Improving DNA capture on micro-arrays by integrated repeated denaturing

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Hybridization of nucleic acids to microarrays is a crucial step for several biological and biomedical applications. However, the poor efficiency and resulting long incubation times are major drawbacks. In addition to diffusion limitation, back-hybridization to complementary strands in solution is shown to be an important cause of the low efficiency. In this paper repeated denaturing in an integrated device has been investigated in order increase the efficiency of micro-array hybridization. The sample solution is circulated from the micro-array chamber over a denaturing zone and back in a closed loop. In addition to the improved binding rate due to flow, repeated denaturing significantly increases the total amount of molecules bound. Our results demonstrate that cyclic repeated denaturing improves the efficiency of hybridization by up to an order of magnitude over a broad range of concentrations studied (1 pM to 100 nM).

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1. Introduction

Biological assays based on the hybridization of nucleic acids have revolutionized the research in biology and medicine since microarrays were introduced in 1995. Analysis of gene expression has opened the way to the understanding of complex biological processes and has led to a new approach to medical diagnostics. Microarrays have been successfully applied to the study of tumors, diseases progression, cellular response to stimuli, and drug target identification. More recently, microarray-based capture of nucleic acids has been employed for target enrichment in next generation sequencing. Instead of sequencing the whole genome, only selected genes (regions of interest, ROI) are analyzed after having been captured via particular target enrichment strategies. Since ROIs typically represent less than a percent of the human genome, efficient targeted enrichment can dramatically reduce the cost and effort of sequencing.

There is a growing need for technologies that selectively isolate genomic sequences with high specificity and efficiency, to maximize the coverage of the target regions with sufficient depth for accurate base calling. To meet these requirements, several solid-phase and solution-phase hybridization solutions have been proposed, yet many issues concerning poor yield, long incubation times, and laborious sample preparation still need to be solved. Long hybridization times are required to obtain high signal-to-noise ratios and to reach thermodynamic equilibrium; incubation times of up to 65h have been reported. Thermodynamic equilibrium is critical for specificity, since considerable cross-hybridization with low affinity targets occurs under non-equilibrium conditions. A further motivation for the long hybridization times of conventional assays is the diffusion limitation of target DNA to the capture probes on the surface. Due to the distribution of the probes over a large surface area long diffusion distances across the surface are required for large molecules with small diffusion coefficients. A binding efficiency of only 0.1% is typically observed after overnight incubation.

To overcome the mass transport limitation, several methods have been proposed and commercialized. In the past few years mechanical or electrical forces have been coupled to microarrays to induce active mixing or to generate flow within the hybridization solution. More recently, the progress of microfluidic technology has led to the development of a variety of microfluidic devices for hybridization assays, as reviewed by Wang et al. A number of microfluidic solutions have been developed, to enhance mass transport of target molecules and thus accelerate the hybridization to the capture probes on the surface. Higher sensitivity, shorter assay time and less sample usage are obtained with this approach. However, these solutions deal with the diffusion limitation of the hybridization reaction only. In fact hybridization to the probes on the surface has to compete with back-hybridization to the complementary strand in solution, which is present at the same concentration as the target strand. Given that this concentration is effectively higher than that of the probes on the surface, the probability of back hybridization in solution is higher than hybridization to the capture probes. Moreover, hybridization in solution is a second order reaction, while hybridization on the surface follows first order kinetics. Consequently, hybridization in solution is typically faster than on the array, and reaction times in solution have been reported to be 20-40 times faster. In current practice samples are denatured at 95 °C prior to their introduction into the hybridization chamber. At that point, all material is in single stranded form. However, with time the effective concentration of single stranded nucleic acids (ss-DNA) will decrease, such that the hybridization to the probes at the surface will slow down and eventually stall or even be reversed when the concentration of ss-DNA has reached its very low equilibrium concentration at the pertinent hybridization temperature.

