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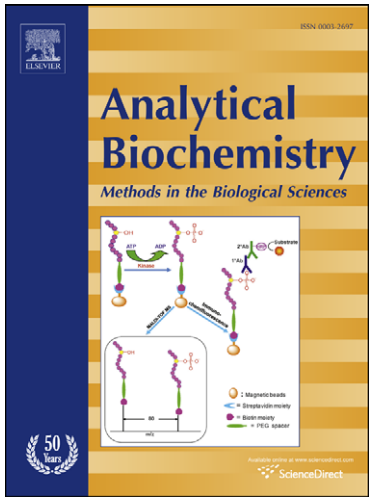

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Sarah Thiollet, Conrad Bessant, Sarah L. Morgan

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Application of multiple response optimization design to quantum dot-encoded microsphere bioconjugates hybridization assay

Sarah Thiollet [a*], Conrad Bessant [b], Sarah L. Morgan [a]

[a]Translational Medicine Group, Cranfield Health, Cranfield University, Cranfield, UK

[b]Bioinformatics Group, Cranfield Health, Cranfield University, Cranfield, UK

[*]Corresponding author. Tel: + 31 61979 8003. E-mail address: s.thiollet@gmail.com (S. Thiollet)

Abstract

The optimization of DNA hybridization for genotyping assays is a complex experimental problem, which depends on multiple factors such as assay formats, fluorescent probes, target sequence, experimental conditions, and data analysis. Quantum dot-doped particle bioconjugates have been previously described as fluorescent probes to identify single nucleotide polymorphisms even though this advanced fluorescent material has shown structural instability in aqueous environment. To achieve the optimization of DNA hybridization to quantum dot-doped particle bioconjugates in suspension while maximizing the stability of the probe materials, a non-sequential optimization approach was evaluated. The design of experiment with response surface methodology and multiple optimization response was used to maximize the recovery of fluorescent probe at the end of the assay simultaneously with the optimization of target-probe binding. Hybridization efficiency was evaluated by the attachment of fluorescent oligonucleotides to the fluorescent probe through continuous flow cytometry detection. Optimum conditions were predicted with the model and tested for the identification of single nucleotide polymorphisms. Design of experiment has shown to significantly improve biochemistry and biotechnology optimization processes. Here, we demonstrate the potential of this statistical approach to facilitate the optimization of experimental protocol that involves material science and molecular biology

Keywords: Design of experiment; Multiple response optimization; Quantum beads; Hybridization; Flow cytometry

Introduction

Similar in function to planar microarrays, suspension array technology (SAT)[1] uses a microsphere as a single element or "spot" array in a two dimensional space [1] with higher speed of fluidic detection and enhanced surface area of hybridization [2]. The combination of the high level of multiplexing of optically encoded microspheres and the high throughput serial analysis of flow cytometry (FCM) instruments provides SAT with a more suitable format than flat microarrays for applications such as disease diagnostics, genetic and bacterial marker identification [3-6]. The performance of such detection system for single-strand DNA (ssDNA) identification and quantification depends mainly on the fluorescent code and intensity of the bead-probe and the sensitivity of DNA hybridization [7,8]. Commercial sets of dyed-microspheres developed for multiplexed DNA detection assay in clinical and research applications are limited since they require multiple excitation sources and specialized instruments for the organic multicolor bead detection [9,10]. They incur high costs with limited flexibility of the bead library enlargement because of the high risk of organic dye spectral overlap [11]. To overcome these limitations, semiconductor nanocrystals or quantum dots (QDs) have been used to encode synthetic microspheres. Due to their remarkable optical properties, different colors of QDs can be integrated in synthetic beads and illuminated with a single excitation light to create unique spectral 'barcodes' [12]. QD-doped microspheres are encoded in terms of color, by the combination of different QDs, and in terms of intensity, corresponding to the amount of each color present in the bead structure. QD-doped particles have the potential to produce an unprecedented number of fluorescent codes, with higher

flexibility, chemical- and photo- stability, and lower limits of detection than conventional fluorescent particles.

In this study, an oligonucleotide hybridization method, presented in Fig. 1 was specifically developed using an innovative set of QD-encoded polystyrene microspheres commercially available, the QDEMs (Crystalplex, USA), and conventional FCM detection. First, the stability of the fluorescent code and physical properties of the probe are crucial for the accuracy of the final results of the suspension assay [2]. The effect of the procedure on the QDEM material was thus a critical parameter for the optimization of the assay since previous studies reported the instability of QD-doped particles in aqueous conditions [13]. Secondly, the hybridization reaction is the other main limitation step of bead-based assays, with similar experimental considerations to planar arrays since the reaction occurs at a solid-liquid interface. Kinetics, target detection specificity and sensitivity in ASO hybridization methods depend highly on the target and probe environment interface [14]. Here, the initial challenge was to optimize a system dependent on a large number of parameters. The selection of more than two or three experimental factors multiplies the number of experiments needed if a sequential method is applied. The traditional one-factor-at-a-time optimization technique also completely ignores all interactions between the different factors. The design of experiments (DOE), described as a strategy to plan research using multivariable statistic methods has been scarcely used in biology [15-18], whereas it is commonly applied to analytical chemistry processes, biosensors and drug delivery system optimization [19-21,14]. This study reports the application of response surface methodology (RSM) and multiple response optimization (MRO) to predict optimum experimental conditions for the hybridization of a short oligo

fluorescent target to QDEM-DNA probe (Fig. 1A). The objectives of the MRO study were to maximize both the hybridization signal detected through continuous FCM and the percentage of stable QDEMs recovered at the end of the assay. Hybridization buffer, time, temperature and oligo target quantity were selected as the main parameters influencing both the hybridization efficiency and the bead stability. The validity of the DOE approach was empirically evaluated with fluorescent target titration and allelic probe specificity assay (Fig. 1A,B). With this strategy, the main factors destabilizing the QDEM were identified. In addition, an optimal combination of the parameters that influence the reliability of the assay in terms of stability and efficiency was determined.

