

Multiplexed microsphere diagnostic tools in gene expression applications: factors and futures

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Abstract: Microarrays have received significant attention in recent years as scientists have firstly identified factors that can produce reduced confidence in gene expression data obtained on these platforms, and secondly sought to establish laboratory practices and a set of standards by which data are reported with integrity. Microsphere-based assays represent a new generation of diagnostics in this field capable of providing substantial quantitative and qualitative information from gene expression profiling. However, for gene expression profiling, this type of platform is still in the demonstration phase, with issues arising from comparative studies in the literature not yet identified. It is desirable to identify potential parameters that are established as important in controlling the information derived from microsphere-based hybridizations to quantify gene expression. As these evolve, a standard set of parameters will be established that are required to be provided when data are submitted for publication. Here we initiate this process by identifying a number of parameters we have found to be important in microsphere-based assays designed for the quantification of low abundant genes which are variable between studies.

Keywords: microspheres, surface chemistry, gene expression, diagnostics, flow cytometry

Introduction

The unprecedented biological resource of the high quality human genome reference sequence, completed in April 2003, created the potential to provide significant advances in human health. The field of functional genomics, where biological function is assigned to genes, has been catalyzed by this database but gene expression profiling relies heavily on data obtained from microarray platforms. Microarray technologies were proclaimed as the key that would unlock a wealth of information (Gerhold et al 2002; Tilstone 2003); however, it has become ever increasingly evident that there are many barriers to researchers in the limitation of current diagnostic options. An underlying assumption made in the application of a microarray is that thousands of probes will discriminately hybridize with target species with identical kinetics in the same environment. In the format of a microarray it is not surprising that this is not observed. Concerns that have arisen are related to the selection of probes which are assembled on the microarray and include poor specificity (false positives); the effect of splice variants; incorrect probe sequences derived from inaccuracies in public sequence data; and statistical issues in data handling (Nadon and Shoemaker 2002; Attoor et al 2004; Rockett and Hellmann 2004). A separate issue relates to the reporting of data obtained from microarrays as a consequence of separate laboratories conducting their array experiments with variable numbers of steps and a variety of conditions. As a consequence, there has been a move to establish the Minimum Information about a Microarray Experiment (MIAME) to allow independent verification at a later date and, in fact, several journals will not consider papers unless they have adopted these standards (Rockett and Hellmann 2004). With increasing pressure to validate and corroborate microarray data, many studies now

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publish microarray data in combination with that obtained from separate techniques such as Northern Blot analysis or quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Careful experimental design and assessment prior to commencing a procedure is recommended (Yang and Speed 2002; Armstrong and van de Wiel 2004).

Microspheres have emerged as an exciting new platform for biologists to adopt into their armory of techniques in the investigation of biomolecule interactions and cellular processes. There are numerous reports that establish that microsphere-based assays can provide reliable data in simple binding and purification applications for a wide variety of biomolecule interactions (Figure 1) (Braeckmanns et al 2002; Kellar and Iannone 2002; Lawrie et al 2003; Yingyongnarongkul et al 2003). In recent years there have been increasing numbers of studies in which microspheres have been used in more diverse applications and it is evident that the range of potential applications is enormous.

Microsphere technology offers several improvements over existing technologies because many copies of microspheres containing identical probes are available and each microsphere represents a self-contained assay. The ability to multiplex (simultaneously screen uniquely identifiable probes) through encoding microspheres (Braeckmanns et al 2002; Lawrie et al 2003) is a significant advantage and there are increasing numbers of studies demonstrating applications

of this type (Carson and Vignali 1999; Xu et al 2003; Martins et al 2004). In fact, very large multiplexed libraries containing millions of different probes can potentially be assembled (Battersby et al 2002). The assays are performed in a 3D liquid suspension (hence these are sometimes termed “suspension arrays”) which provides significantly better kinetics and transport phenomena to support reliability of the results, and there is flexibility to add extra probes to the library without having to make or purchase new arrays. As microsphere-based probes are randomly located in a suspension in a very small volume (unlike the compounds in microarrays and microplates which are in a fixed, known, position on an array), an encoding system is required to allow the rapid identification of the probe structures or reconstruction of the target sequences. Recent reports have described the application of multiplexed, microsphere-based assays in polymorphism genotyping (Ye et al 2001; Xu et al 2003; Bortolin et al 2004; Pickering et al 2004) and gene expression profiling (Fuja et al 2004; Kuhn et al 2004).

