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Single cell typing by on-chip Low Volume PCR (LV-PCR)

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Introduction

Analysis of single or sorted cells mostly obtained by laser capture microdissection [1, 2, 3] has been carried out successfully in the past. Using 34 PCR cycles, Findlay et. al. typed the total number of 226 buccal cells with six STRs and obtained 50% full profiles and 64% acceptable profiles with more than 4 STRs typed successfully. This paper points out that single cell profiling is associated with the risk of PCR artefacts like additional alleles or stochastic effects [3].

Using a fluorescence activated cell sorter, defined numbers of sorted single cells were analyzed in this study by on-chip LV-PCR. In our investigation not only single cells but one to nine sorted cells were typed in up to 88 independent 1 µL-LV-PCRs using a commercial multiplex kit with 11 STRs to observe the changes in typing success and reliability. The use of chemically structured amplification slides makes it possible to perform a PCR reaction in a 1 µL volume. First results concerning the Ampligrid™ platform using commercially available typing kits have already been published [4, 5]. Improvements for PCR efficiency and sensitivity as well as the reduction of possible stutter artefacts and the occurrence of 'drop-in' alleles were described.

The results from single and sorted cell typing should provide another impression about the limitations of the LCN typing process as, compared to diluted DNA, a defined number of target sequences can be amplified and observed without any DNA extraction process influencing the DNA amount or condition in advance.

Material and Methods

The „PCR-Chip"AmpliGrid™ A480F (Advalytix, Germany) was used to perform the 1 µL-LV-PCRs on a Mastercycler (Eppendorf) using an appropriate in situ adapter. Most pipetting steps were done using a Hamilton robot. The AmpFlSTR® SEfiler™ (Applied Biosystems) multiplex STR typing kit was used for all tests performed. PCR reaction mix consisted of 0.5 µL master mix and 0.5 µL H_2O. Master mixes and cycling protocols were set up according to the manufacturers' instructions with 28 cycles (SEfiler™) and 30 cycles (PowerPlexES™), respectively. The individual 1 µL PCR assays were covered each with 5 µL covering solution supplied with the Ampligrid™. Three negative controls and one positive control (female DNA 9947A, Promega, 400 pg/µL) were applied to randomly selected positions on every slide to check for possible contamination or amplification problems. The initial denaturation process during PCR was thought to be sufficient to release the genomic DNA from the cell. Amplifications where e.g. single reactions failed due to evaporation or merging of neighbouring samples where not taken into the evaluation ('verified amplifications').

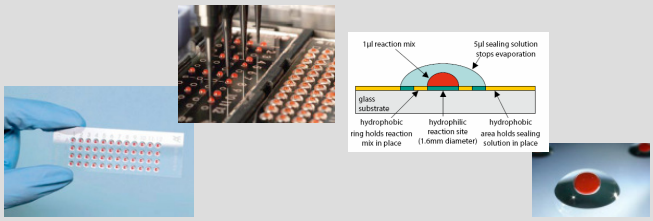

Fig 1: Layout „PCR-Chip" AmpliGrid AG480 (Fa. Advalytix, Brunnthal, Germany)

Single cell sorting as a refinement of flow cytometric bulk cell sorting has been used for the deposition of individual cells on AmpliGrid™ slides for genetic analysis by single cell PCR. We used a MoFlo cell sorter (Dako Denmark A/S) developed by Cytomation (Fort Collins, CO, USA) and its single cell deposition unit CyCLONE which allows to sort on microscope slide arrays. The Instrument settings were: nozzle diameter 70 um, pressure 60 psi, drop drive frequency 103,000 Hz, drop volume 1.4 nl, sheath fluid was HBSS (Hank's balanced salt solution). We selected the cells on the basis of side and forward scatter parameters. In order to ensure that each cell sorted was a single individual; In addition we took care to detect multi-cell clumps or attached debris. Therefore we used pulse processing which is available in the MoFlo. Furthermore, for the single cell sort a low cell concentration and a slower sort rate also reduced the incidence of doublets. Cells do not traverse the saline stream in phase with the droplet generation oscillations of the sorter. To yield exact one cell per spot we used the Single cell Sort mode of our MoFlo and sorted with one deflected drop. The uncertainty in trajectory was reduced if the sorted cells travel downwards at an angle closer to vertical. It was also important to avoid air currents in the flow chamber when sorting the individual droplets onto the Ampligrid™.

Human male lymphocytes from whole blood from a voluntary donor were used for cell sorting. 1, 2, 4, 6, and 9 cells were spotted twice onto 44 Ampligrid™ anchor spots, so that e.g. 88 single cell LV-PCR reactions could be performed resulting altogether in 480 LV-PCR assays including control reactions as described above.