Here a new approach is presented which overcomes the significant limitation of back-hybridization. In this approach the sample is repeatedly denatured during the hybridization process. This is achieved by

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flowing the sample over a hot zone (using a thin film heater) which is integrated in the micro-fluidic cartridge containing the microarray. The temperature of the microarray remains constant at the hybridization temperature. The hot zone is set at the denaturing temperature. When the sample flows over the hot zone the DNA will denature and will be present as ss-DNA at the microarray which is next to the hot zone. Moreover, as the sample circulates in closed loop, at every pump cycle the targets are repeatedly denatured. Therefore, the process can be applied for long hybridization times and small sample volumes. By properly designing the hot zone, flow rates can be maintained that are necessary to overcome diffusion limitation and still have a full denaturation. The residence time in the denaturing area has to be adjusted to the heat transfer through the glass slide and can be as short as 2 seconds, as in ultrafast PCR chips. With this approach the two limitations of microarray hybridization, i.e. diffusion limitation and back-hybridization to the complementary strand in solution, are addressed simultaneously. It will be demonstrated that in this way the hybridization efficiency can be increased by an order of magnitude.

2. Experimental

2.1 Cartridge

Hybridization experiments were performed in microfluidic devices containing microarrays on aminosilane-coated glass slides (Genorama™ SA, ES), see Fig. 1. The microchannel is U-shaped with straight channels (A and B) of 20 mm containing the microarray, and a transverse tract of 5 mm. The channel is 1.2 mm wide and 100 µm high. The channel is laser cut in a double-sided pressure sensitive adhesive tape (PSA), 100 µm thick. One side is attached to the SA slide, which constitutes the bottom of the device. A glass slide, provided with 2 drilled holes (inlet and outlet) serves as cover. A laser cut element of PMMA is mounted on top of the inlet and outlet supporting PEEK tubes for interconnection to the tubing to allow fluid injection/extraction in the microchannel.

The temperature in the microchannel is controlled via a segmented thin film heater. The thin film heater is deposited on a glass substrate and comprises 6 areas of 3 x 6 mm², which are controlled in pairs via 3 integrated thin film sensors. A dedicated electronic set-up and LabView software ensure very fast heating (100°C/s) and precise control of the temperature (< 0.1°C) in the 3 different thermal zones, with minimum crosstalk. Unless elsewhere specified, two contiguous zones are kept at 50°C (hybridization area) and the third zone at 95°C (denaturing area). During the experiments the cartridge is positioned on top of the heater, the U-bend on the denaturing area. Vacuum is applied at the contact surface to ensure good thermal contact.

Fluids are pumped through the cartridge at controlled flow rate via either a syringe pump (11 Plus, Harvard Apparatus, US) or in a closed loop by a peristaltic pump (MasterFlex C/L, Cole-Parmer, US). The peristaltic pump is operated with Tygon tubes, 0.25 mm inner diameter, resulting in a flow rate of at 35 µl/min and a cycle time of 80 s. Unless otherwise specified, the syringe pump operates at a flow rate of 6 µl/min. For comparison, hybridization experiments have been carried out in isothermal conditions, maintaining the entire microchannel at 50°C. For this set of experiments, the sample was denatured at 95°C for 10 minutes prior to the introduction in the cartridge.
2.2 Probes used
Hybridization tests were carried out with two different targets, amplified from the human genome by Polymerase Chain Reaction (PCR). Target 1 is 114 base pairs (bp) in length, target 2 108 bp (primer sequences available on request). In both cases the G-C content of the PCR product equals 47% of the sequence. Both PCR products were fluorescently labeled by amplification with forward and reverse primers coupled to Atto700 dye (Atto-Tec, G) at their 5’ ends (Biolegio, NL). The products were purified over MinElute columns (Qiagen, G). For control experiments, single stranded DNA targets with the same sequence as the hybridizing strand of targets 1 and 2 were synthesized and labeled with Atto700 (Biolegio, NL). The targets were dissolved at desired concentration in hybridization buffer (3 x sodium citrate buffer, SSC, containing 0.1% SDS).

