Universal protocol for grafting PCR primers onto various Lab-on-a-Chip substrates for solid-phase PCR

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SUPPLEMENTARY INFORMATION

1 Materials

1.1 Oligonucleotides

HPLC purified oligonucleotides are obtained from Biomers, Ulm, Germany. *Cy5 primer* (a), 5' - NH_2 - C6 - ATG TAG CCT CAA TCC TGG TGT ATC CGC TTG - Cy5 - 3', which is a Cy5-labelled primer as immobilization control. *Extendable primer* (b): 5' - NH_2 - C6 - TTT TTT TTT TCT GAG CGG GCT GGC AAG GC, which is a solid-phase primer with a fully complementary sequence to the target. *Not extendable primer* (c), 5' - NH_2 - C_6 - TTT TTT TTT TGA TAC ATG GCT GTG AGT TAT CAA TTA CGA, which is a solid-phase primer with a sequence being not complementary to the target. Oligonucleotides as PCR primers for liquid phase PCR: 5'- CTG AGC GGG CTG GCA - 3' (forward primer); 5' - GCC TCC CTC GCG CCA TCA G - 3' (reverse primer). Lyophilized primers are dissolved in bidistilled deionized (DI) water to a final concentration of 10 µM and stored frozen at – 20 °C until use.

1.2 Substrates for DNA immobilization

Foils of COC (300 µm thick, COC Topas 8007 F04, Zeon Europe, Düsseldorf, Germany), PP (300 µm tick, PURELY®-Pharm, Etimex, Dietenheim, Germany) and COP (188 µm thick, ZF 14-188, Topas Advanced Polymers, Frankfurt, Germany) are cut by a scissor into slide format (26 x 76 mm²). Foils fulfill all criteria for the use in the pharmaceutical and diagnostic applications as stated by the suppliers. Furthermore, COP has proven its compatibility for PCR applications. (M. Focke, F. Stumpf, G. Roth, R. Zengerle, F. von Stetten, *Lab Chip* **2010**, 10 3210-3212; M. Focke, F. Stumpf, B. Faltin, P. Reith, D. Bamarni, S. Wadle, C. Müller, H. Reinecke, J. Schrenzel, P. Francois, D. Mark, G. Roth, R. Zengerle, F. von Stetten, *Lab Chip* **2010**, 10 2519-2526) Unmodified glass slides are purchased from Carl-Roth, Karlsruhe, Germany.
PDMS slides are fabricated by depositing a ~ 5 µm thick PDMS layer (Sylgard 184, Arrow Central Europe GmbH, Bietigheim-Bissingen, Germany) on a glass slide. Therefore, PDMS pre-polymer and curing agent are thoroughly mixed at a 10:1 (w/w) ratio. 1.5 mL is pipetted onto a glass slide fixed to the vacuum chuck of a spincoater (Spincoater WS-400B-6NPP/LITE, Laurell Technologies Corp., North Wales, USA). After spinning for 20 seconds at 2200 rpm, slides are cured overnight at 70 °C in an oven cabinet.

2 DNA immobilization

2.1 Immobilization protocol

All materials used as substrates for immobilization of primers are treated with the same surface chemistry. Chemical modification steps are performed at room temperature in a PP slide holder containing up to 5 slides (ZITT Thoma, Freiburg, Germany). Each slide is pre-cleaned by rinsing in ethanol (≥ 99.8 %, Carl Roth, Karlsruhe, Germany), dried by N_2, and stored in a closed slide holder until plasma activation.
Slides are activated by oxygen plasma with a 40 kHz plasma generator (Zepto, Diener electronics, Nagold, Germany) for 1 minute at an oxygen flow of 80 $l \cdot h^{-1}$ and 100 W generator power to generate hydroxyl groups on the surface of the slides.
After plasma activation, slides are immediately transferred into a silanization solution (5 ml/slide) containing 3-aminopropyltriethoxysilane (3-APTES, ABCR, Karlsruhe, Germany), bidistilled DI water (Carl Roth, Karlsruhe, Germany), and ethanol ≥ 99.8 % in ratios of (v/v/v = 5/5/90) of the end-volume. After silanization overnight (12 h ≤ t ≤ 16 h), slides are flushed with 99.8 % ethanol to remove unbound silane residues before immersing for 5 min in 99.8 % ethanol (5 ml/slide). Slides are dried by N_2, put in a slide holder and immediately cured in an oven for 1 h at 70 °C in the presence of humid air (~ 50 % relative humidity).
The 1,4-phenylene diisothiocyanate (PDITC) is coupled to the amine-terminated surface in a solution consisting of 0.1 g PDITC (98 %), 5 ml pyridine (anhydrous, 99.8 %), and 45 ml N,N-Dimethylformamid (≥ 99.8 %) (all from Sigma-Aldrich, Schnelldorf, Germany). After 2 hours of incubation, slides are washed by flushing with ethanol to remove unbound PDITC molecules before immersing 2 times for 5 min in fresh 99.8 % ethanol (5 ml/slide). Slides are blown dry by N_2 and further dried in vacuum at ~ 0.3 bar (0.5 h ≤ t ≤ 1 h), and stored overnight under protective N_2 atmosphere in a slide holder sealed with parafilm. In this way, slide can be stored for at least one month before DNA immobilization.
For immobilization of solid-phase primers, the latter are diluted in 1 × spotting buffer (Nexterion Spot 2 ×, Peqlab Biotechnologie, Erlangen, Germany) to final concentrations of 0.05, 0.10, 0.20, 0.40, 0.80, 1.60 µM for dilution series and 2.00 µM for SP-PCR experiments. Microarrays are printed onto the polymer slides with a TopSpot® system (Biofluidix, Freiburg, Germany) employing a 24 nozzle printhead ejecting an array of 1 nL droplets per print. After printing, slides are immediately placed in a plastic petri dish containing a DI water soaked tissue and sealed with parafilm. After incubation overnight at room temperature in a dark environment, slides are directly transferred into a slide holder containing a 10 % ammonium hydroxide solution (NH_4OH, Sigma-Aldrich, 5 ml/slide) for 30 min for the inactivation of unreacted PDITC groups and removal of unbound oligonucleotides. Slides are directly transferred into fresh 0.1 × sodium saline citrate (SSC, Sigma-Aldrich) containing 0.1 % Sodium Dodecyl Sulphate (SDS ultra-pure, Carl Roth) at 70 °C for 10 min, finally immersed in DI water at 70 °C for 5 min, and blown dry by N_2. Slides are now ready for extension of solid-phase primers and can be stored at room temperature under protective N_2 atmosphere in a slide holder sealed with parafilm for at least 2 month.

