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Dynamic range extension of hybridization sensors

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ABSTRACT

In hybridization based nucleic acid sensors the stringency of hybridization poses a challenge to design and experiment. For a given set of experimental parameters the affinity window of probe–target interaction is always limited and vice versa for a given probe set design, changes in experimental conditions can easily bring some measurements out of detection range. In this paper we introduce and apply a strategy to extend this dynamic range for affinity sensors, sensors which measure the amount of hybridized molecules after equilibrium is reached. The method relies on concepts of additivity of nucleic acids hybridization free energies and on equilibrium isotherms. It consists in combining the measurements from probes with different lengths, by appropriately rescaling the measured signals. We test the validity of the approach on experiments and show that by combining probes with hybridizing regions of length 21, 23 and 25 nucleotides we manage to extend the dynamic range of the intensity signals by a factor of 25. The presented concept is easy to extend, platform free and applies to any hybridization based affinity sensor.

1. Introduction

For the detection of nucleic acid abundance or sequence variations there exists a large set of different methods which can be divided into two classes: hybridization based techniques and enzyme based techniques. The latter are used in e.g. PCR and sequencing devices. The focus of this paper is on hybridization techniques which are appealing due to simplicity: a nucleic acid probe is designed to hybridize to the perfectly matching target molecule with a high efficiency while having a low efficiency for targets containing sequence variations. The principle is open to many technical implementations and miniaturization for use in biosensor devices (Cornett et al., 2013; Janse et al., 2012; Vanden Bon et al., 2014; Tomlinson et al., 2014; Cherstvy, 2013; Mahadhy et al., 2014). However, hybridization has challenges on the side of probe design and dynamic range due to hybridization stringency conditions (Yang et al., 1999; Sharov et al., 2004; Sassolas et al., 2007) which are not easy to optimize especially when parallelization is aimed for. The dynamic range of a sensor, i.e. the range in which the response signal is linear in concentration of the target, is an important concept and the control of it is of high practical value for any sensor type. The presented work considers this issue for DNA affinity sensors, i.e. sensors which measure equilibrated signals without kinetic observations.

We address this issue with in mind the application of DNA genotyping by single nucleotide polymorphism (SNP) detection. A SNP is a DNA sequence variation between humans (or other members of the same species) consisting of a single nucleotide difference. It accounts for the majority of genome variations between people and is functionally highly relevant. The detection of SNPs is important in many research and application domains like genome-wide associations studies, personalized molecular diagnostics and forensic identification (Gresham et al., 2008; McCarroll, 2008; Holbrook et al., 2011; Blakemore and Froguel, 2010; Kling et al., 2012; Freire-Aradas et al., 2012).

To detect SNPs on a given sequence by a hybridization technique one should use a sensor which contains the complementary probes of the possible target variants. In such a setup target molecules hybridize to different probes with different affinities: not only to their perfect complement, but also to probe sequences containing one or more mismatches. To make an optimal differentiation between them, they should all fall within the dynamic range of the detecting device. This is the region in which the measured signal I is proportional to the target concentration: $I \propto c$. The intrinsic limitation, also in practice, is that a biosensor has a limited window of detection in which “too low” or “too high” signals are not exploitable.

In the current work we present a way to face this problem, we define the dynamic range of a hybridization affinity device and we

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introduce a concept by which the range can be extended by quantitatively combining readouts of probes with different lengths. We show an experimental implementation of the principle by means of a microarray experiment in which we use three different probe lengths which results in the extension of the dynamic range by a factor of 25. The concept is however general and more probe lengths will result in larger extension factors.

2. Materials and methods

2.1. Sample preparation and hybridization experiment

The target in the experiment is a single stranded DNA (ssDNA) oligo which consists of a hybridization region and a labeling region. The former is the part of the oligo of interest which hybridizes to the microarray probes, its sequence is presented in Table 1. The latter is added for labeling purposes and consists of poly A spacer together with a barcode sequence 5′-AAA AAAAAAATCACACAGGAAA CAGCTATGACGCCAG-3′.

The microarray experiments were performed using the commercially available Agilent platform and followed a standard protocol with Agilent products, as described subsequently. Each hybridization mixture contains the target sequence at 10 pM together with a Cy3-labeled Barcode (Cy3-5′-AAAAACTGGCGTCA-TAGCTGTTTCCTGTGTGA-3′) diluted in nuclease-free water to a final concentration of 0.5 μM. The solution also contains 5 μl 10 × blocking agent and 25 μl 2 × GEx hybridization buffer HI-RPM. The hybridization mixture was centrifuged at 13,000 rpm for 1 min and the microarray of the 8 × 15K custom Agilent slide was loaded with 40 μl of the mixture. The hybridization occurred in an Agilent oven at 65 °C for 17 h with rotor setting 10 and the washing was performed according to the instructions of the manufacturer. The array was scanned on an Agilent scanner (G2565BA) at 5 μm resolution, high and low laser intensities and further processed using Agilent Feature Extraction Software (GE1 v5 95 Feb07) that performs automatic gridding, intensity measurement, background subtraction and quality checks. The choice for a barcoded indirect labeling design is not essential here, it was made to have a multipurpose assay without the need to label any target sequence.