As capture probes unlabeled synthetic oligonucleotides (Biolegio, NL), 76 nucleotides in length, were inkjet-printed and immobilized on the SA slides via UV-cross linking. The probes comprised of 60 nucleotides fully complementary to the targets, and a 16 thymine spacer on the 5’ end. These oligonucleotides were dissolved at a concentration of 6 µM in PBS with 1.5 M betaine (Sigma Aldrich, B) and inkjet-printed on the SA slides in spots of 150 µm diameter with in-house built inkjet printing equipment. In addition, non-binding capture probes of the same length were printed as negative control. As reference spots Atto700-labeled oligonucleotides (Biolegio, NL) dissolved in PBS with 1.5 M betaine at concentration of 2 µM were printed. After printing the slides were washed and blocked for 1 hour at 50°C in SSPE buffer containing 0.1% Sarkosyl (Sigma Aldrich, B) and 1% herring sperm DNA (Sigma Aldrich, B), before being assembled in the cartridge. Cartridges were stored at 4°C in dark conditions until they were used.

2.3 Benchmarking experiment
SA slides were inkjet-printed as described in 2.2 and used in two commercial hybridization stations. For comparison, slides were assembled in the microfluidic device of 2.1 connected to the peristaltic pump, used in a microarray hybridization chamber with full-surface gasket slide (Agilent), and in the NimbleGen hybridization system with HX1 mixer (Roche, CH). Hybridization was carried out in duplicate, for two different concentrations of probe 1 and probe 2, namely 100 pM and 1 pM. The targets were dissolved in hybridization buffer, denatured at 95°C for 10 minutes and cooled down in ice before being introduced in the commercial systems. No prior denaturation was required for the cartridge. Comparable volumes of solution were used in the cartridge (65 µl) and in the Roche mixer (40 µl), while
500 µl was required for the Agilent chamber. Hybridization was carried out overnight at 50°C. The solution circulated at 35 µl/min in the cartridge, corresponding to an average velocity of 4.8 mm/s. The Roche HX1 mixer operated at 4.3 s of half cycle time for actuation. The Agilent chambers were rotating in the oven at 10 rpm.

2.4 Signal detection and data treatment
The efficiency of hybridization was measured via the fluorescence spot intensity detected in the microchannel. The signal was measured in dry conditions, in both the parallel channels of the cartridge. In end-point measurements the signal was measured before and after a washing step, carried out at room temperature in SSC with 0.2% SDS, by flowing over the washing buffer for 5 minutes at 6 µl/min. No washing step was applied during the kinetic measurements. Fluorescence was measured by an inhouse-built compact confocal scanner based on DVD technology, at a 5 µm spatial resolution, with 1.5 mW of excitation power at the lens surface. The fluorescence data were averaged for each spot by dedicated software. Curve fitting of the experimental data was performed using Origin software, after background subtraction.

3. Results and Discussion

3.1. Effect of back hybridization in solution
To demonstrate how the efficiency of capture on the array is influenced by back hybridization an experiment was carried out in which the sample is stored for different times after denaturing before hybridization is carried out in flow in a microchannel. If back hybridization is significant the signals obtained on the microarray should scale with the storage time. To separate the effects, the storage time was taken longer than the hybridization time on the array. The experiment was performed in duplicate using two different fluorescently-labeled oligonucleotide targets (probe 1 and probe 2), both at 1 nM concentration. The normalized fluorescence data are given in Figure 2 for both probes. As can be seen the signals decrease strongly with storage time. This indicates that hybridization efficiency is strongly reduced during storage and clearly demonstrates the importance of back hybridization for micro-array applications. The total effect will be even bigger than apparent from Figure 2, since the first time point in these graphs is already influenced by back hybridization, as can be inferred from the steepness of the curve at short times.
The measured curves can be described very well ($R^2=0.995$ for probe 1 and 0.993 for probe 2) by the following equation:

$$I = \frac{1}{1 + At}$$

which represents the time dependence of a second order reaction, where $t$ is the storage time and $A$ is the rate constant. From the quality of the fits in Figure 2 one can conclude that the loss of hybridization efficiency can indeed be described by a second order rate equation, assuming that the fluorescence intensity is proportional to the effective concentration of targets. For relatively short hybridization times and partial coverage of capture spots, as in the current experimental conditions, the relation can be assumed linear. From the fits in Figure 2, $A$ has been determined to be $4.4 \times 10^{-4}$ s$^{-1}$ for target/probe 1 and $2.6 \times 10^{-4}$ s$^{-1}$ for target/probe 2. These values correspond to a characteristic time $\tau$ ($1/A$) of 38 and 64 minutes, respectively. This result demonstrates that for the pertinent target concentration of 1 nM approximately half of the targets are no longer available for binding to the capture probes after an hour.

To increase the efficiency of hybridization the system would need a constant supply of freshly denatured targets, which is not provided by state of the art approaches. Here two new approaches are described to achieve this: either (i) the sample is pumped over the microarray to a waste reservoir with a denaturing zone in front of the microarray, or (ii) the sample is continuously recirculated over the microarray and the denaturing zone arranged next to it. For case (i) the total hybridization time is given by the sample volume and the flow rate. This can be limiting the applicability, if only a small sample volume would be
available. In (ii) a pump must be integrated in the device which can operate in a closed loop at small total volume.

The open loop approach allows a direct comparison between normal and integrated denaturing by placing the denaturing heating zone between two identical microarrays as shown in Fig. 1. The sample, containing a mixture of targets 1 and 2 at 1 nM, is pumped through the microchannel at a controlled flow rate (6 µl/min). The flow is maintained for 30 minutes and followed by a wash step. The average residence time in the denaturing area is 18 s.

Figure 3

Figure 3 shows the average endpoint signal intensity for both targets, respectively, in front of and behind the denaturing zone (mind the log-scale). When hybridization occurs without prior denaturation, the signal intensity is low and comparable to the values measured in Figure 2 for long hybridization times. After flowing through the denaturing area, the signal intensity increases by 2 orders of magnitude and approaches the values shown in Figure 2, for short times after denaturation. This experiment thus demonstrates that the loss of hybridization efficiency can be counteracted by exposing the ds-DNA probes to denaturation temperature, while pumping the sample in a microfluidic channel. The timescale of the hybridization experiments was chosen short in accordance with the simple system considered, i.e. only 2 different types of targets at reasonably high concentration of 1 nM.

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3.2. Concentration and time dependence

Having demonstrated the strong effect of back hybridization in solution on the capture efficiency of a microarray at a concentration of 1 nM, one would like to be able to extrapolate the benefit of integrated denaturing for other concentrations.

As described above, two types of reactions occur in hybridization chambers, i.e. the hybridization of single stranded targets, \( sa \), to single stranded capture probes, \( sp \), immobilized on the surface (the desired reaction) and the hybridization of single stranded targets, \( sa \), to a complementary strand, \( sb \), in solution (the competing reaction). Both reactions are reversible and temperature dependent. Assuming that only fully denatured and fully native species are present in solution, in the simple case of one type of target and probe, the reactions can be denoted as follows:

In solution:
\[
\text{sa} + \text{sb} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{ds}
\]

with \( k_a = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{[ds]}{[sa][sb]} = \frac{[ds]}{[sa]^2} \)  