As microsphere-based diagnostics evolve, the concerns surrounding the publication of microarray data should be taken as a sign that a set of standard information should be established as a prerequisite and provided on publication of multiplexed, microsphere-based studies of gene expression profiling. In this report we propose a number of factors for consideration when reporting data obtained for gene

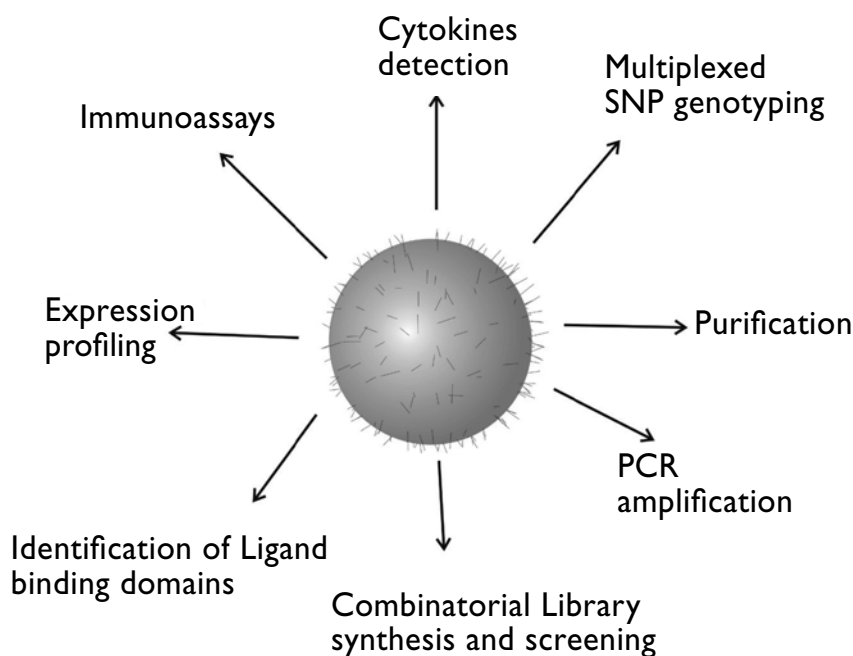


Figure 1 Common applications of microsphere-based analytical tools.

Abbreviations: PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

expression analyses using microsphere-based diagnostics. In the literature a range of terminology is adopted in reporting interactions between biomolecules for analytical purposes, and in this report we will use the terminology of “probe” for the oligonucleotide covalently attached to the surface of the microsphere (sometimes referred to as anti-tag) and “target” for the biomolecule that is fully complementary to the probe and exists in solution.