2.2 Tips and tricks

To obtain spots with high integrity and high homogeneity, we found the following points to be of utmost importance:

1) Between chemical modification steps, slides have to be quickly transferred between slide holders so that they do not fall dry.

2) Before spotting of DNA microarrays, activated slides have to be kept for at least one hour in vacuum to remove solvents like DMF and ethanol, otherwise spots strongly disintegrate and smear (see Figure S1).

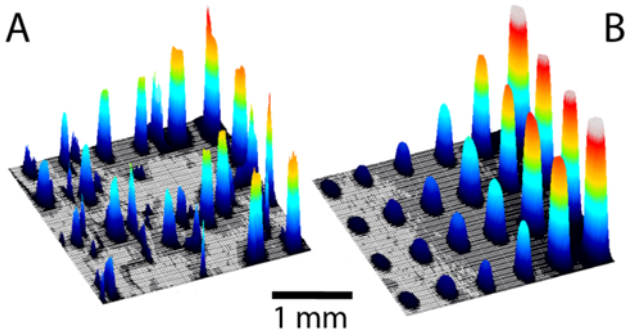

Figure S1. 3-D plot of the scanned dilution series bonded onto a PDITC activated PDMS surface. After surface modification, microarrays are directly printed, resulting in a strong "floating" of spots after washing (A). Flawless microarrays (B) with high integrity and homogeneity of spots are obtained by removing all solvents residues from the coating process for at least one hour in vacuum.

3) The amount of spotted DNA per area must not exceed 8 pmol·cm^{-2}, otherwise the surface binding capacity is exceeded. In such a case, non-bound DNA sticks to the spot surrounding surface during washing, resulting in strong smearing.

4) Slides have to be immediately transferred into petri dish containing a DI water soaked tissue (humid atmosphere). If not, spots desiccate resulting in inhomogeneous spots (doughnut like structure). (V. Dugas, J. Broutin, E. Souteyrand, *Langmuir* **2005**, 21 9130-9136)

5) A stringent washing procedure after DNA immobilization ensures the removal of non-bound or weakly absorbed primers. This is important in regard of PCR-based primer extension.

3 Solid-phase PCR

3.1 Solid-phase PCR protocol

For SP-PCR a reaction mixture of 25 µl contains 2 U of hot start DNA polymerase HotStarTaq Plus, 1 × reaction buffer, with additional 0.5 mM $MgCl_2$, (all from Qiagen, Hilden, Germany), 0.2 mM dATP, dGTP, dCTP, 0.16 mM dTTP, (Jena Bioscience, Jena, Germany), 0.04 mM biotin-11-dUTP (Yorkshire Bioscience, York, United Kingdom), 0.125 µM forward primer, 1.00 µM reverse primer, 0.1 % BSA (w/v), 10^3 template molecules (vector pTYB1, New England Biolabs, Hitchin, United Kingdom). With the employed forward, reverse, and solid-phase primers (for sequences see supporting information) a 308 bp sequence of the vector is amplified. All indicated values are final concentrations.
Before reaction, each printing-block on the slide is sealed with a 25 µl GeneFrame (in situ Rahmen, Peqlab, Erlangen, Germany) and stored overnight at room temperature enhancing the bond between GeneFrame and substrate. For extension of solid-phase primers by SP-PCR, 25 µl of reaction mixture is inserted into each GeneFrame using a filter pipette tip and sealed with the provided plastic lid. Thermocycling is performed in a slide cycler (peqStar in-situ, peqLab Biotechnologie, Erlangen, Germany). For enhancing thermal transfer and leak tightness of the GeneFrame, a custom made spring (d = 1.4 mm, D_a = 16.5 mm, L_o = 43.0 mm, i_g = 6.5 mm, Cr Ni 18-8, Alfred Weigel KG Federnfabrik, Chemnitz, Germany) is placed on top of each frame, which deforms upon closure of the lid applying 10.8 N to the GeneFrame and ensuring close contact between the slides and the thermo-block. Temperature profile of two-step PCR is as follows: 5 min activation at 95 °C, 20 × (94 °C for 30 sec and 60 °C for 45 sec) for fast pre-amplification of template DNA), 30 × (94 °C for 1 min and 60 °C for 3 min) for extension of solid-phase primers. After SP-PCR, GeneFrames are detached and slides are incubated for 5 minutes at room temperature in 5 × SSC, 0.1 % SDS (1 ×), 0.1 x SSC (1 ×), bidistilled DI water (1 ×), and finally blown dry with N_2.