At the surface:
\[
\text{sa} + \text{sp} \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{dp}
\]

with \( k_a = \frac{k_{\text{on}}}{k_{\text{off}}} = \frac{[dp]}{[sa][sp]} \)  

where \( k_{\text{on}} \) is the association rate constant and \( k_{\text{off}} \) is the dissociation rate constant, both assumed equal for the surface and solution reaction. In the absence of back hybridization in solution and taking into account that the overall concentration of capture probes \([sp]\) is very low so that the binding reaction at the surface will not significantly influence the concentration of targets (\([dp]\) \(<\ll [sa]\), ambient assay condition)\(^{28}\) and the displacement reaction of \( sa \) from \( sp \) at the surface by \( sb \) is disregarded, the hybridization of single stranded targets to capture probes on the surface has been found to follow Langmuir kinetics\(^{29}\):

\[
\frac{[dp]}{[sp]}(t) = \frac{k_{\text{on}}[sa]}{k_{\text{off}}[sa] + k_{\text{off}}} \left(1 - e^{-(k_{\text{on}}[sa] + k_{\text{off}})t}\right)
\]

The time constant, \( \tau \), of the reaction is a function of the concentration of target molecules \([sa]\):

\[
\tau^{-1} = k_{\text{on}}[sa] + k_{\text{off}}
\]

By measuring the time dependence of the binding at the surface at different concentrations one can derive \( k_{\text{on}} \) and \( k_{\text{off}} \) from a plot of \( \tau^{-1} \) versus \([sa]\).

In the case of competing back hybridization reaction \([sa]\) becomes time dependent \([sa](t)\) and Eq. 4 no longer holds and the binding kinetics would have to be modeled numerically. To do so one would have to describe \([sa](t)\) in addition for the various starting concentrations. Unfortunately, the direct measurement of back hybridization in solution is not possible at the low concentrations (pM) of interest here due to the lack of sensitivity of analytical techniques to discriminate single- from double-stranded DNA. The indirect approach shown in the section 4.1 is only valid as long as the time scales for both

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reactions can be separated. In view of these limitations no attempt is made for a quantitative modeling of the system, but rather an experimental investigation of the time- and concentration dependence, comparing repeated denaturing with isothermal hybridization conditions.

For the purpose of investigating the effect of denaturing on samples with lower concentration and/or much higher degree of multiplexing, it is necessary to use longer hybridization times. To limit the required sample volumes for long term hybridization, the inlet and outlet of the microfluidic device were connected to a peristaltic pump to create a circulating flow with only 65 µl total volume of sample solution. Hybridization tests were performed under repeated denaturing (RD) conditions (denaturing zone at 95°C on the thin film heater) and isothermal (IT) conditions, at different values of the flow rate, to identify the conditions in which the system operates in a reaction-limited regime. The sample concentration was set to 1 nM. Figure 4 shows the time dependence of the hybridization signal for 3 different values of the flow rate, namely 18 µl/min, 35 µl/min and 60 µl/min in IT conditions in comparison to RD at 35 µl/min. The experimental points have been fitted by an exponential equation of the form of the Langmuir isotherm (Eq. 4): \[ I = I_\infty (1 - e^{-\frac{t}{\tau}}). \]

Figure 4 shows that the value of \( \tau \) can be minimized by tuning the flow rate \( Q \) and the minimum was observed for \( Q = 35 \) µl/min. This value has been chosen for all the subsequent experiments as the condition for reaction-limited regime. The same trend was observed when \( Q \) was varied in RD conditions. The observed increase in \( \tau \) with high flow rates may be due to an increased stringency for binding by the strong shear forces: the shear rate corresponding to a flow of 60 µl/min is \( \sim 200 \text{ s}^{-1} \). Figure 4 also demonstrates that the flow rate does not influence the signal intensity at equilibrium strongly, as observed also by other authors.\(^{20-22}\) Conversely, the results in Fig. 4 show that repeated denaturing has a pronounced effect on the maximum signal intensity that can be achieved, which is
determined by the availability of single stranded targets in solution. Despite the external denaturing step prior to the start of the experiment, integrated denaturing leads to a strong increase in binding at the surface. It is interesting to see that the apparent $\tau$ is hardly changed. We note that the signal intensity for non-binding probes was at the background level for both RD and IT conditions, which indicates that RD does not increase non-specific binding.