Materials and methods

Silica microspheres were synthesized from 3-mercaptopropyltrimethoxysilane (MPTMS) by an emulsion method developed in our laboratory (Johnston et al 2005) and subsequently sedimented to produce a narrow size distribution ($5 \pm 1 \mu\text{m}$). These microspheres were subsequently modified by the synthesis of a 3-aminopropyltrimethoxysilane (APS) shell to produce a surface containing amine functionalities. These reactions were carried out in triplicate on approximately 4×10^7 microspheres using $50 \mu\text{l}$ of 25% ammonia solution with variable concentrations of APS (0.1, 1, 5, and 10% v/v) in ethanol (Spectroscopic grade, Ajax Chemicals) to result in a final volume of 1 mL. The reaction vessels were shaken for 90 minutes at 1400 rpm at room temperature in the dark and the microspheres were washed 3 times and suspended in ethanol. The concentration of amines produced as a result of this modification was assessed by performing a ninhydrin test which relies on the reaction between ninhydrin and primary amines. The Ruhemann's purple product adsorbs at 570 nm and the absorbance can be read using a spectrophotometer and the number of amines quantified. Bifunctional carboxylic linker species, typically adipic acid, were coupled to these amine functionalities using diisopropyl carbodiimide (DIC, Aldrich, MO, USA). $250 \mu\text{L}$ of a 20 mg/mL solution of adipic acid in tetrahydrofuran (THF, EMD, USA) was added to the particle pellet along with $5 \mu\text{L}$ DIC and agitated for 12 hours prior to washing first with THF and then with ethanol. The oligonucleotide probes were attached to the carboxylic acid moieties by washing and suspending 4×10^7 microspheres in $50 \mu\text{l}$ of 0.1 M 2-morpholino ethane sulfonic acid (MES). $5 \mu\text{l}$ of $50 \mu\text{M}$ amine modified 40-mer ($5'$ -NH₂-TTGCTGGCACAGGAGGTGACAGTGGTTGAGGGCCAGGAAG 3'; $T_m = 73^\circ\text{C}$; named “csf1r”) (Geneworks, Australia) which possesses a hexamethyl (C6) spacer between the amine group and the 5' end of the probes was added to the microspheres and vortexed. $50 \mu\text{l}$ of carbodiimide (EDC) solution (50 mg/ml in 0.1 M MES) was added to the mixture and incubated for 20 min at 25°C with continuous shaking at 1400 rpm (Eppendorf Thermomixer

Comfort Agitator and Incubator 5355). A second aliquot of $50 \mu\text{l}$ of freshly prepared EDC was then added and incubated for another 20 min at 25°C with continuous shaking. The EDC addition process was repeated a further 2 times. The microspheres were then washed 3 times with 1 ml of a solution containing both 0.1 M MES and 0.1% sodium dodecyl sulfate (SDS, ICN Biomedical OH, USA). The oligonucleotide coupled microspheres were then resuspended in 1 mL of 4XSSC (4 X 150 mM NaCl, Chem-Supply, Australia, 15 mM sodium citrate, Aldrich, USA)+0.1% SDS. Oligonucleotide probes coupled to silica microspheres were incubated in a hybridization buffer 4XSSC/0.1%SDS with the specified concentration of complementary Cy5-modified target oligonucleotide (Geneworks, Australia). The hybridizations were all performed in triplicate for 60 min at 65°C . Microspheres were maintained in suspension by agitation in an Eppendorf Thermomixer. A series of variants of the csf1r sequence was examined as probes against the 40-mer complementary target including 10-mer (GTGACAGTGG; $T_m = 32^\circ\text{C}$); 20-mer mid (AGGAGGTGACAGTGGTTGAG; $T_m = 55^\circ\text{C}$); 20-mer end (AGTG GTT GAGGGCCAGGAAG; $T_m = 55^\circ\text{C}$). These hybridizations were also performed at 65°C .

The number of bound targets was assessed by passing the microspheres through a high-performance flow cytometer (DakoCytomation, Ft Collins, CO, USA) equipped with a 635 nm red diode laser to excite the Cy-5 label on the target oligonucleotide. Emitted photons were detected on a photomultiplier tube (denoted FL6) located behind a narrow bandpass filter ($670 \pm 15 \text{ nm}$). The data were analyzed with the Summit software (Dakocytomation Ft Collins, CO, USA) where the median fluorescence intensity of the histogram observed for the microsphere population was used as the statistic to compare fluorescence intensities.

Results and discussion

In order to identify the factors that can vary in performing microsphere-based gene expression experiments in separate laboratories, consideration must begin with the simplest level of hybridization: a single probe species attached to a single microsphere exposed to a single fully complementary target molecule of equivalent length. Assuming constant buffer conditions, the only factors influencing the efficiency of hybridization will be the proximity of the reaction temperature to the T_m (melting temperature) of the probe sequence and the distance between the probe and target. An increase in complexity arises at the next stage, which is to introduce several variables including the number of probes

on each microsphere, the concentration of target molecules, and the relative lengths of probe and target species, illustrated schematically in Figure 2.

