Spacer Length, Label Moiety Interchange and Probe Pair Orientation in a Homogeneous Solid-Phase Hybridization Assay Utilizing Lanthanide Chelate Complementation

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Abstract

We have studied parameters affecting DNA hybridization and lanthanide chelate complementation based signal formation in a separation-free solid-phase assay suitable for spatial multiplexing. This binary probe assay system consists of two probes labeled either with a europium carrier chelate or a light harvesting antenna ligand. One probe was immobilized on the microtiter well bottom in spot format while the other probe was free in solution. The probe concentration used in spotting, spacer length, and the choice and orientation of the either 3´ or 5´-end immobilized probe had significant impact on signal-to-background (S/B) ratios. The highest ratio was achieved by saturating the spot with the 5´-end immobilized antenna ligand probe separated from the solid support with a 25 nucleotide poly dT spacer. The dynamic range of the assay for synthetic *Pseudomonas aeruginosa* heat shock protein *groES* gene target was three orders of magnitude and the detection limit of 18 pM was obtained. The *groES* target sequence was also amplified from isolated genomic DNA of *P. aeruginosa* both with and without co-amplification of synthetic internal assay control (IAC) followed by successful detection of the *groES* amplification product although the co-amplification of IAC resulted in slightly decreased *groES*

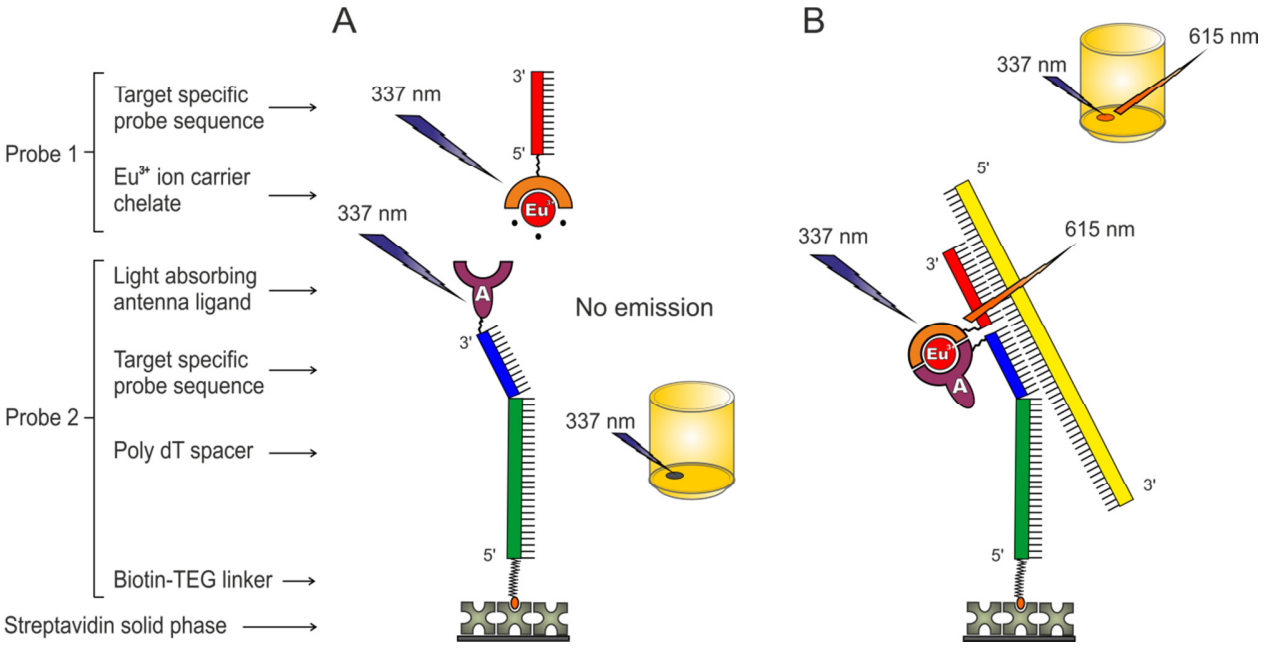
signal. The homogenous detection of spatially resolved probes on solid-phase could enable alternative way of multiplexing to closed tube nucleic acid assays.

Keywords

Lanthanide chelate complementation, europium, homogeneous, separation-free, hybridization, time-resolved fluorescence

1 Introduction

The increasing interest in fast, reliable and economic diagnostic tools has created a need for miniaturized multiplexed assay concepts where several different parameters can be detected simultaneously in a single sample. The possibility to simplify the assays and make them less error prone has directed the interest further to separation-free assay formats. Karhunen *et al.*[1] have developed a homogenous nucleic acid assay utilizing lanthanide chelate complementation technology (Scheme 1). In this technology two inherently nonluminescent label moieties, a light harvesting antenna ligand (sensitizer) and a nonluminescent lanthanide ion carrier chelate, form a luminescent complex when in close proximity. Formation of mixed chelate complex is a prerequisite for energy transfer to occur from the sensitizer to the lanthanide ion carrier chelate. Multiplexing by spatial differentiation of multiple targets is made possible by immobilizing the probe containing one of the label moieties on a solid support[2]. In this paper we describe a detailed study of the homogeneous solid-phase method using a sequence from *Pseudomonas aeruginosa* heat shock protein gene, *groES*, as the model target.