3.2 Tips and tricks

1) The primer sequences are terminally anchored to the substrate via a 6-atom carbon spacer connected to an additional poly(10)-dT-spacer. As reported by Guo et al. a poly-dT spacer can enhance priming by a factor of 20. (A. Halperin, A. Buhot, E. B. Zhulina, *Langmuir* **2006**, 22 11290-11304; Z. Guo, R. A. Guilfoyle, A. J. Thiel, R. F. Wang, L. M. Smith, *Nucleic Acids Research* **1994**, 22 5456-5465)

2) The concentrations of aqueous forward and reverse primers are set to an asymmetric ratio of 1 to 8, with 0.125 and 1.00 µM, respectively. By this means, the forward primer is the limiting factor of the aqueous PCR reaction and is depleted after several cycles. Thus PCR is driven onto the immobilized forward primer, shifting PCR from liquid to solid-phase.

3) In the first 20 cycles, 45 seconds of annealing and extension time is applied to prefer the aqueous PCR-reaction, where in the last 30 cycles this time is extended to 3 minutes to enhance base pairing and favor the solid-phase reaction.

4) The length of the aqueous forward primer is shortened by 4 bases at the 3' end compared to the immobilized forward primer (for sequences see section 4), reducing its melting temperature by ~ 8 °C. Khan et al. reported that this measure shifts the reaction to the solid phase (energetically favored) by which the solid-phase load can be increased by a factor of 10. (Z. Khan, K. Poetter, D. J. Park, *Analytical Biochemistry* **2008**, 375 391-393)

4 Staining of immobilized PCR-products

Extension of solid-phase primers was proven by staining the incorporated biotin-dUTP moieties with fluorescently labelled streptavidin by incubating each array for 5 min at room temperature with 75 µl of staining solution: 5 $\mu g \cdot ml^{-1}$ streptavidin-Cy5 (VWR international, Bruchsal, Germany) in 100 mM NaPi buffer (di-natrium-hydrogenphosphat-dihydrat and natrium-dihydrogenphosphate-monohydrate, pH-value of 7.2), complemented with 0.1 % Tween 80 (reinst, Carl Roth) to reduce unspecific absorption and ensure low contact angle on the slides. All indicated values are final concentrations. To remove the unbound streptavidin-Cy5 conjugate, slides are washed for 5 min at room temperature in 100 mM NaPi buffer, pH = 7.2, followed by 5 min in 10 mM NaPi buffer, pH = 7.2, and blown dry with N_2.

The selected staining approach yields several advantages. First, biotin-dUTP is incorporated into the DNA by the polymerase with higher efficiency compared to fluorophor labeled dUTPs. Second, each streptavidin molecule contains 3 to 9 Cy5 fluorophors, yielding a high fluorescence signal-to-noise ratio compared to other detection methods. Cy5 as detection fluorophore yields in a reduced fluorescent background signal of the substrate compared to the fluorophore FAM. (F. Erdogan, R. Kirchner, W. Mann, H. H. Ropers, U. A. Nuber, *Nucleic Acids Res* **2001**, 29; Y. Sun, R. Dhumpa, D. Bang, J. Hogberg, K. Handberg, A. Wolff, *Lab Chip* **2011**, 11 1457-1463; M. von Nickisch-Rosenegk, X. Marschan, D. Andresen, F. F. Bier, *Analytical and Bioanalytical Chemistry* **2008**, 391 1671-1678)

5 Scanning and analysis

Polymer slides are attached to a standard glass slide with a drop of water as a capillary "adhesive" providing a planar hybrid polymer-glass. Such slides are scanned at different exposure times using a darkfield fluorescence scanner (Bioanalyzer Microarray BA 4F/4S, LaVision, Goettingen, Germany) using emission and detection filters appropriate for Cy5. Scanning resolution is 5 µm. The according 12-bit tiff images are analysed with the software GenePix Pro® 7 and ImageJ.