It is evident that when the target concentration is relatively low, increasing the number of probes on the surface of a microsphere will increase the number of bound targets on each microsphere and maximize the sensitivity of the reaction. The same optimization in the limit of detection can be achieved by decreasing the number of microspheres, thereby increasing the number of target molecules bound to the surface of each microsphere and increasing the relative fluorescent intensity. The impact of varying the number of microspheres (with constant probe concentration) exposed to a fixed target concentration demonstrated that the sensitivity of the assay could be increased by reducing the number of microspheres (Figure 3).

The surface concentration of probes on a microsphere does not independently control the sensitivity of the assay; in fact, it is a combination of the surface concentration of probes and the number of microspheres supporting the probes that are fully complementary to the target. While many studies report the number of microspheres used in an assay, very few report the average surface concentration of probes per bead.

In a multiplexed assay, there may be an effect of the volume of microspheres in the hybridization mixture impacting on the accessibility of the target to the microsphere-bound

probe. In identifying the level of information that should be prerequisite in reporting microsphere-based hybridizations, it would seem that the probe density is significant. This parameter depends on the identity of the microsphere and related chemistry reactions performed to couple the probe to the surface. Microspheres developed from a range of materials have been reported as suitable for gene expression studies, but the most common are polystyrene (Carson and Vignali 1999; Ye et al 2001; Bortolin et al 2004; Fuja et al 2004; Martins et al 2004; Pickering et al 2004) and silica (Battersby et al 2002; Kuhn et al 2004). The surface of these microspheres is typically carboxylated to enable the covalent attachment of oligonucleotide probes. However, a range of linker molecules is adopted to optimize the interaction between the probe and target molecules, including polyethylene oxide (to minimize nonspecific binding) and alkyl chains to improve specificity (Shchepinov et al 1997) through reduction of steric hindrance. The number of probes attached to each microsphere is of highest significance in assays where the potential target concentration is very low (<0.1 pg/ μ l), as in the case of low-abundance genes. It may be necessary to perform a microsphere number titration to optimize the mean fluorescence observed after hybridization to cDNA (Fuja et al 2004). The ability to control the loading of probes on the microsphere surface is critical in tailoring the microsphere-based diagnostic to potential applications. The surface loading of initial functionalities to which the probes

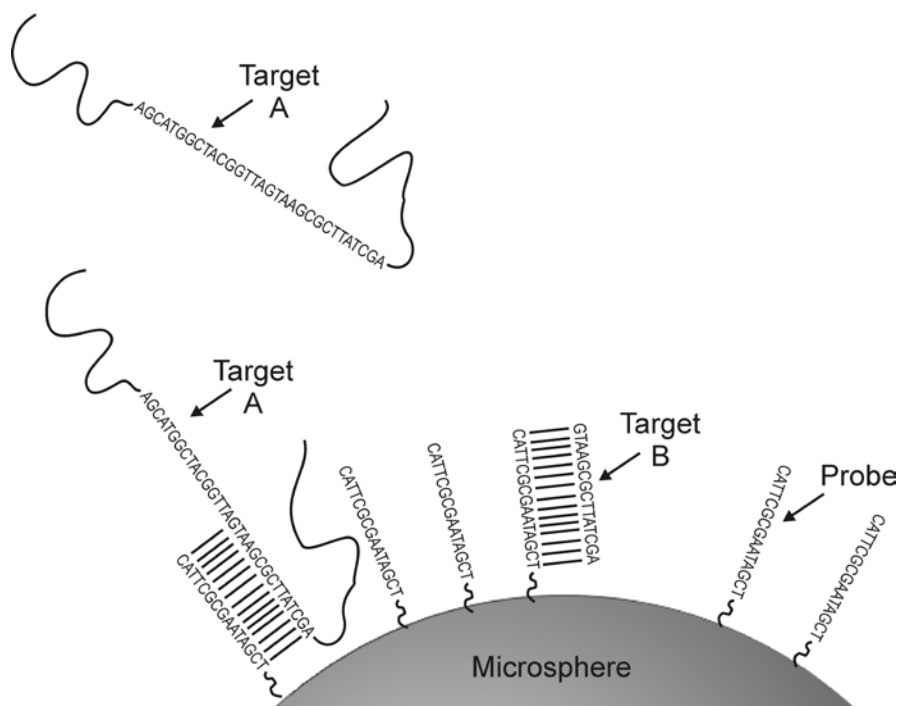


Figure 2 Schematic representation of a microsphere-bound probe-target hybridization occurring on the surface of a microsphere. Target A is significantly longer than Target B.