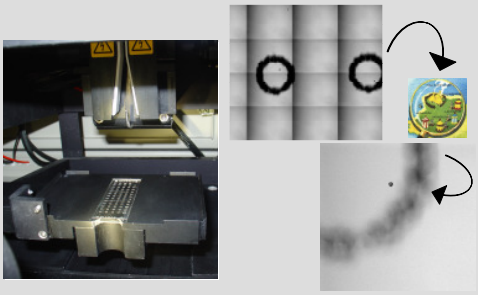

Fig 1: Single cell sorting on a MoFlo cell sorter and microscopic control of the sorted cells in the Ampligrid™ slides anchor spots

Results and Discussion

- A total number of 1,894 cells were typed in 423 separate LV-PCR assays.
- Table 1 summarizes the typing results of the amplified sorted cells. In addition to these failed amplifications the number of negative amplifications where no PCR product could be detected decreased from 21 to three when more than one cell was amplified. One additional negative amplification could be observed when six cells were typed.
- Apparently, quite large peak heights can be achieved even when only a small number of cells are typed. When the number of detectable targets is considered (23 per profile including amelogenin) the typing success rate increased from 21.7% in single cell amplifications up to 95.7% for typing nine cells.
- Even with four cells more than two thirds of the targets were detected as well as the first full profiles obtained. These profiles are quite imbalanced but complete.
- With nine cells per PCR a total number of 73 full profiles (84.9%) out of 86 LV-PCRs could be obtained.
- A total number of 97 additional alleles were recorded in all sorted cell experiments performed (data not shown). The largest number of additional alleles was found for SE33, followed by D19 and VWA. Concerning the location, the majority of additional alleles is found in -1 and +1 repeat positions, whereas alleles that are more distant from the original alleles are comparably rare.
- The data (not shown) obtained throughout this study led to the assumption, that partial or full dropout of amelogenin is more frequent than expected.

Table 1. Results of 'sorted cells' experiments using LV-PCR. [1]from 88 assays performed

Cells amplified / assay	1	2	4	6	9	Total
Verified amplifications[1]	76	87	87	87	86	423
Diploid cells amplified	76	174	348	522	774	1894
No. of negative amplifications Rate in %	21 27.6	3 3.5	-	1 1.2	-	
Detectable targets (incl. Amelogenin)	1748	2001	2001	2001	1978	9729
Targets detected (excl. additional alleles)	379	787	1405	1711	1895	6264
Typing success rate in %	21.7	39.8	70.2	85.5	95.7	64.4
No. of full profiles obtained Rate in %	-	-	4 4.6	14 16.1	73 84.9	

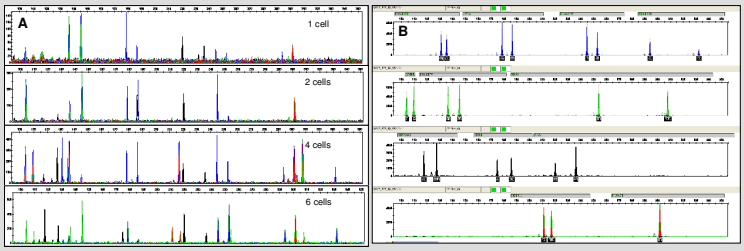

Fig. 2: Non representative SE-Filer example profiles (A) obtained from 'sorted cell' experiments and (B) example (full) profile obtained typing 9 cells (~50-60 pg) in a single on chip LV-PCR

The amplification of DNA from single and sorted cells by on-chip LV-PCR proves to be a suitable and also appropriate way to get a valuable impression of the STR typing process. A remarkable value (27.6%) for negative amplifications could only be observed in the single cell assays.

As a defined amount of target molecules are available the evaluation benefits from this advantage over diluted DNA samples. Only ~6 or ~12 pg of DNA amplified in 28 cycles result in quite high signal intensities up to 300 rfu and reasonable DNA profiles. This also leads to the assumption that a significant amount of DNA from LCN samples might get lost during the standard DNA extraction process as only 6 or 9 sorted cells gave full DNA profiles in ~85% and ~95% (Table 1), respectively. Looking at these results one would expect that more than such a small number of cells are present on touch DNA evidence swabs obtained from surfaces of a crime scene. Findlay et al. [2] report a 9% amplification failure rate typing 226 buccal cells isolated by micromanipulation procedures. Unfortunately the paper gives no information about any extraction process and PCR was done on only six STRs in 34 cycles and with modified primer concentrations. Especially these amplification modifications make a direct comparison difficult, but as well they might be a reason for the higher overall success rate that also includes full profiles in 50% of the cells typed. In contrast, additional alleles were observed more often and may be result of the high number of PCR cycles used.

The results for the present single and sorted cell experiments comprise high values for the occurrence of additional alleles. The fact that the cells were subjected to PCR without a separate DNA extraction procedure might have had an influence on the condition of the DNA in terms of accessibility e.g. for primers and polymerase.

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