Scheme 1 A schematic illustration of the homogenous solid-phase lanthanide chelate complementation technology. The 3´-end light harvesting antenna ligand labeled oligonucleotide probe (blue) with a poly dT spacer (green) containing a biotin at the 5´-end is attached into the streptavidin coated solid support (microtiter well). The 5´-end europium ion carrier chelate labeled oligonucleotide probe (red) is free in solution. (a) In the absence of the target no luminescent complex is formed and there is no post-excitation signal. (b) When the target (yellow) is present the parallel hybridization of the two probes into the target brings the label moieties in such a close proximity that a luminescent complex is formed and the europium luminescence can be measured at 615 nm after excitation at 337 nm. The unbound probes need not to be removed because they are nonluminescent.

Europium ion has a total of 9 coordination sites from which six to seven coordination sites are involved in the carrier chelate (DOTA-Eu^{3+}) formation leaving two to three coordination sites free for additional bonding. In water, these free coordination sites are occupied by quenching solvent molecules, which are replaced with a light-harvesting antenna ligand in the chelate complementation[1]. In the assay of Karhunen *et al.*[2] the oligonucleotide probe with the europium ion carrier chelate at the 3´-end was immobilized into a streptavidin coated microtiter well through a 5´-end biotin moiety while the antenna ligand probe was free in solution. In our work we have investigated the effect of label moiety interchange in the immobilized and free oligonucleotide probes on the assay performance.

Even though solution phase reactions are kinetically favoured to solid phase reactions[3] the advantage of using a solid support is the possibility for spatial identification of the bound targets in multiplexed arrays. By separating the immobilized probe from the solid support with a spacer brings the probe closer to the solution state enabling more freely interactions with target molecules. After evaluating the effect of spacer length, charge, and hydrophobicity, Shchepinov *et al.*[3] concluded the length of the spacer as having the highest impact on hybridization yields which can be increased up to two orders of magnitude by introducing spacers between the solid support and the immobilized oligonucleotide probes[4]. Karhunen *et al.*[2] had 6 nucleotides (36 atoms) as spacer in the immobilized oligonucleotide probe which was within the limits of the optimal spacer length of 30–60 atoms determined by Shchepinov *et al.*[3] and correlated with the poly dT spacer of 5 nucleotides used by Livache *et al.*[5]. However, Guo *et al.*[6] considered a spacer consisting of 6 dT nucleotides to be insufficient. The benefits of spacers are generally admitted although their optimal design seem to vary and is highly dependent of the different experimental conditions[7]. In our research we have investigated the effect of spacer length on S/B ratios by introducing from 0 (0 atoms) up to 67 thymidines (402 atoms) into the 3´-end immobilized probe. Moreover, we have used biotin with a mixed polarity triethylene glycol (TEG) linker to introduce additional 15 atoms into the spacer and to reduce steric hindrance between the biotin moiety and the oligonucleotide, thereby providing streptavidin easier access to the biotin for capture and immobilization of the oligonucleotide. In addition we have evaluated the effect of probe pair orientation on hybridization as well as the effect of surface density of the immobilized probes and hybridization conditions. Interference of non-target DNA in *groES* detection was evaluated with synthetic oligonucleotides. The detection of *groES* amplification product and interference of non-target DNA were evaluated by amplification of *groES* target sequence from isolated *P. aeruginosa* genomic DNA both with and without co-amplification of synthetic internal assay control (IAC) templates.

2 Experimental

2.1 Materials

Probe and primer oligonucleotides (Table 1) were purchased from Biomers.net (www.biomers.net, Ulm, Germany) and the synthetic target and template sequences were from Integrated DNA Technologies (www.idtdna.com, Leuven, Belgium). Two target specific probe sequences for *groES* detection were designed based on the genomic sequence of *P. aeruginosa* heat shock protein gene *groES* originally obtained from the National Center for Biotechnology Information

(NCBI, http://www.ncbi.nlm.nih.gov, gene accession number M63957). The target specific probe sequences were designed on the anti-sense strand between nucleobases 55 and 82 with one nucleobase separating the two sequences. The synthetic *groES* target used in the assay optimization contained one nucleobase outside the combined probe binding sequence at both the 3´ and 5´-ends. *P. aeruginosa* (ATCC® Number 27853™) was originally from American Type Culture Collection (Rockville, MD), plate cultured and the genomic DNA was isolated using NucleoSpin® Tissue kit from Macherey-Nagel GmbH & Co. KG (www.mn-net.com, Düren, Germany). The concentration of the genomic DNA was determined by measuring the absorbance at 260 nm. IAC was designed by artificially mutating (< 50 % similarity) *Carassius auratus auratus* (gold crucian carp) isolate DG18-1 D-loop gene originally obtained from DNA Data Bank of Japan (DDBJ, http://getentry.ddbj.nig.ac.jp, gene accession number EU697145). All reagents were ACS laboratory reagents and from Sigma-Aldrich (www.sigmaaldrich.com, St. Louis, MO) unless stated otherwise. Low fluorescent streptavidin coated 96-well microtiter plates used for immobilization of biotinylated probes were from Kaivogen Oy (www.kaivogen.com, Turku, Finland) and DMF was from Acros Organic (www.acros.com, Geel, Belgium). Labeled oligonucleotides were stored and immobilized in oligo storage buffer consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl and 10 µM EDTA. Standard hybridization buffer contained 50 mM Tris-HCl (pH 7.75), 600 mM NaCl, 0.1% (v/v) Tween® 20, 0.05% (w/v) NaN_3 and 100 µM diethylenetriaminepentaacetic acid (DTPA) (Merck, www.merck.com, Darmstadt, Germany).

Table 1 Synthetic oligonucleotide sequences and modifications for the amplification and detection of *groES* and IAC.