are coupled should be provided as part of their specification or be quantified. A desirable option is the ability to select the surface concentration of probes according to the application and potential target concentration. An example is provided in Figure 4 where the concentration of amine functional groups can be controlled through the modification of the surface of silica microspheres with a coat of APS. The final number of primary amines available on the surface of the microspheres was dependent on the concentration of APS and it would appear that this will translate into a potential route to controlling the surface concentration of probes.

While many groups report the initial “loading” or concentration of the functional groups available on the surface of their microspheres to which their probes will be coupled, they assume a 100% efficiency of conversion into a concentration of probes on the surface after several coupling reactions. The popular application of carbodiimide chemistry to react probes onto the functionalities present on the surface of the microspheres (Xu et al 2003; Bortolin et al 2004; Fuja et al 2004) introduces a major source of variability, as this coupling reaction is well known to be inefficient with the carbodiimide required to be present in great excess initially and in some cases to be added in multiple aliquots. Indeed, several coupling reactions may be adopted to add tag or linker molecules in between the microsphere surface and the probe species. It is evident that there is a need for researchers to quantify the final probe concentration that has been used in

a gene expression assay. Recent studies have incorporated an innovative approach to address this issue by adopting either a 18-mer poly (dT) linker (Xu et al 2003) or a luciferase tag sequence as part of the probe (Ye et al 2001) to determine the coupling efficiency.

This strategy raises the second major issue in developing microsphere-based hybridizations: variations between the structure of the probe sequence and the target sequence including relative length and region of complementarity. Several studies demonstrate the ability to discriminate single nucleotide polymorphisms using Luminex microsphere-based systems (Ye et al 2001; Bortolin et al 2004; Pickering et al 2004). Specificity is critical and the ability to detect single-base mismatches (point mutations) should be demonstrated for the lowest abundant targets in an experiment by incorporating both complete match and single mismatch probe. A common factor in many reports of microsphere-based oligonucleotide hybridizations is that the probe sequences are often significantly shorter than the target genes. Steric issues may play an important role in the hybridization efficiency (even assuming there are no hairpin structures formed in either sequence). An investigation into the effect of the length of the probe sequence attached to the microsphere on the efficiency of hybridization revealed that, for a 40-base oligonucleotide target molecule, the probe of the same length gave the maximum fluorescence (Figure 5). In this study, the microsphere number remained constant at 20 000