Materials and methods

Oligonucleotides and QD technology

Conjugation probes were designed with: (i) a 5' amino group for coupling to carboxylated microspheres, (ii) a 5' six carbon spacer, (iii) a universal sequence in addition to a 18-mer polyadenine sequence. The hybridization probe for the fluorescent titration consisted of an 18-mer polythymine holding a 3' Cy3 (Fig. 1A). Two allelic probes were designed with Primer3 [22] for the identification of a human SNP on the Y chromosome: dbSNPs accession number rs9000, G/C allele frequencies of 0.7/0.3 (dbSNP, www.ncbi.nlm.nih.gov/projects/SNP/). The component (iii) was replaced by the specific allelic sequence for each probe. PCR primers were designed with Primer3: forward primers 5'-AATACAGAACTGCAAAGAAAC and reverse primer B-CTAAGTATGTAAGACATTGAACG amplifying a 96–bp product, where B denotes the biotin label on the 5' end of the reverse primer. A streptavidin linked to fluorophore cyanine 5 (Cy5-SA) (Sigma Chemical Co, Poole, Dorset, UK) was used as a reporter dye to identify the hybridized complex (Fig 1B). All the oligos were purchased from Thermo Electron (Bremen, Germany). Coupling and hybridization experiments using organic dye were performed in the dark.

Carboxylated polystyrene microspheres of 5 μm diameters (± 10%) encoded with TriLite™ nanocrystals, and non-encoded microspheres (referred to as blank or 0QDEM) were purchased from Crystalplex (Pittsburgh, PA, USA). QDEM solutions were quantified as previously described using a Neubauer haemocytometer (Reichert, Bright-line®, NY, USA) [23]. Samples were run through a Coulter Epics XL-MCL flow cytometer equipped with an air-cooled 488 nm argon-ion laser (Beckman Coulter, Brea, CA, USA).

Coupling of QDEMs to oligonucleotides

The coupling method was adapted from Spiro et al. [9]. Briefly, 1 µL of QDEM (10^4/µL) was conjugated by amino-carboxy coupling to 60 pmol of coupling oligo in 20 µL of imidazole (pH 7.0, Fisher Scientific Ltd, Loughborough, Leicestershire, UK); 2 µL of fresh carbodiimide activators, *i.e.*,1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (10 mg/ml in nuclease free water (H_2O), Sigma) and sulfo-*N*-hydroxysuccinimide (10 mg/ml in H_2O, Sigma), were added to the mix and incubated for 30 min at room temperature (RT) shaking at 500 r.p.m. Bioconjugates were washed (RT, 4 min, 400 r.p.m.) once in 300 µL of imidazole (pH 7.0), twice in the storage buffer (1x TBS, 1% BSA, 0.01% azide, pH 7.0), and stored in 15 µL at 4°C. All bead solutions are centrifuged at 1133x g for 4 min and resuspended by 15 s of vortex and 20 s of sonication. Three different negative controls were used: QDEM stock solution and QDEM conjugated to H_2O evaluated the fluorescent noise of the FCM and the effect of the procedure on the QDEM emission; the fluorescent background due to non-specific binding was estimated by incubating QDEM with fluorescent probes but without the carbodiimide activators. Negative controls were performed for each batch experiment.

Hybridization

QDEM bioconjugates (1 µL of QDEM ($9x10^3$/µL)) were resuspended in 15 µL of prewarmed 6x standard saline citrate (6x SSC, 0.5% SDS, pH 7.0, Sigma) and hybridized with various target concentrations, temperatures and times of incubation (Table 2). Hybridization mixes were washed at RT in 400 µL of 0.5x SSC, 0.05% SDS for 4 min, then twice in 400 µL of storage buffer successively for 2 and 1 min, and finally in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0, Sigma)

for 1 min. Samples were analyzed in 400 µL of TE on the flow cytometer (Fig. 1A).

A standard hybridization titration curve was constructed using QDEMs encoded with 525 nm nanocrystals (525QDEMs) with 0 to 65 µM increasing range of fluorescent oligo (Fig. 1A(2)), and with the optimum conditions found with the design of experiment, DOE [24]. Untreated 525QDEMs incubated directly with the fluorescent oligo were used as negative controls for each oligo quantity tested. Fluorescent background was evaluated for each target concentration by using QDEMs instead of QDEM bioconjugates in the hybridization experiment.

To evaluate the sensitivity and specificity of the assay, the titration of two SNP allelic probes was undertaken using the same hybridization protocol and a fluorescent-streptavidin reporter dye (Cy5-SA, Sigma) was used to identify the hybridized target (Fig 1B). QDEMs conjugated to the allelic probes were encoded with 525 and 575nm QDs, *i.e.*, (525;575), with ratio intensities of (1:0), (0:1) (Crystalplex). (525;575)QDEM fluorescent codes were detected with the detector channel 1 and 2 (FL1 and FL2) of the FCM instruments simultaneously with the hybridization signal (Cy5 emitting at 670 nm) detected in the channel 4 (FL4) (Fig. 1B(2)). Target concentrations ranged from 0 to 50 nM.