Probe[a]	Label	5´ Modification	Sequence 5´→ 3´	3´ Modification	dT's in Spacer
ImT0_5' Ant	Antenna	Aminolink C6	GTC GTT ATC CGT C	Biotin-TEG	0
ImT13_5' Ant	Antenna	Aminolink C6	GTC GTT ATC CGT CTT TTT TTT TTT TT	Biotin-TEG	13
ImT25_5' Ant	Antenna	Aminolink C6	GTC GTT ATC CGT CTT TTT TTT TTT TTT TTT TTT TTT TT	Biotin-TEG	25
ImT25_5' Eu	DOTA-Eu^{3+}	Aminolink C6	GTC GTT ATC CGT CTT TTT TTT TTT TTT TTT TTT TTT TT	Biotin-TEG	25
ImT67_5' Ant	Antenna	Aminolink C6	GTC GTT ATC CGT CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT	Biotin-TEG	67
ImT25_3' Ant	Antenna	Biotin-TEG	TTT TTT TTT TTT TTT TTT TTT TTT TCT CTG CAT GAT CG	Aminolink C6	25
3' Eu	DOTA-Eu^{3+}	none	CTC TGC ATG ATC G	Aminolink C6	n/a
5' Eu	DOTA-Eu^{3+}	Aminolink C6	GTC GTT ATC CGT C	none	n/a
3' Ant	Antenna	none	CTC TGC ATG ATC G	Aminolink C6	n/a

Oligonucleotide	Sequence 5´→ 3´
groES target	CGA CGG ATA ACG ACG CGA TCA TGC AGA GG[b]
groES forward	GCC GTT GAA ACC ACT GTT TGG AAG T
groES reverse	CCT GCG GTC TTG GTC TCT TCC TCG
IAC sense	CCG ACT TCA GGA CCG CAA TCA GAC CCT CTT GCT AAG TTA GCA AGA ATG GCG CTG TAT GTC GTA AGC GCA CCC ATG GTG GAT GGA TGT CGG CGC GGT CAG ATT
IAC anti-sense	AAT CTG ACC GCG CCG ACA TCC ATC CAC CAT GGG TGC GCT TAC GAC ATA CAG CGC CAT TCT TGC TAA CTT AGC AAG AGG GTC TGA TTG CGG TCC TGA AGT CGG
IAC forward	CGA CTT CAG GAC CGC AAT CAG AC
IAC reverse	CTG ACC GCG CCG ACA TCC

[a] Probe name includes information of the length of the poly dT spacer (Tn) in the immobilized (Im) probe and into which end the antenna ligand or europium ion carrier chelate is attached.
[b] Probe binding sequences underlined.

2.2 Instrumentation

Labeled probes with a molecular mass less than 20 kDa were purified with reverse-phase high-performance liquid chromatography (HPLC) (Shimadzu Corp., www.shimadzu.com, Kyoto, Japan). Biotinylated probes were spotted with GeSiM Nanoplotter NP 2.1 (GeSiM Gesellschaft für Silizium-Mikrosysteme mbH, www.gesim.de, Grosserkmannsdorf, Germany). EnVision® Multilabel Plate Reader (PerkinElmer, www.perkinelmer.com, Waltham, MA) was used to measure the luminescence in time-resolved fluorescence mode.

2.3 Procedures

2.3.1 Labeling of oligonucleotide probes

The probe oligonucleotides were labeled with isothiocyanate-activated form of either the ion carrier chelate DOTA-Eu^{3+} ((2,2',2''-(10-(3-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tri(acetate)europium(III))[8] or the light harvesting antenna ligand (4-((isothiocyanatophenyl)ethynyl)pyridine-2,6-dicarboxylic acid)[1] using published methods[2]. The labeled probe oligonucleotides (probes) with a molecular mass less than 20 kDa were purified with reverse-phase HPLC using a 150 mm × 4.6 mm Aeris Peptide 3.6 µm XB-C18 column from Phenomenex (www.phenomenex.com, Torrance, CA) and a linear acetonitrile gradient (from 14% to 30% acetonitrile in 7 min) in a 50 mM triethylammonium acetate (TEAA) buffer pH 7.0 with a flow rate of 1.0 mL min^{-1}.

2.3.2 Spotting on the wells

Biotinylated probes were diluted in the oligo storage buffer to a final concentration of 1–64 µM and spotted (13 drops, ~230 nL per drop) with GeSiM Nanoplotter NP 2.1 on the streptavidin coated well, one spot of ~700 µm in diameter per well. After spotting the wells were left in the instrument (room temperature (RT), 70% relative humidity) for minimum of 30 min after which they were used directly in the assay or stored at 4 °C in an aluminium foil bag with a desiccant.

2.3.3 General assay setup

The non-immobilized probe and target were diluted in hybridization buffer and added onto the spotted wells as triplicates in a total volume of 60 µL. After a 1 min shaking at RT, the wells were covered with a tape and incubated 20 min at 50 °C without shaking followed by a 15 min incubation at RT with shaking. After incubations the luminescence was measured in time-resolved mode with an EnVision® Multilabel Plate Reader (laser excitation at 337 nm, emission measured at 615 nm, 10 flashes, 2 mm measurement height) from the bottom of the well as a 5 × 5 (2 mm × 2 mm) or 10 × 10 (4.5 mm × 4.5 mm) raster (0.5 mm between the measurement points). The luminescence of the spot was calculated as an average of four highest luminescence signals on the spot area and background (no target) subtracted unless stated otherwise.