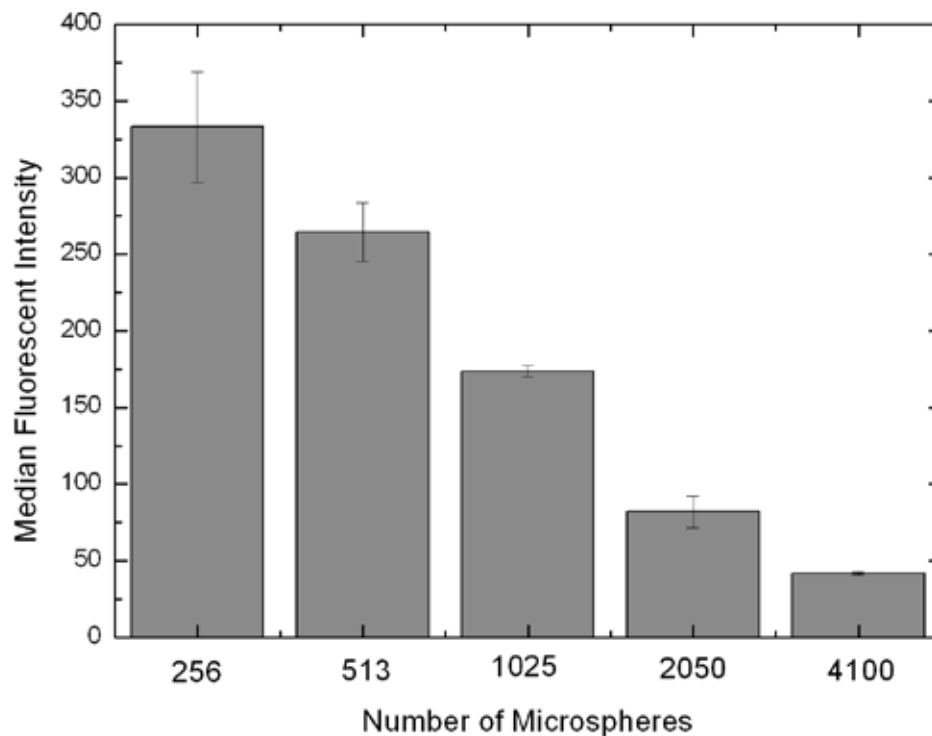


Figure 3 The influence of the number of microspheres on the sensitivity of an assay with a fixed target concentration 30 pM.

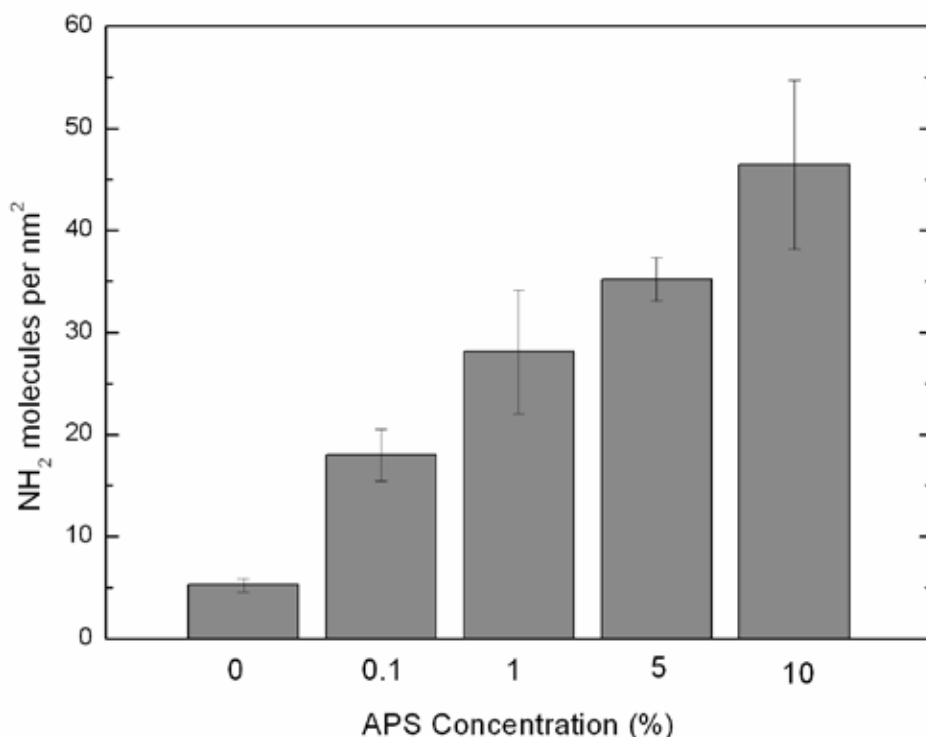


Figure 4 The application of the ninhydrin test to quantify the concentration of amine groups available on the surface of a silica microsphere.
Abbreviations: APS, 3-aminopropyltrimethoxysilane.

microspheres/hybridization reaction and the hybridizations temperature was slightly lower than the T_m of the 40-mer sequence but above that of the shorter sequences.