Design of experiment - DOE

Response surface methodology - RSM

The hybridization signal and fluorescent probe stability were optimized with RSM, and facilitated by the software Statgraphic Centurion XV (StatPoint, Herndon, VA, USA). RSM uses the fit of a polynomial equation to experimental data to describe the behavior of a data set and the relationship between experimental factors and responses with the objective to provide statistical

predictions. With this method, the optimal settings of a set of numerical experimental factors can be predicted. RSM is based on factorial design and involves at least three levels of each experimental factor. The main steps in the application of the RSM to our experimental system were: (1) to select the independent factors that had a major impact in the bead-based assay. Screening studies were used to choose the factors and the experimental range under investigation within the objective of this research; (2) to chose the experimental design methodology or model that will provide the experimental matrix to be executed in the laboratory; (3) to fit the polynomial function with the experimental data obtained and perform the mathematical–statistical treatment of the DOE; (4) to evaluate the model using the analysis of variance (ANOVA) statistical results; (5) to verify if a displacement in direction to the optimal region is necessary; and (6) to obtain the optimum values for each studied variable.

Four primary experimental parameters (X) of the hybridization procedure were considered in this study: (X_0) hybridization buffer, (X_1) temperature, (X_2) time and (X_3) single-strand target oligo quantity (Table 1). The hybridization efficiency was quantified using a fluorescent oligo specific to the QDEM-probe. The experimental factors were expected to impact on the two response variables (Y) analyzed: (Y_1) the fluorescent hybridized signal on the bead surface measured with the median of fluorescent intensity (MFI), and (Y_2) the QDEM structural stability was measured as the percentage of events (%Events) recovered at the end of the procedure. Hybridization buffer, being a categorical factor (not quantitative), was excluded from the RSM strategy and evaluated individually with preliminary screening experimental tests (data not shown).

Central composite design with two-level factorial design, central points, and star points, [2^3 + star], was employed to study the relationship between the parameters influencing the three independent factors left (X_1 in degree Celsius (°C), X_2 in minutes, and X_3 in mole) and the two response variables of the hybridization assay (Y_1 in RMFI and Y_2 in %Events). The [2^3 + star] design was expressed in standard units where -1 and +1 represented respectively the low and high level of each factor (Table 1). Table 2 presents the planned design of all 14 experiments with the different combinations of the three independent factors at different levels. For each experiment, three replicates were undertaken to estimate assay variability

Multiple response optimization approach - MRO

The experimental factor settings that maximized simultaneously the desired characteristics expected for the two responses studied were investigated using multicriteria methodology and the desirability function (or Derringer function), which is the most currently used multicriteria methodology in MRO analytical procedures [25]. We applied this approach available with Statgraphic Centurion XV (StatPoint), to optimize the hybridization signal and the bead stability. To use MRO, the RSM data are first analyzed for each response variable to create a model for each separately. The desirability function (d(y)) is then constructed based on the values obtained for each optimized response. The MRO approach assumes that the response values equal to (y) can be modeled through the desirability function d(y), where the desirability ranges from 0 to 1.

Data acquisition, processing and analysis

The median fluorescent intensity (MFI) and the geometric mean were calculated on gated population using WinMDI2.8 software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). Three replicates were run per experiment.

Quantification and titration curve

The QuantiBRITE PE kit (Becton Dickinson BD biosciences, Oxford, UK) was used as previously described [26] to evaluate the molecules of equivalent soluble fluorochrome (MEF). Corrected MFI, MEF (respectively RMFI in arbitrary unit (a.u.) and RMEF in oligo), and the hybridization signal (HybS, RMFI in a.u.) were calculated by subtracting the background signal detected with the negative controls from the positive samples. The oligo density (D_o) corresponded to the RMEF divided by the microsphere surface (in oligo/μm^2) [27]. The relative QDEM recovery was calculated as the percentage of events recovered (%R) in the gate at the end of the procedure divided by the percentage of QDEMs before treatment. Data are presented as the mean of replicates ± the standard error of the mean (± SEM). Curves and statistics were calculated with GraphPad Prism software version 5.01 (GraphPad Software, San Diego, CA, USA).

Analysis of variance ANOVA

The RSM data (%R and the RMFIs) were analyzed separately to optimize the experimental parameters specifically for each response. The data set of each response was first manually validated to ensure confidence in the agreement across replicates and the identification of outliers, and secondly screened to verify that the expected range of values had been obtained and the best results identified. After validation, data were imported in the software (Statgraphic centurion XV).

The predicted values were compared with the observed (experimental) best values and parameters influencing the process and the hybridization responses were identified.

ANOVA results for each RSM experiment determined which of the temperature, time, and target concentration had a significant effect on the increase of the RMFI and on the %R. The model was validated with the lack-of-fit test that determined whether the model created for a response was adequate to describe the observed data, at the 95% confidence level (p-value ≥ 0.05). The lack-of-fit test compares the variability of the current model residuals to the variability between observations at replicate settings of the factors. The R-squared statistics with the coefficient of determination (r^2) indicated how the model explains the variability of the response studied. These statistical tests were also used to conclude if factors time, temperature, and target concentration significantly affected or not the resultant response values in an additive manner.

Response surface analysis

The response surface equation constructed by the software for each response was then evaluated and plotted. Hence, the model provides the optimum responses with the combination of the parameter levels that maximize the response studied over the range of values used to build the model. The model also predicts the responses for any conditions within the experimental space. The graphical tools used to validate the model were as follow: (i) the normal plot, which distinguished between the positive and the negative effects; (ii) the actual versus predicted values plot; and (iii) the two dimension (2-D) contour plot and 3-D response plot, which showed the behavior of the response studied as a function of two significant

factors while the other significant factor was held constant, generally at its centre point value or at the optimum value.

Hybridization plot

Finally, the hybridization signal exclusively due to the oligos hybridized on the QDEM bioconjugates was plotted and a linear regression analysis was performed on the initial range of the titration where MEF values increased. The hybridization detection limit (in molar concentration) was determined as follows: the lowest quantity of Cy3-oligo detected in the hybridization assay was divided by the suspension volume of the hybridization experiment (15 μL).