2.3.4 Incubations

The single-stranded DNA target is prone to formation of internal structures due to its flexibility, especially in high salt conditions[6]. The first incubation at elevated temperature was considered opening up these internal structures in probes and target thus increasing the hybridization efficiency when the temperature was lowered below the melting temperature (T_m) of the probes on the second incubation. In diagnostic assay development there is always strive for faster and simpler test

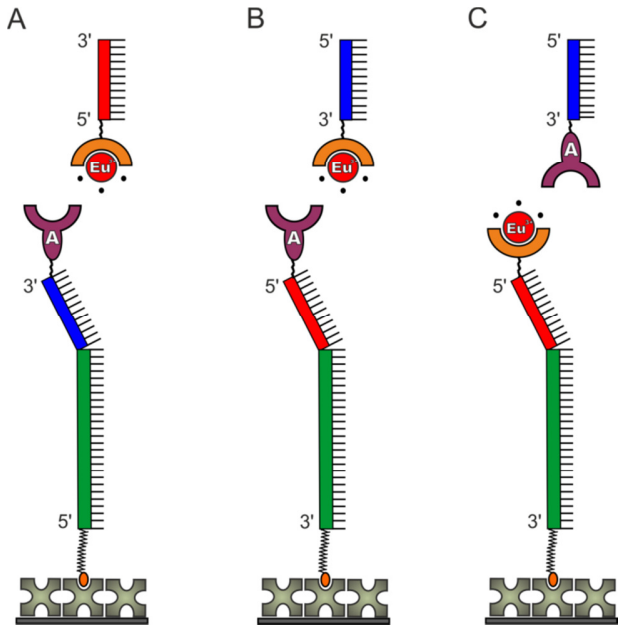
protocols so the necessity and effect of the first incubation on assay performance was evaluated. The first 20 min incubation was done at RT, 50 °C, 60 °C or left entirely out followed by a 15 min incubation at RT with shaking. The wells were spotted with 16 µM ImT25_3´Ant probe. In hybridization assay 50 nM 5´Eu probe combined with 0, 0.0316, 0.316, and 3.16 nM target were used. Luminescence was measured as a 10 × 10 raster (4.5 × 4.5 mm).

2.3.5 NaCl concentration in hybridization buffer

The repulsive electrostatic interactions between negatively charged oligonucleotides can be reduced by increasing the salt concentration in hybridization buffer[9,10]. In order to investigate the effect of NaCl concentration in our assay the 5´Eu probe (50 nM) and target dilutions (0, 0.0316, 0.316, and 3.16 nM) were made by using hybridization buffers containing 0, 150, 300, 600 or 900 mM NaCl without DTPA. The wells were spotted with 16 µM ImT25_3´Ant probe. Luminescence was measured as a 10 × 10 raster (4.5 × 4.5 mm).

2.3.6 Probe pair orientation and label moiety interchange

The 5´-end antenna ligand labeled 3´-biotin-TEG oligonucleotides ImTn_5' Ant (where n was 0, 13, 25 and 67) for the spacer length test were immobilized through the biotin at the 3´-end. The effect of the 180° probe pair flip (Scheme 2a and 2b) was tested by spotting either ImT25_3´Ant or ImT25_5´Ant probes (10 µM spotting concentration) on the well. In hybridization assay respective 5´Eu and 3´Eu probe concentrations were 50 nM and target concentrations 0, 0.1, 1, and 10 nM. For label moiety interchange test the 3´-end biotinylated oligonucleotide was labeled either with antenna ligand (ImT25_5´Ant) or europium ion carrier chelate (ImT25_5´Eu) and the respective solution based oligonucleotide with the complementary label moiety (Scheme 2b and 2c). The probe and target concentrations were the same as with the probe pair orientation test. Luminescence was measured as a 5 × 5 raster (2 × 2 mm).



Scheme 2 Schematic presentation of the probe pair orientation (a and b) and label moiety interchange (b and c) test setups. In the probe pair orientation test the antenna ligand probe was immobilized on the streptavidin plate either through the 5´ (a) or 3´-end (b) biotin-TEG linker and separated from the solid support with a poly dT spacer (green). The europium ion carrier chelate probe was in solution. In the label moiety interchange test the 3´-end immobilized probe was 5´-end labeled either with the antenna ligand (b) or the europium ion carrier chelate (c) and the 3´-end labeled probe was in solution.

2.3.7 Spacer length

Hybridization of the oligonucleotide probes to target should be as free as possible from the interference of the solid support[3] so the 3´-end immobilized probe was separated from the well surface with poly dT spacers of 0, 13, 25 or 67 nucleotides. The wells were spotted with 32 µM ImT0_5´Ant, ImT13_5´Ant, ImT25_5´Ant, and ImT67_5´Ant probes. In hybridization assay 3´Eu probe concentration was 50 nM and target concentrations were 0, 0.01, 0.04, 0.16, 0.625, 2.5, and 10 nM. Luminescence was measured as a 5 × 5 raster (2 × 2 mm).

2.3.8 Spotting concentration

The surface density of the immobilized oligonucleotide probe is known to affect both hybridization and the interaction between the solid support and probe[4,6]. To find the optimal spotting concentration 1, 4, 16, 24, 32, and 64 µM ImT25_3´Ant probe was spotted on the wells resulting in theoretical maximum of 3, 12.5, 50, 75, 100, and 200 fmol antenna ligand probe per spot. In hybridization assay 50 nM 5´Eu probe and 0, 0.00316, 0.0316, 0.316, 3.16, and 31.6 nM targets were used. Luminescence was measured as a 10 × 10 raster (4.5 × 4.5 mm) to observe possible spot spreading.

2.3.9 Detection limit and hook effect

To define the detection limit and dynamic range of the optimized assay 24 µM ImT25_3´Ant probe was spotted into the wells. In hybridization assay 50 nM 5´ Eu probe and 0–3160 nM target were used. Limit of detection was calculated as background mean (0 nM target, n = 12) plus 3 times the standard deviation of the background. The hook effect was tested by using a vast excess of the target. Luminescence was measured as a 5 × 5 raster (2 × 2 mm).