The maximum signal was anticipated for the equivalent length target sequence at this temperature, and discrimination in the binding of shorter sequences whose T_m were lower than the hybridization temperature was observed. A comparison was made between a 20-mer probe sequence being complementary to a central region of a 40-mer oligonucleotide target sequence (Figure 5, 20-mer, mid) and a 20-mer probe sequence being complementary to the end of a 40-mer target sequence (Figure 5, 20-mer end). It was revealed that there was increased sensitivity to the probe hybridizing to the end of the target sequence. This preliminary study demonstrated that steric effects will influence the efficiency of a microsphere-based assay even for short sequences and this effect will be magnified as the sequences are lengthened. In fact, many studies use PCR amplification to increase the target concentration in the assay and to incorporate a unique label on the target species.

Many factors are beyond the scope of this report to examine in depth, including optimization of the signal obtained for bound target species by amplification of targets by PCR (Xu et al 2003), or the use of dendrimer labels (Fuja et al 2004; Lowe et al 2004). The optimal assay would be to achieve single molecule detection of a target by a capture

probe, but there remains a substantial amount of development required in these systems to achieve this goal.

In summary, from the increasing number of studies reporting the application of microspheres in gene expression studies, it is evident that microsphere-based hybridizations are a viable method for performing sensitive and specific quantification of gene abundances. However, several factors may introduce variability in the data reported in separate laboratories and we recommend statements of several parameters, including the final probe concentration; the number of microspheres per assay; the T_m for the capture probe; the hybridization temperature; and, if available, the length of both the probe and the target sequence, and the mode of amplification of signal for target sequence.

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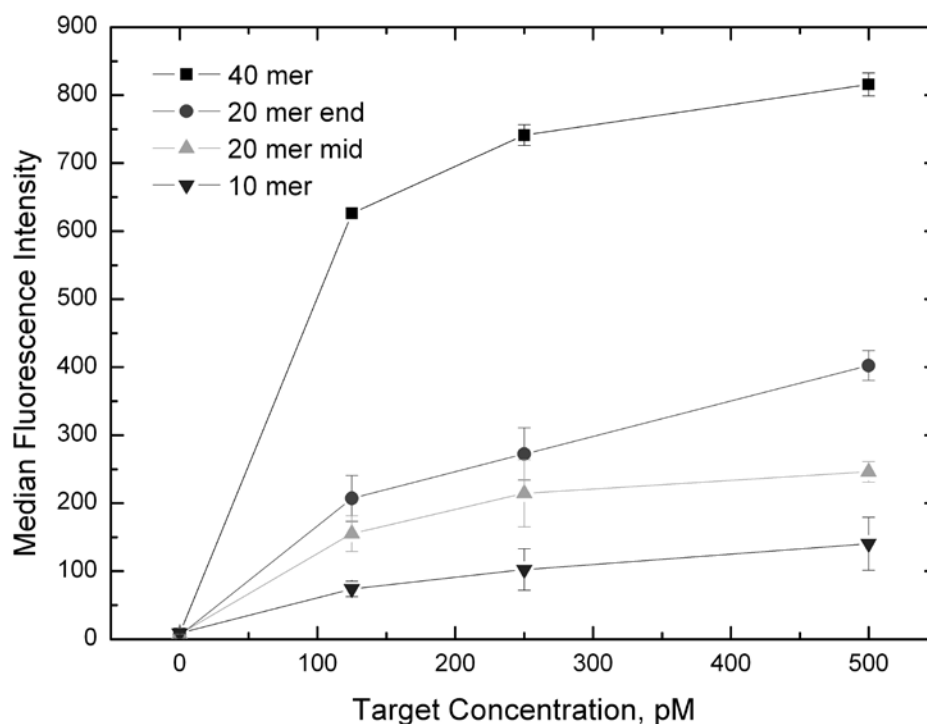


Figure 5 Examination of the influence of probe length and specificity on hybridization to a 40-mer oligonucleotide target. The difference between the two 20-mer probe sequences is that one was fully complementary to the middle sequence of the target mid and the other was fully complementary to the end of the target sequence end.

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