Results

Response surface design

The hybridization conditions of the QDEM suspension assay were optimized through experimental design, with the RSM using central composite design with a two-level factorial design, central points, and star points [28]. The selection of factor ranges was based on the literature [27,29] and preliminary experiments (data not shown) (Table 1). Hybridization target ranges corresponded to the expected quantity of a sensitive hybridization assay [23]. An experimental study of the QDEM material performed in our laboratory (data not shown) identified a low stringent sodium citrate buffer (6x SSC) as the most suitable hybridization buffer to develop a QDEM bead-based assay. The design matrix of the three experimental factors along with the two responses studied is presented in Table 2, which also illustrates the experimental points undertaken for the DOE with 6x SSC buffer.

Analysis of variance (ANOVA) for the fitted model

The results of the response-surface model fitting are illustrated in Table 3 with the analysis of variance (ANOVA). The ANOVA analysis tests the adequacy and the significance of the model. Table 3 showed that the R^2, the coefficients of determination of the %R and the RMFI were respectively 0.72 and 0.81. Hence, the constructed model explained ~81% of the variability in the hybridization signal data and ~72% of the stability data. ANOVA table also describes the statistical significance of the models (*F* values) and the lack-of-fit test. No significant lack-of-fit was found. The models designed were thus adequate to describe the percentage of recovery and observed hybridization signal at the 95% confidence level since both responses *p*-value were ≥ 0.05 (Table 3).

Response plots for hybridization and stability optimization

Fig. 2A and B show the 2-D contour plot and 3-D response surface plot resulting from the fitted equation of the model which investigate the interactions between variables (oligo quantity, temperature, and incubation time) and also determine the optimal values of each variables for the maximization of the two responses analyzed (hybridization signal and percentage of bead recovery). The response surface design predicted the response curvature and resulted in a maximized hybridization at ~420 a.u. of fluorescence with 10 to 80 min incubation at 49-51°C, and a maximized stability with ~73% of bead recovery for 50 to 60 min of incubation at 49-51°C. The response surfaces were the lowest at high temperature (55°C) and short incubation times (30 min). Fig. 2A and B show that high temperature and short time of incubation were more detrimental for QDEM stability and hybridization efficiency than low temperature and longer time of incubation. The variation between the lowest and highest predicted values of the hybridization signal and the QDEM recovery was ≥ 10%. Therefore the DOE predicted values were found significantly relevant to optimize the experimental conditions (Supplementary data).

Multiple response optimization design

The MRO was used to optimize the two desirable responses analyzed in the assay. The desirability functions (d(y)) for the maximization of the hybridization signal and the QDEM recovery were described by the following equation:

$$d_{\mathrm{HybS}} = \begin{cases} 0 \\ \left(\dfrac{\mathrm{RMFI_{calc}} - \mathrm{RMFI_{min\ obs}}}{\mathrm{RMFI_{max\ obs}} - \mathrm{RMFI_{min\ obs}}} \right) \\ 1 \end{cases} \quad (1)$$

$$d_{\%\mathrm{R}} = \begin{cases} 0 \\ \left(\dfrac{\%\mathrm{R_{calc}} - \%\mathrm{R_{min\ obs}}}{\%\mathrm{R_{max\ obs}} - \%\mathrm{R_{min\ obs}}} \right) \\ 1 \end{cases} \quad (2)$$

Where RMFI and $\%R_{\mathrm{calc}}$ were the predicted values of the optimized hybridization signal and the percentage of bead recovery; $[\mathrm{RMFI} / \%\mathrm{R}]_{\mathrm{min\ obs}}$ were the minimum RMFI / %R values obtained from the experiments, below which, the response was unacceptable, and $[\mathrm{RMFI} / \%\mathrm{R}]_{\mathrm{max\ obs}}$ were the maximum RMFI / %R values obtained from experimental data, above which, the desirability was at its maximum. Table 4 illustrates the analysis options defined for the MRO analysis of the different RSM.

The desirability of the two responses (equation 1, and 2) were combined and a single composite function *D* was created by the software (Statgraphic, VA, USA). All responses were considered to be equally important: the weight of the responses for computational analysis was similar and an equivalent impact coefficient was given to the responses in the MRO. By default, values of the impact coefficients were set to three (Statgraphic). The composite function was the geometric mean of the separate desirabilities given by:

$$\mathrm{D} = \left\{ \mathrm{d_{HybS}} \times \mathrm{d_{\%R}} \right\}^{1/2} \quad (3)$$

Oligo quantities in the femtomolar ranges were chosen in this DOE in order to optimize the sensitivity of target detection. Table 1 presents the factors' levels used in the DOE with 6x SSC hybridization buffer.

The optimum conditions determined by the MRO maximized the percentage of recovery at ~77%, and the hybridization signal at 450 a.u. with a *d*-value of ~0.9. To obtain these optimum values the hybridization conditions needed the incubation of 1,341 pmol of the Cy3-oligo target for 20 min at 58ºC. The desirability function was plotted as a response surface plot, which illustrated the optimum point of the model (Fig. 2C). The desirability function predicted conditions of hybridization with the lowest desirability at 0.0 whereas the responses were maximized with a desirability of *d* ~0.99. The ratio calculated in Table 4 showed that the input of the MRO was significant to define optimum conditions since differences of almost 45% were observed between the lowest and highest desirability values.

