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Short communication

Lambda exonuclease pre-treatment for improved DNA-chip performance depends on the relative probe-target position

Anja Brinker[a], Holger Schulze[b], Till Bachmann[b], Robert Möller[a,c,*]

[a] Jenaer Biochip Initiative, Friedrich Schiller University, Institute of Physical Chemistry, Helmholtzweg 4, Jena, Germany
[b] Division of Pathway Medicine, College of Medicine and Veterinary Medicine, The University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, Scotland, UK
[c] Institute of Photonic Technology, Albert-Einstein-Str. 9, 07745 Jena, Germany

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ABSTRACT

The hybridisation characteristics of DNA targets to solid phase bound probes, e.g. in DNA microarrays, depend on the probe-target position and on target renaturation if a dsDNA target is used. We investigated a lambda exonuclease treatment of a PCR amplified dsDNA target to produce ssDNA with regard to probe-target position, treatment duration and inactivation time towards its impact on fluorescence or electrical signals on two DNA-chip formats. Surprisingly, the achieved amplification factors varied by three orders of magnitude, i.e. 2–1074 fold signal enhancement, depending on the relative probe-target position and readout scheme. The presented results can be used to design future studies involving lambda exonuclease preanalytic treatments.

1. Introduction

Today DNA microarrays are a standard tool for the analytics of nucleic acid targets. They provide a versatile platform to analyse large sets of targets in a highly parallel manner. As the standard fluorescent target labelling requires sophisticated equipment for readout various alternative formats were designed, e.g. electrical readout (Leinberger et al., 2010; Moeller and Fritzsche, 2005; Schena et al., 1995; Stears et al., 2003). It is a well known fact that hybridisation of nucleic acid targets in solution to solid phase bound probes is critical and competes unfavourable with complementary targets in solution. In addition, long PCR produced dsDNA targets are thermodynamically much more stable than comparably shorter probe-target hybrids (Boissinot et al., 2007; Holloway et al., 1993; Peytavi et al., 2005). The result is generally decreased hybridisation efficiency and consequently a resulting higher limit of detection. As previously described, the length and sequence of the dangling end of the target strand was found to have a great impact on signal intensity and decreased specificity of probe-target hybridisations (Riccelli et al., 2002; Stedtfeld et al., 2007). It was mentioned that a long dangling end provides a better access to its complementary strands in solution what leads to renaturation and therefore to a decreased hybridisation efficiency or even no signal detection is possible at all (Peytavi et al., 2005). One possibility to reduce or to circumvent the problem of renaturation is the production of single-stranded DNA for the hybridisation. In previous studies the production of single-stranded DNA, using asymmetric PCR, LATE-PCR, RCA, exonuclease etc., for hybridisation was already described (Pierce et al., 2005; Tang et al., 2009; Zhao et al., 2008). We decided to use a lambda exonuclease pre-treatment since the existing PCR-systems could be used without any modifications and additional time requirements, e.g. for the development of new protocols or longer process times. Lambda exonuclease is an enzyme that degrades one strand of double stranded DNA (dsDNA) in 5′–3′ direction only if a phosphate is present at 5′ position (Higuchi and Ochman, 1989; Mitsis and Kwagh, 1999). The use of exonucleases was reported with great variance in process time and/or enhancement efficiency (Holloway et al., 1993; Karnani et al., 2009; Nikiforov et al., 1994; Reske et al., 2007). Signal enhancements up to 15 times, after 5 min digestion, were mentioned (Boissinot et al., 2007). A comparison of the already described results for an exonuclease treatment and the reached enhancement efficiency is not possible as the probe-target position was often not specified and not investigated. If the probe-target position is good, which means that the target has a short overhang to the solution, a strong enhancement of signal intensity could not be expected. On the other hand, a high signal enhancement can be reached by removing the non-target strand, if the target-strand has a long overhang to the

* Corresponding author at: Jenaer Biochip Initiative, Friedrich Schiller University, Institute of Physical Chemistry, Helmholtzweg 4, Jena, Germany.
Tel.: +49 3641 206306; fax: +49 3641 206399.
E-mail address: robert.moeller@ipht-jena.de (R. Möller).

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solution and therewith a suboptimal position to the probe. With no information given about probe-target position, a direct comparison of the efficiency of the used exonuclease treatment is not possible. The aim of this work was to give the operator the opportunity to assess if the use of a lambda exonuclease pre-treatment is suitable for its application and if a benefit is expectable for specific chip-based applications. Therefore, we have tested the digestion and hybridisation of three different PCR products. For each system three probes were used for the on-chip hybridisation. One probe was selected where the target strand has a long overhang to the medium (x_3′ probes), one with a sequence complementary to a sequence in the centre of the target (x_c probes) and one probe where the target has a short overhang to the medium (x_5′ probes) after hybridisation. Hence it should be clarified how strong the effect of renaturation depends on the probe-target position and therefore if a signal enhancement could be expected for different probes. Also, the advantages or disadvantages of this additional process step for a chip-based optical and electrical detection were analysed. The applied systems are used for the detection of *Mycoplasma mycoides* subspecies *mycoides* small colony type (*Mmm*SC). *Mmm*SC is the etiological agent of contagious bovine pleuropneumonia (CBPP), a severe respiratory disease affecting cattle and buffalo, which is characterised by lesions in the lungs and thorax of affected animals (Bashiruddin et al., 1999). The development of a fast and sensitive method for the detection of epizootic diseases would be favourable as time is critical in the case of an epizootic outbreak. Therefore, the development of platforms which enable on-site-testing is essential.