The effectiveness of RD was investigated for different concentrations by monitoring the signal intensity and kinetics of hybridization until a steady state was observed. From the steady-state signals an apparent dose response curve (DRC) is established. Hybridization experiments with ss-DNA probes were used as a benchmark. Figure 5 depicts the DRCs, i.e. the intensity values read on the scanner upon reaching the steady state (i.e. after over-night hybridization), vs. concentration for all the conditions analyzed.

![Figure 5](image_url)

First of all one can notice the strong increase in intensity with RD as compared to IT for both probes at all concentrations, as long as the signal is above the detection limit. The DRC for RD conditions is very close to the benchmark of ss-DNA in which no back hybridization can occur. This means that with the used set up and conditions the denaturing is very effective. Consequently, a considerable gain in hybridization efficiency can be made, compared to current state of the art even if diffusion limitation has been relieved by active flow. At high concentrations the RD curve does not match the ss-DNA benchmark fully. This can be explained by the residence time between the hot zone and the array (The cycle time was 80 s). For high concentrations the back hybridization will be fastest because the reaction rate is expected to scale with $[sa]^2$, see Eq. 2.

Since for concentrations lower than 1 pM the experimental approach is insufficiently sensitive, extrapolation to lower concentrations is attempted. For that purpose the kinetics of back hybridization should be described as a function of concentration of targets, $[sa]$. For thermal jump experiments Wetmur derived an expression for the back-hybridization of ds DNA for conditions close to equilibrium:
\[ \tau^{-1} = \sqrt{2k_{on}k_{off}C_0 + k_{off}^2} \text{ with } C_0 = 2[sa] + 2[ds] \]  

An essential difference to Eq. 5 is that in Eq. 6 \( \tau \) no longer depends on a time-varying concentration, \([sa]\), but on the constant total nucleotide concentration, \( C_0 \). Since the kinetic parameters are not accessible directly, they are derived from the DRCs of the single stranded targets, where \( K_D = c_{50} \), with \( c_{50} \) the concentration that produces half of the maximum in the DRC. This is assuming that \( k_{on} \) and \( k_{off} \) for the hybridization at the surface are equal to those for hybridization in solution. Since the reaction rate, \( R \), for back hybridization was determined from the experiments shown in Fig. 2 with the aid of eq. 1, by choosing \( R = 1/\tau \) one can determine both \( k_{on} \) and \( k_{off} \), knowing \( C_0 \). Here the validity of the equation derived by Wetmur is not evident, however, for lack of a better expression this approximation is taken.

From the fit of the DRC of ss-DNA in Fig 5, \( K_D = 8.1*10^{-10} \) M for probe 1 and \( K_D = 1.1*10^{-9} \) M for probe 2 is obtained. Since for \([sa] = 1 \) nM the value of \( \tau^{-1} \) is known, the values of \( K_D \) can be used to solve Eq. 6, and thus obtain \( k_{on} \) and \( k_{off} \) for back-hybridization. For probe 1 \( k_{on} = 2.9 \times 10^5 \) M\(^{-1}\)s\(^{-1}\), \( k_{off} = 2.3 \times 10^4 \) s\(^{-1}\), for probe 2 \( k_{on} = 1.4 \times 10^5 \) M\(^{-1}\)s\(^{-1}\) and \( k_{off} = 1.5 \times 10^4 \) s\(^{-1}\). The reliability of these values was assessed by comparing the experimental DRC with the calculated curve using \( k_{on} \) and \( k_{off} \). A good match is obtained. Since \( k_{on} \) and \( k_{off} \) are concentration independent, they can be substituted in Eq. 6 to calculate the characteristic time of back-hybridization for different values of \( C_0 \), and the result is shown in Table 1.