Hybridization titration with the predicted optimum conditions

To validate the optimized conditions and investigate the sensitivity found in the assay, a hybridization titration was undertaken. QDEM bioconjugates (~8000) were hybridized in the optimum condition defined by the DOE, in 6x SSC, 0.5% SDS (pH 7.0) hybridization buffer at 49ºC for 1 hour with increasing quantity of indirect fluorescent probe ranging from 0.5 fmol to 1000 pmol (Fig. 3) and the number of fluorescent probes hybridizing to the QDEM bioconjugates was evaluated with the QuantiBRITE PE Calibration kit (BD Bioscience). A linear quantitative relationship ($r^2 = 0.92$) between targeted oligo and RMEF values was found over the dynamic range of DNA quantities where RMEF increased (0.5 fmol to 400 pmol). The limit of detection of 29 pM was obtained with the

hybridization of ~8000 QDEM bioconjugates (5 μm diameter) to 0.5 fmol of Cy3 oligo target. From 800 pmol to 1000 pmol of targeted probe the maximum hybridization signal was lowered by ~11% to 22% (Fig. 3).

Evaluation of assay specificity

QDEM probes were used in a duplex assay (Fig 1B(1)) with ~100 fmol Y-SNP PCR product (Fig. 4). PCR samples were obtained in a range of 400 to 800 ng and diluted to the concentration required. The quantity of PCR products was chosen from the titration results and aimed to provide a significant hybridization signal with a high sensitivity and discrimination level in the linear quantity range. The two QDEM- fluorescent probe and the reporter dye fluorescent signals were successfully discriminated with FCM detection. The hybridization signal detected in FL4 and represented by the fluorescent intensity of Cy5-SA attached to biotinylated PCR products was significantly higher for the matching QDEM-probe populations. The matching allele was detected with ~239 a.u. of FL4 RMFI for 50 nM of PCR target. A background signal < 30 a.u. was recorded with the non complementary probe (Fig. 4).

Discussion

Experimental approach and design modelling

A crucial step in the identification of allele-specific detection methods is the specific hybridization of the probes to the DNA target. The main objective of this study was to develop an optimized DNA hybridization method in a liquid format using QDEM-probe and FCM detection (Fig. 1). DNA hybridization requires highly optimized conditions for the probes to specifically anneal to their corresponding allele, especially in complex mixtures.

First, a 18-mer poly(T) sequence was chosen to evaluate the hybridization efficiency as a way to mimic the potential hybridization to a short targeted sequence with low content of G/C nucleotides (Fig 1A). The number, the composition, and the type of bases composing the sequence as well as the position of the SNP mismatch base influence the probe hybridization temperature, or melting temperature. The hybridization decreases when the G/C content is lower, presumably reflecting the lower affinity of the oligo for their PCR target. The A/T interactions being weaker than the G/C liaisons, the optimization of hybridization with short poly(T) oligo to a poly(A) probe sequence would therefore potentially assure the specificity of the experimental conditions adapted to high affinity DNA-DNA interactions [30].

Previous studies used fluorescent oligos complementary to a specific probe sequence identifying a particular gene to optimize the hybridization procedure on the microsphere surface [4,31]. Fulton et al. [31] initial work to develop the first commercialized multiplex bead-based assay (FlowMetrix™ system, Luminex Corp, Austin, TX, USA) used a single oligo that identify a specific allelic sequence from a housekeeping gene. This group tested the impact of oligo

concentration, time and temperature of incubation with three separate experimentations to define optimum conditions for the multiplex bead-based assay. This approach was limited as it used a specific allelic sequence to test the effect of target concentration on hybridization properties [32]. The experimental approach we chose, using a Cy3-poly(A) sequence is believed to be more adapted to highly optimize hybridization assay for broader applications. Further, previous studies reported that shorter probes had higher affinity and discrimination properties [33]. Typically, 17-mer probes are used to obtain unambiguous SNP genotypes. Hence, a short oligo sequence of 18 bp was chosen here for its higher hybridization specificity properties [32].

The application of a sequential approach for the optimization process was the second main limitation of Fulton et al.'s study [31]. The DOE approach used here not only predicted optimum conditions for a maximum of hybridization and bead recovery using minute quantities of DNA target with a model that significantly fitted the data (Table 3), but it also described how factors impacted on the QDEM hybridization assay. Experimental design was shown to consume less time, effort and resources than one-factor-at-a-time optimization procedures while providing a large amount of information on the experimental process [15].

Computational method benefit

RSM using central composite design enabled the production of surface plot responses that help to understand the variation of the responses in relation with the variation of the conditions studied. Then, the titration of QDEM bioconjugates was performed with the predicted optimum conditions to evaluate the MRO and the impact of the DNA target quantity on the hybridization signal (Fig. 3). The DOE approach improved the efficiency of hybridization optimization through iteration

process: information about the molecular assay was gained with a minimum of experimental work. Here, the use of DOE largely cut down the optimization cost by reducing the number of experiments using expensive reagents such as fluorescent probes and quantum dots materials.

Further, the linear approach to optimize one-factor at a time was not adapted to the hybridization assay developed here, and to molecular biology assay in general, where complex molecular interactions are under study. Data gathering and data interpretation was facilitated through RSM, which mathematically modeled the relationship between factors and responses described as the impact of incubation time, and probes concentration on the fluorescent material and the hybridization efficiency.

The final MRO model showed poor correlation with the validation study. However, the difficulty of optimizing the hybridization process using sensitive material as QDEMs was nevertheless overcome and precise optimum experimental conditions were defined to apply the assay to SNP target detections. The DOE predicted optimum conditions with a competitive DNA target detection limit in the range of previous results obtained in QD-doped particle genotyping based assay optimized with one-factor at a time methods [34]. In addition, the computational model took into account the bead stability in the optimization process, a factor that was completely eluded in the other bead based assay and QD-encoded particle assay using traditional optimization process [9,31,32,34]. Finally, the model used here optimized the variance of hybridization signal and bead stability rather than the responses itself which reduces the variance and therefore improved the robustness of the assay [35].