2.3.10 Detection of amplification products and interference from non-target sequences

Interference from non-target DNA was tested by introducing a single-stranded synthetic IAC oligonucleotide into the assay. In hybridization assay 50 nM 5´ Eu probe, 0 or 10 nM synthetic *groES* target and 0 or 10 nM synthetic IAC oligonucleotide were used.

The assay functionality with *groES* amplification products and the *groES* signal interference from co-amplified non-target amplification products were tested by amplifying[2] a 99 nucleotide *groES* gene sequence from isolated genomic *P. aeruginosa* DNA with a synthetic 98 nucleotide IAC template using previously optimized (results not shown) primer concentrations (0.6 µM *groES* forward / 6 µM *groES* reverse, 1 µM IAC forward / 0.1 µM IAC reverse). In the amplification 0 (negative template control, NTC) and 1×10^3 copies of the genomic DNA of *P. aeruginosa* and 0, 1×10^4 and 1×10^5 copies of double-stranded synthetic IAC were used as templates. The amplification products were diluted 1:5 in the assay. The amplification of the IAC was confirmed by testing the IAC amplification products separately (results not shown).

3 Results and discussion

3.1 Incubations

The first incubation at 50 °C was supposed to open up possible internal structures of the target and probes. Removing this incubation halved the luminescence signals (ESI 1) and changing the incubation temperature to RT or 60 °C lowered the signals by 30% and 12–25%, respectively. Different incubation temperatures and times had no effect on the background signals so the increase in luminescence signals resulted in higher S/B ratios (Fig. 1). The incubation protocol used by Karhunen *et al.*[2] with the first incubation at 50 °C performed best. Target molecules migrate to the probes on the solid support by diffusion so parameters that speed up the diffusion rate, such as elevated temperature and mixing, affect directly the hybridization yields. The diffusion rate can also be speeded up by using targets smaller than 100 bases in size[11].

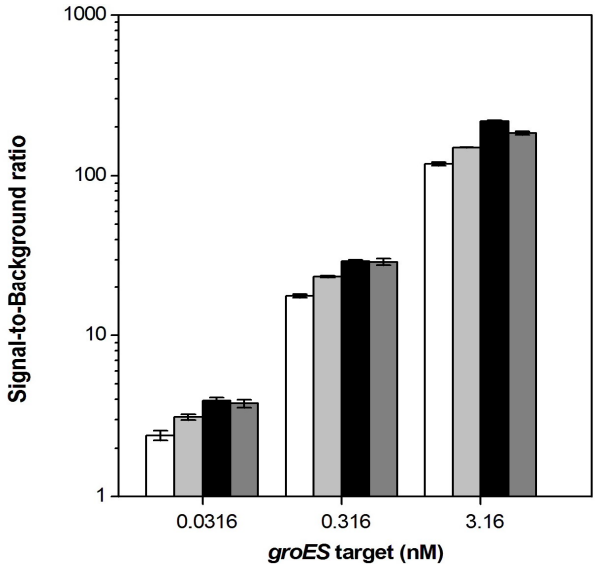


Fig. 1 The effect of the first incubation on S/B ratios. Four alternatives for the first incubation were tested; no incubation (white), 20 min incubation at RT (light gray), 50 °C (black), and 60 °C (dark grey), followed by the latter 15 min incubation at RT with shaking before measurement of the luminescence. Error bars indicate the standard deviation (n = 3).

3.2 NaCl concentration in hybridization buffer

Excluding NaCl from the hybridization buffer led to increased background and almost complete loss of specific signal (ESI 2). The specific signals increased up to 300 mM NaCl hybridization buffer concentrations. With high (300–900 mM) NaCl concentrations in hybridization buffer there were no remarkable differences in specific signals. The 300 mM NaCl in hybridization buffer was sufficient to suppress electrostatic repulsion, but increasing the NaCl concentration to 600 mM

still enhanced the S/B ratios due to the lower background (Fig. 2). DTPA chelates free europium and excluding DTPA increased the background signals and led to 10-fold lower S/B's.

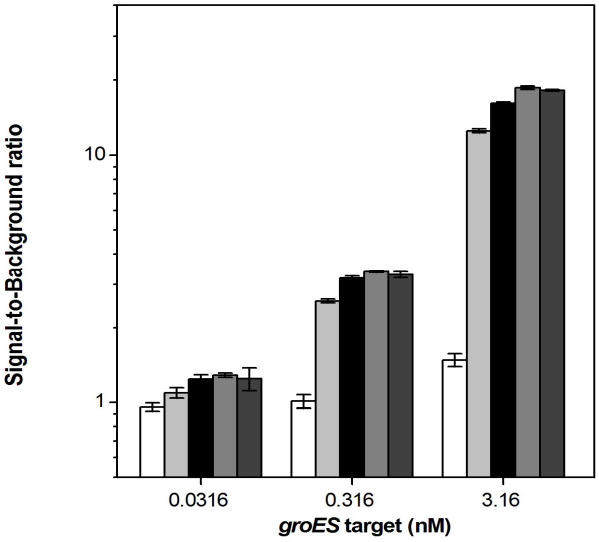


Fig. 2 The effect of NaCl concentration in hybridization buffer. The S/B ratios with five hybridization buffer NaCl concentrations; 0 mM (white), 150 mM (light grey), 300 mM (black), 600 mM (dark grey), 900 mM (grey). Error bars indicate the standard deviation (n = 3).