<table>
<thead>
<tr>
<th>( \tau )</th>
<th>100 nM</th>
<th>10 nM</th>
<th>1 nM</th>
<th>100 pM</th>
<th>10 pM</th>
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<tr>
<td>probe 1</td>
<td>4 min</td>
<td>14 min</td>
<td>39 min</td>
<td>65 min</td>
<td>71 min</td>
<td>72 min</td>
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<tr>
<td>probe 2</td>
<td>8 min</td>
<td>25 min</td>
<td>65 min</td>
<td>102 min</td>
<td>110 min</td>
<td>110 min</td>
</tr>
</tbody>
</table>

**Table 1**: Characteristic time in minutes for the depletion of sa in solution, calculated for probe 1 and 2 from Eq. 6, for different values of the starting concentration \( C_0 \).

The depletion of sa in solution during the hybridization time can now be obtained by substituting for \( A \) the values of \( \tau^{-1} \) from Table 1 in Eq. 6.
Figure 6 depicts the percentage of single-stranded targets \([sa]\) vs. time for probe 1. The graph clearly shows a significant depletion for all values of \(C_0\), even though the extent of depletion is higher when the starting concentration is high. Notably, for \(C_0 \leq 100\) pM the depletion in relative terms is independent of the starting concentration. This is caused as at low concentration \(C_0\) \((k_{\text{off}} \gg k_{\text{on}}C_0)\) i.e. \(\tau\) is determined by \(k_{\text{off}}\) thus the dissociation reaction becomes dominant. The same is observed for probe 2.

Figure 6 demonstrates that back-hybridization reduces \([sa]\) in solution during the assay time and thus the amount of targets available for binding to the capture probes on the array. This effect is present for any value of the starting concentration and is one of the major causes for the low efficiency of hybridization assays. According to these considerations, the performance of capturing on array can be improved by counteracting back-hybridization and thus the depletion of single stranded targets in solution. We expect improvement in the capturing on array for the entire range of concentration under consideration. The device introduced here acts in this direction, with the ultimate goal of maintaining \([sa]_t \gg [ds]\) throughout the capturing process.

3.3. Applications of integrated denaturing

The performance of the microfluidic cartridge with integrated repeated denaturing was compared to two commercial hybridization stations, Agilent and NimbleGen (Roche). Both Roche and Agilent systems operate by actively mixing the hybridization solution, although different mixing strategies and volumes are used. Given that target enrichment assays for next gen sequencing are performed at low target
the performance of repeated denaturing versus conventional hybridization solutions was tested for target enrichment 100 pM and at the lowest detectable concentration (1 pM).

Fig. 7 shows that the hybridization signal is improved by RD in the cartridge, compared to both commercial systems. The benefits of RD are particularly evident at 1 pM, where hybridization in the cartridge gains a factor 10 with respect to the Agilent system. At the same concentration, no signal was detected on the slides combined with the Roche system. Therefore, in a subsequent experiment the comparison was repeated at 100-fold higher concentration to obtain also in the latter system a hybridization signal. Note that in this experiment the concentration of targets is fixed, while in another comparison the amount of targets in solution was kept equal for both commercial systems. The higher signal obtained in the Agilent chamber with respect to the Roche mixer points to a more effective distributive mixing which is achieved by the Agilent system with the air bubble in a tumbling system. Notably, at 100 pM the signal intensity obtained in the cartridge in IT conditions (Fig. 5) equals the value measured in the Agilent system. Thus convection in our cartridge is highly efficient while on top of that in-flow denaturation contributes to a further ten-fold increase in performance compared to the commercial system. Notably, the gain is high at low concentration, in agreement with the observations in the section 4.2. Typically, however, most of the actual quantitative hybridization studies or target enrichment assays involve low concentrations of a very large number of targets (sheared DNA fragments). Such systems are very complex due to the large number of possible cross hybridizations between targets and between non-targets and probes. The effective concentration governing back hybridization in solution is much higher than the concentration of perfect match pairs. Therefore one can assume that the repeated denaturing approach should be very effective as well. However, to test the benefits of repeated denaturing on a commercial microarray slide a microfluidic device needs to be designed which can cover a full micro-array. Since with fluorescence detection no distinction can be made between perfect matches and mismatches a more elaborate analysis is required. In the end a real test will be the determination of the fraction which is on target as determined by a next-gen sequencing run.
4. Conclusions