Evaluation of the model

The optimum hybridization signal and bead recovery was obtained with the incubation of a volume of 8000 QDEM-probes in 6X SSC buffer, 1.3 pmol of target oligo, incubated at 49ºC for 58 min in the dark. The number of fluorescent probes hybridizing to the QDEM bioconjugates was evaluated in the optimum conditions with the QuantiBRITE PE kit (BD Biosciences). A linear quantitative relationship ($r^2 = 0.92$) between targeted oligo and RMEF values was found (Inset Fig. 3). A detection limit of 29 pM was calculated for the fluorescent target which was lower than the 4 nM target DNA detection reported with QD-based solid microarrays [36] and almost as sensitive as the 20 pM limit of detection reported by Cao *et al.* [12] using QD-doped polystyrene microspheres. The factors influencing the hybridization detection limit include: the detection method, the bead diameter, the carboxylation coverage, the coupling efficiency, the probe and target length, and the method to evaluate the sensitivity. The detection limit of hybridization obtained with QDEM bioconjugates was satisfactory for bead-based assay requirements [24]. The results obtained by Cao *et al.* [12] could be explained by a twenty times higher microsphere surface of detection (100 μm diameter). From 800 pmol to 1000 pmol of targeted probe the maximum hybridization signal was lowered by ~11% to 22% (Fig. 3). Steric hindrance, competition effect, lower diffusion of ssDNA, and negative forces repulsion could explain the diminution of the signal. The quenching of Cy3 fluorophore and QDEM emission spectrum could also lead to a global decrease of the fluorescence emission at high concentrations of fluorescent probe. The phenomenon of saturation described at high oligo quantities (400 to 1000 pmol) could induce the plateau followed by a decrease of the hybridization signal observed in Fig. 3. Hence, the diminution of

the hybridization signal can be explained by a lower diffusion of ssDNA in the suspension and a saturation of the QDEM hybridization sites [37].

The determination of the DNA template genotypes for the SNP was undertaken by signal-to-noise analyses and as expected, the relative hybridization signal was higher to the signal-to-noise (> 100 a.u.) for the matching allelic-probe. The average signal-to-noise for non specific hybridization signal with the mismatching allelic-probes was ~6.5 (Fig. 4) compared with a ratio ≥ 7 for Luminex bead-based assay applied to the detection of single-base changes in cystic fibrosis transmembrane conductance regulator gene [32,38]. Non-specific hybridization signal contributing to the noise can vary upon probe sequence, mismatches (some less destabilizing than others), and the flanking regions [39]. The factors influencing the differences of sensitivity observed here can also be explained by the type of detection method, beads diameter, carboxylation coverage, coupling efficiency, probe and target length, and the method chosen to evaluate the sensitivity.

Conclusion

Here, the potential of the design of experiment approach was shown to facilitate and to improve experimental protocol in suspension array. The development of complex bioassay systems promise to benefit from this approach in order to reduce experimental cost while enhancing optimization processes. The work presented here is an initial demonstration of the sensitivity and specificity of DNA detection using QDEM as fluorescent tag conjugated to short oligo probes. The improvement of the DNA detection limit and the average signal-to-noise ratio for SNP detection will be needed to demonstrate the detection capacities of commercialized QDEMs for multiplex SNP genotyping and its application to molecular diagnostics.

Footnote

[1]Abbreviations used: SAT, suspension array technology, QD, quantum dot; QDEM, quantum dot-encoded microsphere; SNP, single nucleotide polymorphism; ssDNA, single-stranded DNA; ANOVA, analysis of variance; DOE, design of experiment; RSM, response surface methodology; MRO, multiple response optimization; FCM, flow cytometry; (R)MFI, (corrected) mean fluorescence intensity; (R)MEF, (corrected) molecule of equivalent of fluorophore; HybS, hybridization signal

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Figures

Fig. 1. (A) Hybridization optimization and (B) the allelic specific oligonucleotide assay. Flow cytometry (FCM) data were collected as the mean of fluorescence intensity (MFI) with Logarithmic scale (Graph A2,B2). (A1) Hybridization optimization and titration was performed with fluorescent oligonucletotide tagged with Cy3 emitting at 570nm and detected in fluorescent channel 2 (FL2); (A2) hybridization (Hyb) signal and the % of hybridized QDEMs recovered at the end of the experiment were optimized; and a Cy3-oligo titration assay was used for validation. (B1) Two SNP probes were tagged with 2 different QDEM fluorescent codes (green, yellow) detected respectively in FL1 and FL2; (B2) SNP allele matching the QDEM probe was identified with Cy5-streptavidin reporter dye emitting at 665 and detected in FL4.

Fig. 2. Response surface plot corresponding to the optimization of (A) the QDEM recovery response (in percentage (%) of events), and (B) the Cy3 hybridization signal response in RMFI (arbitrary units, a.u.); and (C) to the desirability function of the MRO. Three-dimensional response surface and 2-dimensional contour plots as a function of time (in min) and temperature (Temp, in °C). Response plots (A) and (B) show the interaction between temperature and time for the two responses; and the quantity factor was held at its central point value, 500pmol, for both graphs. The temperature, time, and oligo quantity (in fmol) were optimized for the two responses analyzed simultaneously with MRO, the desirability function $d(y)$ expressed the desirability of the response value equal to y on a scale of 0 to 1 (in a.u.) is indicated in the legend on the right side of graph. The pink dots represent the actual experimental points or observed data.

Fig. 3. Hybridization titration of QDEM-probes to Cy3-oligonucleotides. Corrected molecule of fluorophore (RMEF, in number of oligos) function of oligo quantity (n_{oligo}, in fmol). X-axis in log scale. Inset: linear regression plot for 0 to 4 x 10^5 fmol of oligo. Data are presented as RMEF ± standard error (± SEM) of 3 replicates.

