystems) and 0.1 µL of primer L29 (10 pmol/µL) was pipetted to the aqueous phase through the covering solution. Cycle sequencing was performed on AmpliSpeed slide cycler as described in table D.

AmpliSpeed ASC200D slide cycler (Advalytix)

Capillary electrophoresis (CE) analysis

After sequencing, the reaction mix and sealing solution were transferred to a conventional tube and 20 µL double distilled water was added. This mix was loaded onto a Sephadex® G-50-Superfine purification column (Sigma) to remove the sealing solution as well as non-incorporated fluorescent dyes. CE analysis was performed on the ABI Prism® 3700 Genetic Analyzer[3] (Applied Biosystems).

C *Table C: Amplification programme, heating rate: 1°C/s*

Temperature	Duration	
95°C	10 min	
94°C	40 sec	
56°C	30 sec	32 cycles
72°C	30 sec	
72°C	10 min	
RT	∞	

D *Table D: Sequencing programme, heating rate: 1°C/s*

Temperature	Duration	
96°C	80 sec	
96°C	30 sec	
50°C	30 sec	25 cycles
60°C	4 min	
RT	∞	

Results

Results of the sequencing reactions are shown in figure 4. Similar analysis was done to identify heteroplasmies among different single cells and individuals at the Institute of Legal Medicine, Freiburg.[2] Results were presented by Dr. S. Lutz-Bonengel at the 20th Congress of International Academy of Legal Medicine in Budapest 2006.

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Automation of 1 µl AmpliGrid AG480F amplification reaction on Hamilton MICROLAB STAR[LET], SW version 4.1

Discussion

The sequencing application on AmpliGrid has been tested successfully for PCR products after single cell PCR as well as standard plasmid DNA amplification. Using on-chip low-volume (LV) amplification of mtDNA and subsequent on-chip LV-cycle sequencing, this application is highly sensitive and robust enough to allow reliable analysis of DNA amounts representing the single cell level (Lutz-Bonengel *et al*., 2006).

While conventional PCR platforms require cell lysis before DNA amplification from single cells to avoid losing template DNA (Pierce *et al*., 2002). No additional cell lysis step is necessary when using the AmpliGrid technology. This results in significant reductions in working time and the amount of consumables, making the amplification of DNA from single cells much more convenient.

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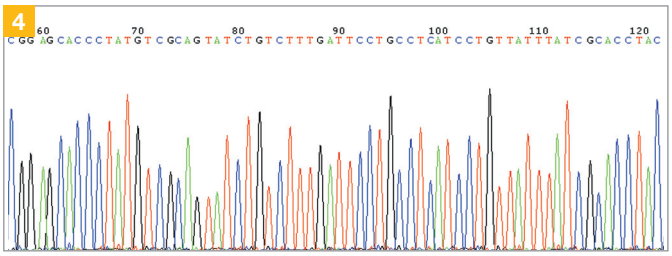

Sequencing results

REFERENCES

Lutz-Bonengel S, Sänger T, Heinrich M, Schön U. Low volume amplification and subsequent sequencing of mitochondrial DNA on a chemically structured chip. Int. J. Legal. Med. (2004), Vol. 121(1) p. 68–73

Pierce KE, Rice JE, Sanchez JA, Wangh LJ. QuantiLyse™. Reliable DNA amplification from single cells. Bio Techniques (2002) Vol. 32 p. 1106-1111

[1] FACS sorting of cells was done by Dr. J. W. Ellwart, GSF - National Research Center for Environment and Health

[2] Primers were provided by Dr. S. Lutz-Bonengel, Institute of Legal Medicine, Albert Ludwig University, Freiburg

[3] Sequencing was done at SequiServe, Vaterstetten, Germany

[4] Dr. S. Lutz-Bonengel, Institute of Legal Medicine, Albert Ludwig University, Freiburg

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