For nucleic acid binding procedures with double-stranded target material, back-hybridization in solution is one of the major causes of low efficiency. Freshly denatured nucleic acids can efficiently bind to probes on a substrate, but hybridization to their complements in solution continuously reduces their availability in the assay. This phenomenon is present for the full range of starting concentrations studied. The relative target depletion is strongest at high concentrations, but for practical applications in genetic testing the depletion is most critical at low concentrations.

A new microfluidic device has been presented with integrated heating stage, which allows in-flow denaturation of the targets during the hybridization assay. The sample is circulating in a closed loop in a microchannel, built on a microarray. The solution is cyclically flown over the denaturing stage, and therefore constantly supplemented with single-stranded targets. This approach can effectively counteract the action of back-hybridization over a very broad range of concentrations. The method does not alter intrinsic reaction kinetics, but significantly increases the amount of bound target. Together with active flow conditions to ensure reaction-limited binding kinetics, this improves the efficiency of hybridization by 10 times compared to commercially available hybridization stations.

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References


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Figure captions

Figure 1. (a) Sketch of the microfluidic cartridge. The microchannel is U-shaped with parallel channels of 20 mm length, and a transverse tract of 5 mm. The channels are 1.2 mm wide and 100 µm high. The cartridge is used in combination with a thin film heater, comprised of 3 independent zones. The two parallel channels are maintained at hybridization temperature (50°C – in grey) and the U-bend area (highlighted in black) is set at denaturing temperature (95°C). (b) Picture of the cartridge and multi-zone heater with flex interconnects.

Figure 2. Normalized average spot intensity on the micro-array vs. storage time before hybridization. Hybridization time is 30 min.

Figure 3. Average spot intensity of the micro-array in front of (before) and behind (after) the denaturing zone after in-flow hybridization for 30 min. In-flow denaturation increases the signal intensity by 2 orders of magnitude for probe 1 and 2, at 1 nM concentration.

Figure 4. The time evolution of the signal intensity on array for different values of the flow rate (18 µl/min, 35 µl/min and 60 µl/min) under isothermal conditions. The τ values in the table are derived from a fit of the experimental points with the Langmuir equation as indicated in the text. The best performance (= lowest τ) is observed for Q=35 µl/min. The curve measured at Q=35 µl/min in RD conditions is reported for comparison.

Figure 5. Dose response curve for target 1 (left) and target 2 (right) under RD and IT conditions. The dose-response curves for the ss-DNA targets are shown for comparison. For C₀ ≤ 100 pM, the RD curves shows a good match with the corresponding ss-DNA, demonstrating that the counteraction of RD to back-hybridization is highly efficient at low concentrations. For C₀ ≥ 100 pM, the RD curves recover at least 70% of the ss-DNA signal. The lines are merely to guide the eye.

Figure 6. Modeling of the depletion of [sa] in solution during the hybridization in isothermal conditions for different values of the starting concentration. The graph shows that [sa] is continuously reduced during the assay time at any starting concentration. For C₀ ≤ 100 pM the curves coincide.

Figure 7. The target capture performance with repeated denaturing in the cartridge is compared to that of the Agilent and Roche hybridization stations. Experiments were carried out overnight under the same conditions, with target 1 and target 2 at 1 pM (A) and 100 pM (B).