Fig. 4. Hybridization titration of QDEM matching and non-matching probe to the SNP target. Hybridization signal detected in FL4. Data presented as the linear regression plot of RMFI ± SEM (the mean of fluorescence intensity (MFI) detected with the positive samples minus the MFI of negative control corresponding ± SEM).

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(a.u.: arbitrary units; Default: values over which response variables were optimized separately)

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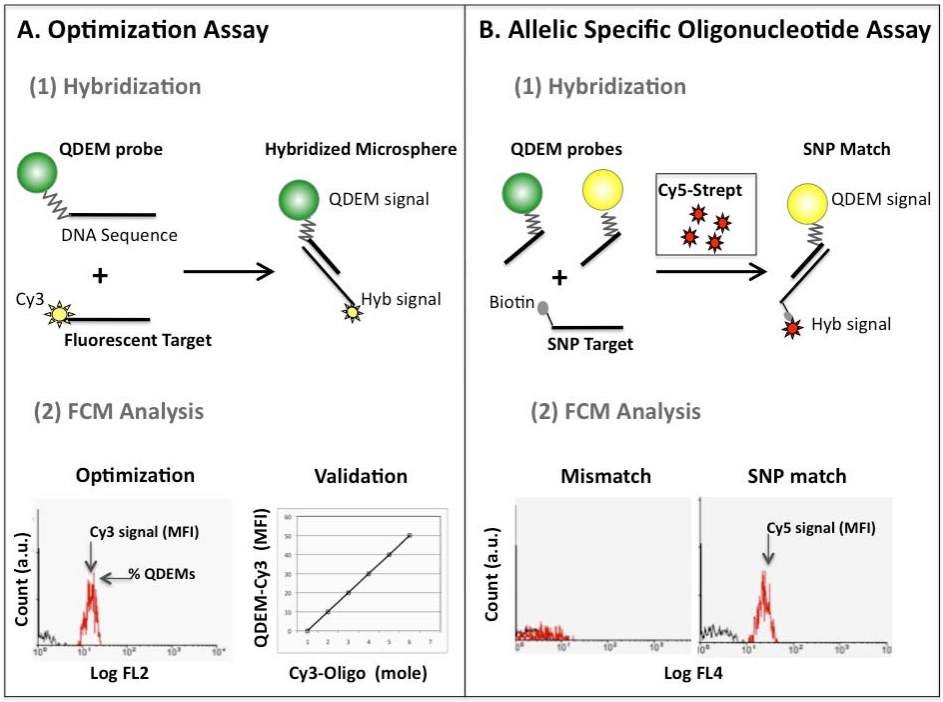