The results presented in this paper show, that the use of a lambda exonuclease digestion could be highly effective to enhance hybridisation efficiency on dependence of probe-target position. Also, we could enable the electrical detection of the pathogen of interest by the use of a lambda exonuclease pre-treatment without additional time requirements in comparison to the conventional thermal denaturation before hybridisation.

2. Materials and methods

2.1. Materials and reagents

A list of the used materials and reagents can be found in the supporting information. DNA and the sequences for primers and probes were provided by the FLI (Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, Jena). The oligonucleotides are listed together with the controls in Table 1S (see supplementary material). The primers used to amplify a target of 285 bp from *Mmm*SC GTP-binding protein elongation factor lepA (system a), a target of 260 bp for the amplification of *Mmm*SC lipoprotein lppQ (system b) and a target of 227 bp from the *Mmm*SC hypothetical transmembrane protein (system c). Probe-target positions for the three systems are shown in Table 2S (see supporting information). For optical detection a Cy3 labelled spotting control (PK-Cy3) and for electrical detection a biotin-labelled oligonucleotide (PK-biotin) was used to check the spotting quality and to facilitate orientation on the array. Furthermore a negative control was spotted. The sequences of the controls were unrelated to sequences amplified by the used primers. All probes had an amino modification at their 5′ end to facilitate terminal immobilisation to the chip surface.

2.2. Array fabrication

For fluorescence detection arrays were spotted on Schott Nexterion Slides E epoxy modified glass slides (Schott, Jena, Germany) using a Microgrid II (Biorobotics, Cambridge, UK). All capture probes were spotted in triplicates. For an optimal immobilisation the slides were incubated in a humidity chamber for 15 min at 37 °C and dried at 60 °C for 30 min. For a covalent binding of DNA-probes the slide surfaces were exposed to 350 kJ/cm^2 using a UV crosslinker (UVP Upland, CA, USA). After UV-crosslinking unbound DNA molecules and salt residues were removed from the slides by washing them in 0.1× Saline Sodium Citrate (SSC)/0.5% sodium dodecyl sulphate (SDS) for 10 min and an additional washing step from 30 s in distilled water at room temperature. Finally, the slides were dried by centrifugation.

For electrical detection glass chips with screen printed electrodes were used. The chips were modified with 3′-glycidoxypropyltrimethoxysilane (GOPS) and afterwards amino-modified probes were bound. The chips and the modification of the surface are described elsewhere in detail (Moeller et al., 2001; Schueler et al., 2009). All capture probes were spotted in quadruplicates.

2.3. Sample preparation

For PCR reactions 25 ng genomic DNA, extracted from the type strain PG1 of *Mycoplasma mycoides* subspec. *mycoides* small colony (*Mmm*SC), were added to the reaction mixture with a total volume of 25 μl. Details are given in the supplementary material. For fluorescence detection the above described conditions were used and the reverse primer was labelled at the 5′ end with Cy3 and for electrical detection a 5′ biotin-label was used. For the pre-treatment with lambda exonuclease, the forward primers were labelled with phosphate on their 5′ end. Before hybridisation amplicons were digested in 1× exonuclease puffer with 15 U of lambda exonuclease at 37 °C for different times and were inactivated at 75 °C for 10 min. Samples which were used for hybridisation without pre-treatment with lambda exonuclease were denatured for 5 min at 95 °C and then cooled on ice for 2 min.

2.4. Hybridisation and readout

In the case of fluorescence detection the hybridisation of labelled PCR products was performed in 54 μl of 3× SSPE, 0.02% SDS for 60 min at 55 °C with constant rotation using an Agilent microarray hybridisation chamber system and oven (Agilent, Santa Clara CA, USA). Afterwards, the slides were washed with 2× SSC, 0.2% SDS, with 0.2× SSC and finally with 0.1× SSC, each time at room temperature, before they were dried by centrifugation. Slides were scanned with a Tecan LS Reloaded microarray scanner (Tecan, Maennedorf, Switzerland). Fluorescence signal intensities were analysed by use of the Quantarray software (Perkin Elmer, Waltham Massachusetts, USA). The resulting raw data were further processed using Excel (Microsoft, Redmond, WA). A mean intensity value for each capture probe was calculated from the three replicate spots for each probe.

For the electrical detection, hybridisation on DNA-chips was performed in a microfluidic device with 30 μl of 3× SSPE, 0.02% SDS for 5 min at 55 °C as described previously (Schueler et al., 2009). The chips were washed with 2× SSC, 0.2% SDS, with 0.2× SSC and finally with 0.1× SSC, each time at room temperature. For the electrical detection an enzyme-induced silver deposition was performed as described elsewhere (Moeller and Fritzsche, 2007) for 3 min and the conductivity measurement was conducted with a custom-built device (Urban et al., 2003). After the enzymatic silver deposition two additional silver enhancement steps, for 2.5 min each, were performed.

3. Results and discussion

The experiments to investigate the digestion time, inactivation time, and probe-target position on the signal enhancement