3.3 Probe pair orientation and label moiety interchange

When the probe pair was flipped 180° with antenna ligand probe immobilized at the 3´-end instead of the 5´-end both the specific signals (ESI 3) and S/B ratios (Fig. 3) decreased 2 to 3 fold. The 3´ and 5´-end linker chemistries differed slightly which might contribute to more favorable conformation and signal increase in the case of 5´-end immobilization. In most DNA assays on solid supports the oligonucleotides are 5´-end immobilized mostly due to the more straightforward 5´-end modification and immobilization methods[6,12] although neither Guo *et al*.[6] nor Shchepinov *et al*.[3] mentioned any differences in assay performance with the 3´-end and 5´-end immobilized probes. Peplies *et al*.[13] reported 1.4–1.8 -fold increase in signal with the 3´-end instead of the 5´-end immobilized probes, but with longer spacer the signal increase was only 1.1-fold. The differences were thought to depend on the variable lengths of the target nucleic acid tails outside the probe binding site as with the 5´-end immobilized probe the longer tail of the target nucleic acid was oriented towards the solid support creating an unfavorable steric situation. In our assay the synthetic *groES* target sequence extended only one base

outside the binding site of the probes on both ends so no target dependent differences in steric crowding were expected. Despite of the lower performance of the 3´-end immobilized probes this setup was used for testing the effect of linker lengths and it was also considered suitable for testing the effect of label moiety interchange. When the antenna ligand probe was immobilized instead of the europium ion carrier chelate probe the background signal decreased 4.6 times leading to 2–4 times higher S/B ratios. The quenching of europium luminescence in aqueous solution is not absolute and in spite of the low molar absorptivity of europium ion it can be weakly excited directly with a high power laser pulse.

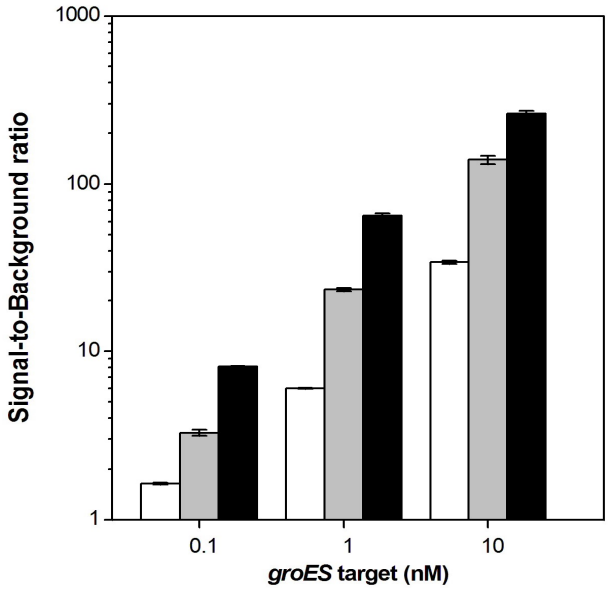


Fig. 3 Probe pair orientation and label moiety interchange. Europium ion carrier chelate probe (ImT25_5´Eu) was immobilized at the 3´-end (white) and antenna ligand probes (ImT25_5´Ant and ImT25_3´Ant) were immobilized either at the 3´-end (light grey) or 5´-end (black). Error bars indicate the standard deviation (n = 3).

3.4 Spacer length

Introduction of a poly dT spacers to the immobilized probes lowered the background signal by 24% and increased the specific signals by 17–69% (Fig. 4a). The average signal increase with T13 was 29%, with T25 40% and with T67 44%. The differences between luminescence signals and S/B ratios (Fig. 4b) with antenna ligand probes containing T25 or T67 spacer were such miniscule compared to the higher price of the longer T67 spacer probe that the T25 spacer was considered sufficient for the further assay optimization. Separation of the immobilized target specific probe sequence from

the solid support was assumed to increase spatial freedom of the probe and decrease the crowding and possible nonspecific binding on the surface. The size of the target affects both the steric crowding and repulsive charges so the optimal spacers might need some adjustment with longer target sequences such as typical amplification products.