Table 1
Sequence of hybridization region of the target and of examples of probes immobilized on the surface of the microarray. The probes (a–c) are perfectly matching the target sequence, however they differ in length (given in the left column). The probes (d–i) are sequences with mismatches.

L	Hybridization region target sequence
25	5′– CTTGCCTACGCCACCAGCTCCAACT – 3′
L	**Microarray probes sequence**
25	5′– AGTTGGAGCTGGTGGCGTAGGCAAG – 3′ (a)
23	5′– GTTGGAGCTGGTGGCGTAGGCAA – 3′ (b)
21	5′– TTGGAGCTGGTGGCGTAGGCA – 3′ (c)
…	……
25	5′– AGTTGGAGCTGGCGGCGTAGGCAAG – 3′ (d)
23	5′– GTTGGAGCTGGCGGCGTAGGCAA – 3′ (e)
21	5′– TTGGAGCTGGCGGCGTAGGCA – 3′ (f)
…	……
25	5′– AGTTGGAGATGGTGGTGTAGGCAAG – 3′ (g)
23	5′– GTTGGAGATGGTGGTGTAGGCAA – 3′ (h)
21	5′– TTGGAGATGGTGGTGTAGGCA – 3′ (i)
…	……

2.2. Probeset design

In the microarray experiment a large number of different probes were used (in total 1836). The design of the probeset combines nucleotide variations and probe length variations. The former is comparable to designs previously used to detect SNPs (Hooyberghs and Carlon, 2010). The probes carry up to two mismatches with respect to the target sequence. A few examples of sequences of this large probeset are shown in Table 1. Sequences (a–c) are perfectly matching the target, but they differ in lengths and overlap the hybridizing region with $L=25$, $L=23$ and $L=21$ nucleotides. (d–i) are examples of probes containing mismatches. Also these probes have three different lengths: $L=21$, 23 and 25. The mismatching nucleotides are underlined.

The constraint in designing the probe sequence is that the minimal distance between two mismatches is of 4 nucleotides and the mismatches have a minimal distance of 4 nucleotides to the ends of the hybridizing region (unless for L21 probes that have up to 3 nucleotides distance to the ends of the hybridizing region). This follows the strategy discussed in Hadiwikarta et al. (2012). As mismatches are sufficiently far apart their effect in the affinity should be considered as additive. By selecting them sufficiently far from the ends of the hybridizing region one can neglect end effects (it is known that mismatches close to the end of the double helix are less destabilizing compared to mismatches in the bulk Hadiwikarta et al., 2012; SantaLucia and Hicks, 2004; Letowski et al., 2004).

2.3. Langmuir isotherm and dynamic range

When nucleic acid targets hybridize on immobilized surface probes its equilibrium state can be described by the Langmuir isotherm (Carlon and Heim, 2006; Held et al., 2003; Hooyberghs et al., 2009). Assuming that the fraction of hybridized probes in a microarray spot are proportional to the measured signal intensity, for a DNA double helix in thermodynamic equilibrium, the isotherm can be written as

$$I = \max\left(I_0, A\frac{ce^{-\Delta G/RT}}{1 + ce^{-\Delta G/RT}}\right) \quad (1)$$

where I is the detected intensity signal, I_0 the lower detection limit of the readout, A the maximum intensity when a spot is saturated by target molecules, c the target concentration in solution, R the ideal gas constant, T the experimental temperature and ΔG the hybridization free energy which determines the affinity of target–probe duplexes in a sequence dependent way. In hybridization experiments the temperature and the target concentration are usually fixed, hence it is instructive to analyze the dependence of spot intensities on the free energy of the target-probe duplex. Pane A of Fig. 1 gives a visualization of Eq. (1) for realistic values of the physical parameters. From this equation one can read that when the target concentration is very high or the duplex affinity is very strong, i.e. $ce^{-\Delta G/RT} \gg 1$, the signal will hit the saturation limit thus $I \approx A$. If the concentration is very low or the affinity is very weak, i.e. $Ace^{-\Delta G/RT} < I_0$, the signal cannot drop below the detection limit thus $I = I_0$. The inequalities

$$I_0 < Ace^{-\Delta G/RT} \ll A \quad (2)$$

define the dynamic range of the hybridization sensor. Within this range a device is in its working regime and Eq. (1) can be approximated by

$$I \approx Ace^{-\Delta G/RT} \quad (3)$$

In Fig. 1 this function is plotted as a dashed line. Within the dynamic range (2) the measured signal intensities are responsive