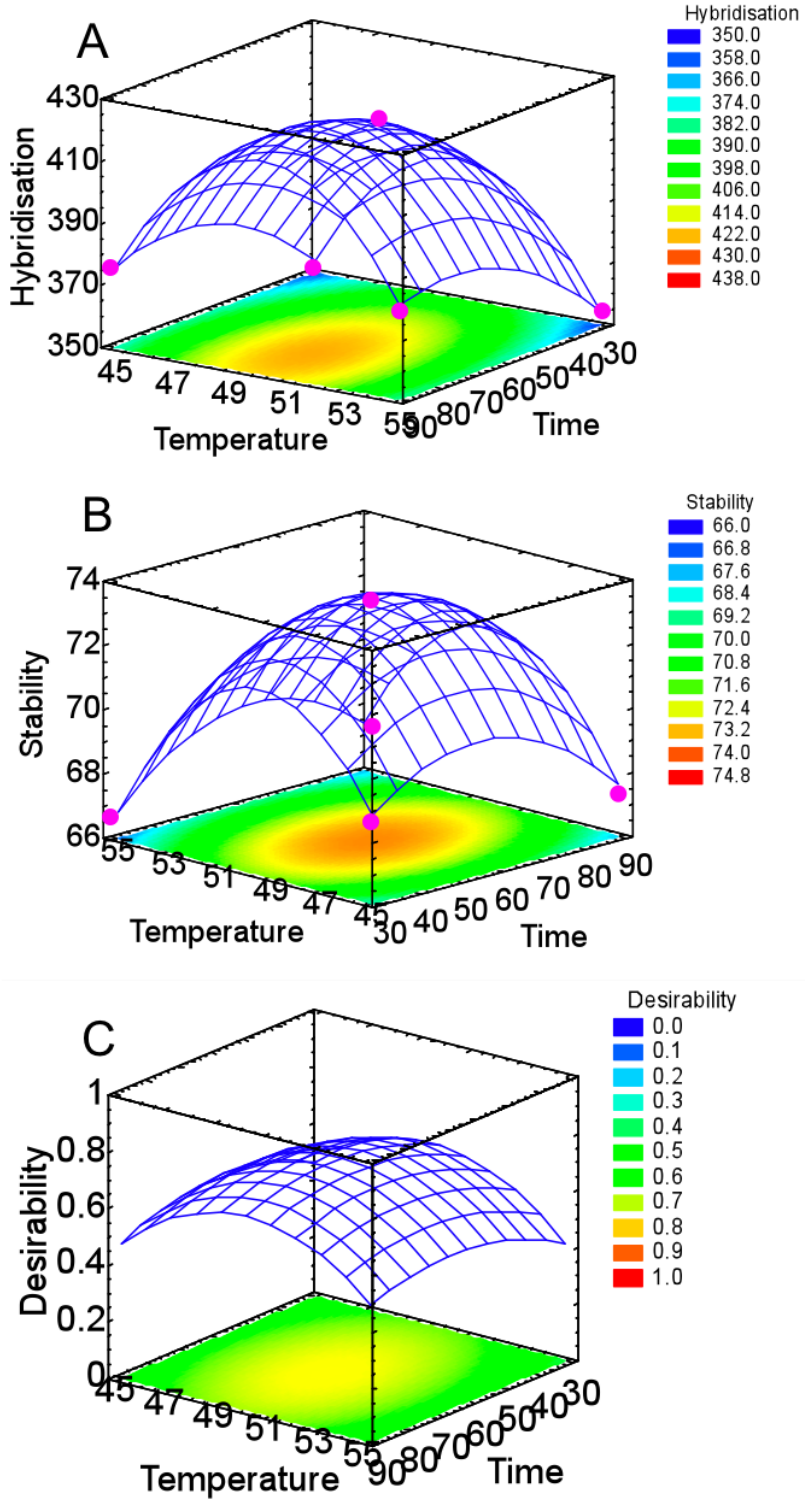

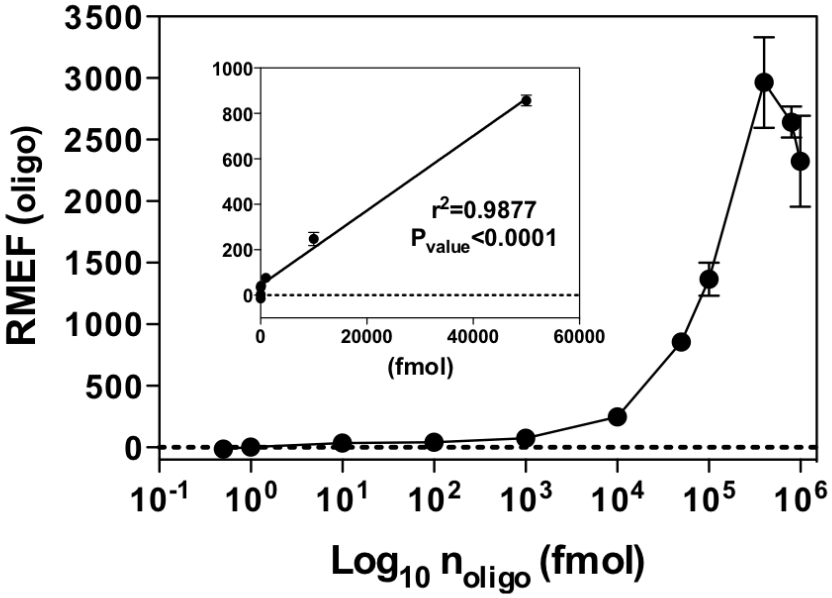

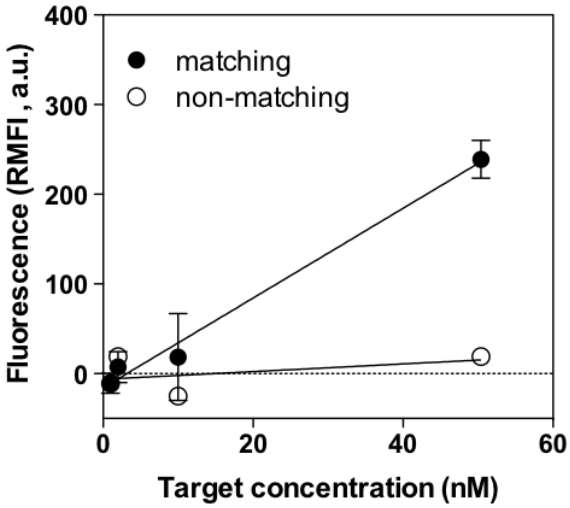

Table 1

Experimental ranges and levels of the independent variables.

Independent variable (X)	Range and Level		
	– 1	0	+ 1
X1 Temperature (ºC)	45	50	55
X2 Time (min)	30	60	90
X3 Oligo quantity (fmol)	0.5	50.25	1000

Table 2

Design matrix of the 2^3 full-factorial central composite design of the three experimental factors along with the two responses (Hybridization Signal, HybS, and the percentage of recovery, %R).

Run no.	X1	X2	X3	Temperature (ºC)	Time (min)	Oligo (fmol)	HybS (RMFI a.u.)	%R (%Events)
1	–1	–1	–1	45	30	0.5	419.8	72.8
2	+1	–1	–1	55	30	0.5	-25.0	67.5
3	-1	+1	–1	45	90	0.5	426.3	70.3
4	+1	+1	–1	55	90	0.5	435.0	77.1
5	–1	–1	+1	45	30	1000	476.0	76.2
6	+1	–1	+1	55	30	1000	503.0	71.7
7	–1	+1	+1	45	90	1000	456.5	75.1
8	+1	+1	+1	55	90	1000	-105.7	56.7
9	–2	0	0	41.59	60	500.25	71.0	57.9
10	+2	0	0	58.41	60	500.25	461.0	54.1
11	0	–2	0	50	9.55	500.25	479.2	50.4
12	0	+2	0	50	110.45	500.25	450.5	64.7
13	0	0	–2	50	60	0	223.3	69.1
14	0	0	+2	50	60	1340.73	437.5	79.0

Table 3

Analysis of variance (ANOVA) lack-of-fit and coefficient of determination (R^2) for the selected model (Hybridization Signal, HybS, and the percentage of recovery, %R).

Response studied	R-squared	Lack-of-fit		Conclusion
		F-Ratio	P-Value	
HybS	0.81	7.09	0.1282	Adequate
%R	0.72	1.95	0.3726	Adequate

Table 4

Responses variables and criteria of analysis for multiple response optimization[a].

Responses	Predicted values		Ratio (in %) (1-L/H)*100
	Highest	Lowest	
HybS (RMFI, a.u.)	420	350	17%
%R (%)	73.5	66.5	10%
Desirability	0.9	0.5	44%

[a] a.u.: arbitrary units; Default: values over which response variables were optimized separately.