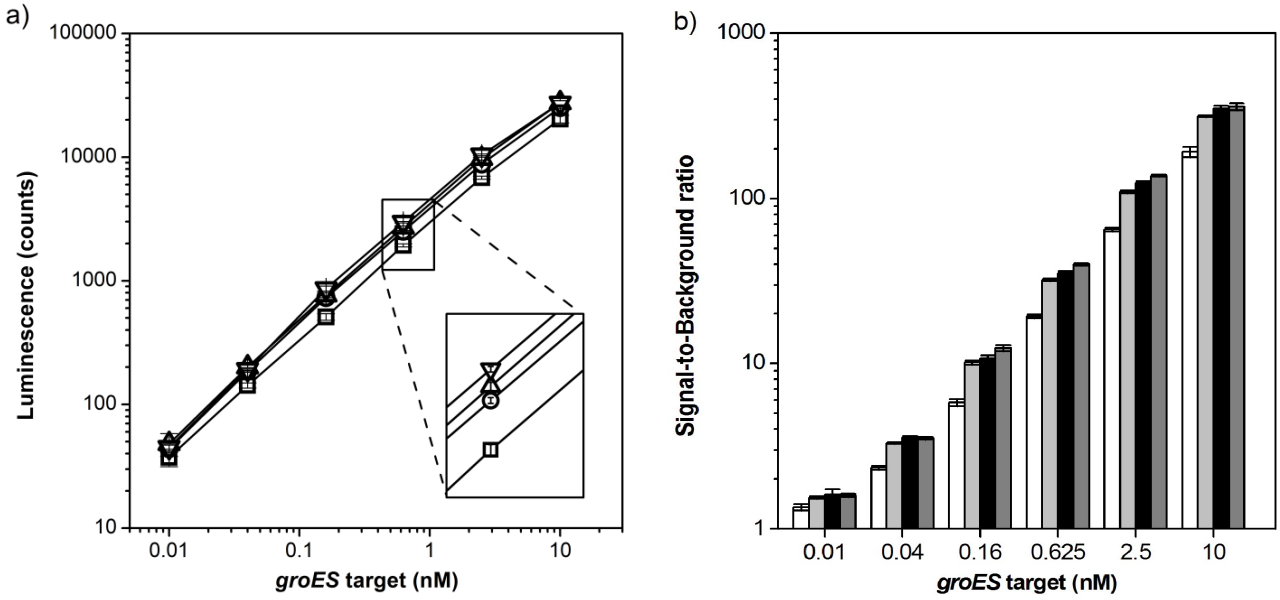


Fig. 4 The effect of poly dT spacers on (a) luminescence signals and (b) S/B ratios. The immobilized probe was separated from the well surface with poly dT spacers of 0 (squares alt. white bars), 13 (circles alt. light grey bars), 25 (up triangles alt. black bars) or 67 (down triangles alt. dark grey bars) nucleotides. Error bars indicate the standard deviation (n = 3).

3.5 Spotting concentration

There were no significant differences in luminescence signals or S/B ratios between antenna ligand probe spotting concentrations of 16, 24, and 32 µM (50, 75, and 100 fmol/spot) (Fig. 5) so the spot was considered close to saturated with the 16 µM (50 fmol/spot) antenna ligand probe concentration. The spots spread with higher spotting concentrations (ESI 4) and the background signals increased. With the spotting concentrations of 1 and 4 µM the signals were 6–20% and 21–43% from the signals of the saturated spots, respectively, and the lower binding capacity of the spots was observed especially with high target concentrations. With 64 µM (200 fmol/spot) spotting concentration and high target concentrations (3.16 and 31.6 nM) both the luminescence signals and S/B ratios were higher than with the spots considered

already saturated which gave an impression for improved capacity. Wells however were not washed after the spotting and hence there might have been unbound biotinylated oligonucleotide probes dried on the spot which came into the solution during hybridization assay and were bound outside the spot causing spot spreading and higher background. The spot diameter was smaller than the measuring area of EnVision® Multilabel Plate Reader and thus the possible spot spreading affected directly the signals. The short target sequence and biotin-streptavidin attachment of the immobilized probe were the most likely reasons we did not see any decline in assay performance due to steric hindrance caused by overcrowding of the spot area even though coating concentrations beyond the surface saturation point were used.

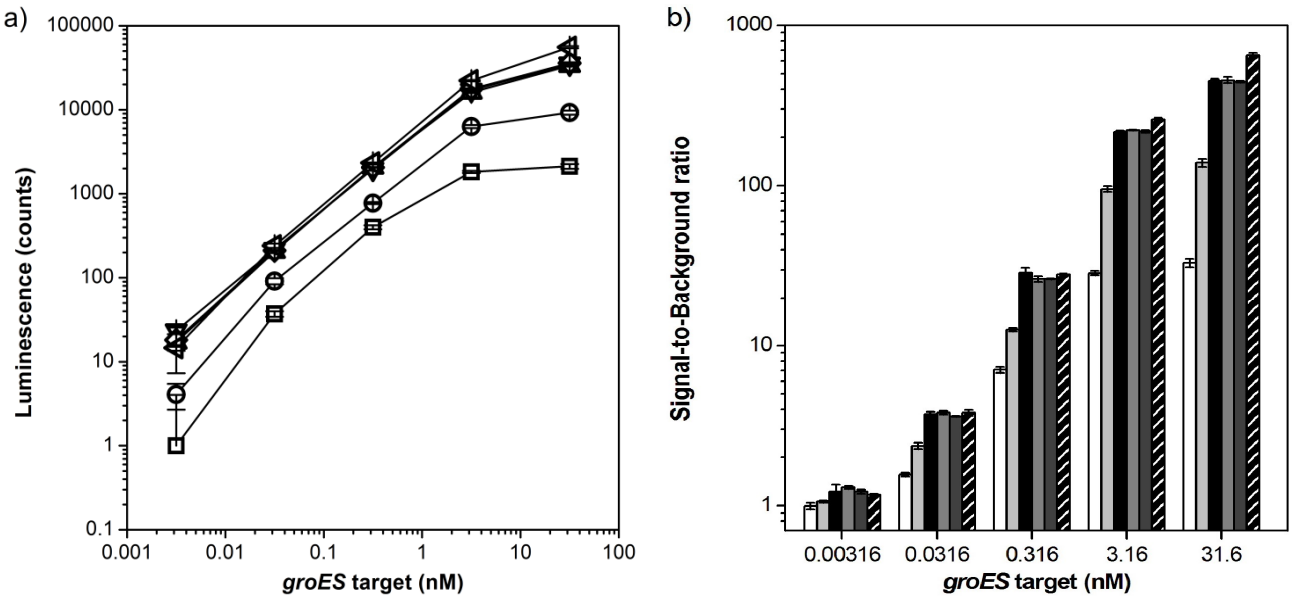


Fig. 5 The effect of spotting concentration on (a) luminescence signals and (b) S/B ratios. The spotting concentrations were 1 µM (squares alt. white bars), 4 µM (circles alt. light grey bars), 16 µM (up triangles alt. black bars), 24 µM (down triangles alt. dark grey bars), 32 µM (diamonds alt. grey bars) and 64 µM (left triangles alt. striped bars). The corresponding molar amounts of spotted antenna ligand probes were 3, 12.5, 50, 75, 100, and 200 fmol per spot, respectively. Error bars indicate the standard deviation (n = 3).

3.6 Detection limit and hook effect

The detection limit of 18 pM (background + 3SD, n = 12) (Fig. 6) was close to a 20-fold improvement compared to the assay developed by Karhunen *et al.*[2] with a detection limit of 0.32 nM for single synthetic target. The dynamic range was

three orders of magnitude. A hook effect was observed with decreased signal at the high target concentration range. When there was an excess of target over the solution phase europium ion carrier chelate probe, some targets bound into the immobilized antenna ligand probe lacked the europium ion carrier chelate probe.

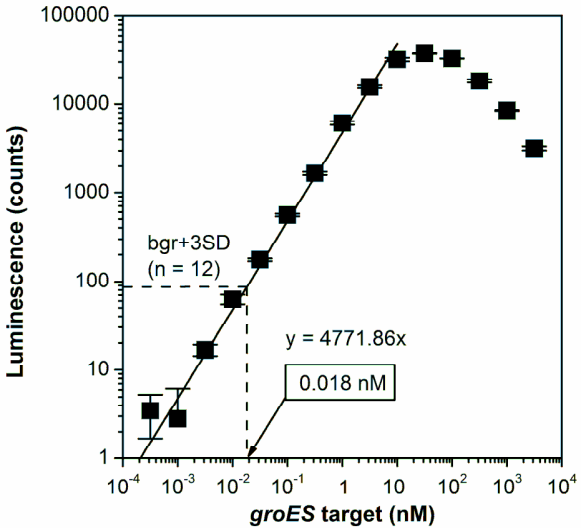


Fig. 6 Titration of the synthetic *groES* target with optimized homogeneous lanthanide chelate complementation based nucleic acid assay (24 µM ImT25_3´Ant, 50 nM 5´Eu). Limit of detection, 0.018 nM (background + 3SD, n = 12), is presented as dotted line. Dynamic range of the optimized assay was three decades. Error bars indicate the standard deviation (n = 3).

3.7 Detection of amplification products and interference from non-target sequences

The presence of a 10 nM synthetic non-target IAC oligonucleotide in the *groES* assay reduced the S/B ratios by 7% for the *groES* target (Fig. 7). When 1×10^4 and 1×10^5 copies of synthetic double-stranded IAC template were co-amplified with 1×10^3 copies of genomic DNA of *P. aeruginosa* the *groES* S/B ratios decreased 21 and 28 %, respectively, compared to the assay with *groES* amplification products alone. In the co-amplification of *groES* and IAC the two parallel amplification reactions competed from the same reagents leading to decreased amount of *groES* amplification product and thus resulted in lower *groES* signals. In case of simultaneous amplification and detection of several different sequences the effect of co-amplified sequences is an important parameter to take into consideration.

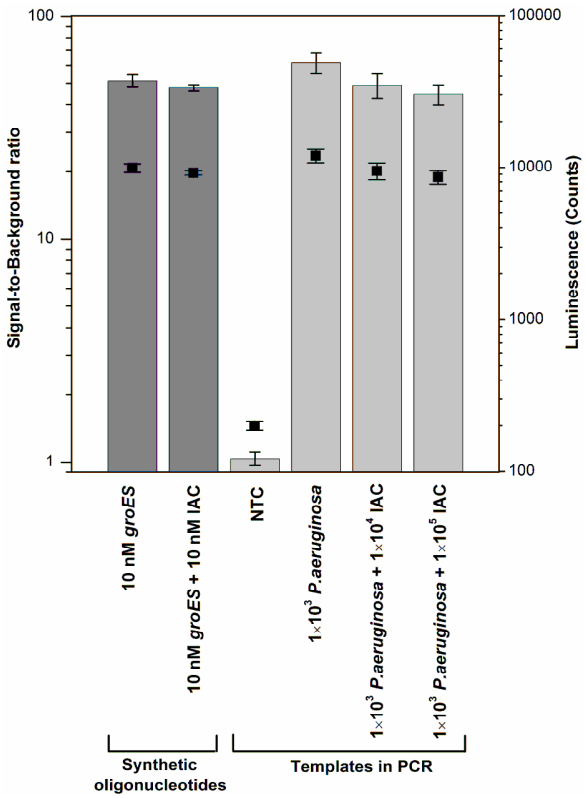


Fig 7 The interference of non-target sequences in the *groES* assay. In the assay interference test synthetic *groES* target (10 nM) was tested both with and without synthetic IAC oligonucleotide (10 nM) (grey). In the combined co-amplification and assay interference test 0 (NTC) and 1×10^3 copies of genomic DNA of *P. aeruginosa* were co-amplified with 0, 1×10^4 or 1×10^5 copies of double-stranded synthetic IAC followed by testing of the *groES* amplification products (light grey). *groES* luminescence signals are marked with squares. Error bars indicate the standard deviation (n = 3).

4 Conclusions

In this study we demonstrated the importance of assay optimization by greatly increasing the assay sensitivity by choosing the best performing probe pair orientation and sufficient spacer, using high probe density spots and minimizing the background signal by interchanging the label moieties. These results can be utilized with different probe-target combinations of similar construction. With this assay we were able to qualitatively show if the *groES* target was present

both with and without a non-target IAC in the amplification reaction and/or in the assay. The number and amounts of different sequences in the same amplification reaction affect the amplification and the detection of individual target. With this study we obtained essential information for future development of sensitive separation-free multiplexed array-based nucleic acid assays. The homogenous detection and spatial identification of different targets could enable higher degree of multiplexing to be combined with integrated target amplification than currently available with multicolor fluorescent probes.

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Appendix A. Electronic Supplementary Information (ESI)

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