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An Aptamer-based Sensor for Aqueous Unchelated Gadolinium(III)

--Manuscript Draft--

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Abstract:	A method for determining the presence of unchelated trivalent gadolinium ion (Gd3+) in aqueous solution is demonstrated. Gd3+ is often present in samples of gadolinium-based contrast agents as a result of incomplete reactions between the ligand and the ion, or as a dissociation product. Since the ion is toxic, its detection is of critical importance. Herein, the design and usage of an aptamer-based sensor (Gd-sensor) for Gd3+ are described. The sensor produces a fluorescence change in response to increasing concentrations of the ion, and has a limit of detection in the nanomolar range (~100 nM with a signal-to-noise ratio of 3). The assay may be run in aqueous buffer at ambient pH (~7 - 7.4) in a 384-well microplate. The aptamer sensor is relatively unreactive toward other physiologically relevant metal ions such as sodium, potassium, and calcium ions, although it is not specific for Gd3+ over other trivalent lanthanides such as europium(III) and terbium(III). Nevertheless, the lanthanides are not commonly found in contrast agents or the biological systems, and the sensor may therefore be used to selectively determine unchelated Gd3+ in aqueous conditions.
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TITLE:
An Aptamer-based Sensor for Unchelated Gadolinium(III)

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SHORT ABSTRACT:

The use of polydeoxynucleotide (44-mer aptamer) molecules for sensing unchelated gadolinium(III) ion in an aqueous solution is described. The presence of the ion is detected via an increase in the fluorescence emission of the sensor.

LONG ABSTRACT:

A method for determining the presence of unchelated trivalent gadolinium ion (Gd^{3+}) in aqueous solution is demonstrated. Gd^{3+} is often present in samples of gadolinium-based contrast agents as a result of incomplete reactions between the ligand and the ion, or as a dissociation product. Since the ion is toxic, its detection is of critical importance. Herein, the design and usage of an aptamer-based sensor (Gd-sensor) for Gd^{3+} are described. The sensor produces a fluorescence change in response to increasing concentrations of the ion, and has a limit of detection in the nanomolar range (~100 nM with a signal-to-noise ratio of 3). The assay may be run in an aqueous buffer at ambient pH (~7 – 7.4) in a 384-well microplate. The sensor is relatively unreactive toward other physiologically relevant metal ions such as sodium, potassium, and calcium ions, although it is not specific for Gd^{3+} over other trivalent lanthanides such as europium(III) and terbium(III). Nevertheless, the lanthanides are not commonly found in contrast agents or the biological systems, and the sensor may therefore be used to selectively determine unchelated Gd^{3+} in aqueous conditions.

INTRODUCTION:

The increasing importance of magnetic resonance imaging (MRI) in clinical diagnosis, which is limited by the inherent sensitivity of the technique, has resulted in the rapid growth of research into the development of novel gadolinium-based contrast agents (GBCAs)[1]. GBCAs are molecules that are administered to improve the image quality, and they typically have the chemical structure of a trivalent gadolinium ion (Gd^{3+}) coordinated to a polydentate ligand. This complexation is of critical importance as unchelated Gd^{3+} is toxic; it has been implicated in the development of nephrogenic systemic fibrosis in some patients with renal disease or failure[2]. Consequently, detecting the aqueous free ion is instrumental in ensuring the safety of GBCAs. The presence of unchelated Gd^{3+} in GBCA solutions often is the result of an incomplete reaction between the ligand and the ion, dissociation of the complex, or displacement by other biological metal cations[3].

Among the several techniques currently used for determining the presence of Gd^{3+}, those relying on chromatography and/or spectrometry rank highest in terms of versatility and applicability[4]. Among their strengths are high sensitivity and accuracy, the ability to analyze various sample matrices (including human serum[5], urine and hair[6], wastewater[7], and contrast agent formulations[8]), and the simultaneous quantification of multiple Gd^{3+} complexes (a listing of studies prior to 2013 is described in a comprehensive review by Telgmann *et al*)[4]. The only drawback is that several of these methods require instrumentations (such as inductively coupled plasma-mass spectrometry)[4] that some laboratories may not have access to. Within the context of novel GBCA discovery at the research and proof-of-concept levels, a relatively more convenient, rapid, and cost-effective spectroscopic-based method (such as UV-Vis absorption or fluorescence) may serve as a valuable alternative. With these applications in mind, a fluorescent aptamer-based sensor for aqueous Gd^{3+} was developed[9].

The aptamer (Gd-aptamer) is a 44-base long single-stranded DNA molecule with a specific sequence of bases that was isolated through the process of systematic evolution of ligands by exponential enrichment (SELEX)[9]. To adapt the aptamer into a fluorescent sensor, a fluorophore is attached to the 5' terminus of the strand, which is then hybridized with a quenching strand (QS) via 13 complementary bases (Figure 1). The QS is tagged with a dark quencher molecule at the 3' terminus. In the absence of Gd^{3+}, the sensor (Gd-sensor), comprised of a 1:2 mole ratio of Gd-aptamer and QS respectively, will have minimal fluorescence emission due to energy transfer from the fluorophore to the quencher. The addition of aqueous Gd^{3+} will displace the QS from the Gd-aptamer, resulting in an increase in fluorescence emission.

[place Figure 1 here]

There is at present, one commonly used spectroscopic-based method for detecting aqueous Gd^{3+}. This assay uses the molecule xylenol orange, which undergoes a shift in the maximum absorption wavelength from 433 to 573 nm upon chelation to the ion[10]. The ratio of these two absorbance maxima can be used to quantify the amount of unchelated Gd^{3+}. The aptamer sensor is an alternative (may also be complementary) to the xylenol orange assay, as the two methods have different reaction conditions (such as pH and composition of the buffer solutions used), target selectivities, linear ranges of quantification, and detection modalities[9].

PROTOCOL:

Note: Molecular biology grade water is used in all buffer and solution preparations. All disposable tubes (microcentrifuge and PCR) and pipet tips are DNase- and RNase-free. Please consult the material safety data sheet (MSDS) for all chemicals prior to use. Use of appropriate personal protective equipment (PPE) is strongly recommended.

1. Preparation of the aptamer stock solutions

1.1) Purchase 2 strands of polydeoxynucleotide commercially. Order both strands with purification via high performance liquid chromatography (HPLC).

Strand 1 (Gd-aptamer):
5'-/56-FAM/AGGCTCTCGGGACGACCAGTTGGTCCCGCTTTATGTGTCCCGAG-3'

Strand 2 (QS):
5'-GTCCCGAGAGCCT/3Dab/-3'

1.2) Dissolve each strand in water to make 100 μM individual stock solutions of the Gd-aptamer and the QS.

1.3) Store these solutions at -20 ºC. The solutions are stable thus far, for 3 years.

1.4) To minimize freeze-thaw cycles, store the stock solutions in 10 μL aliquots.

2. Preparation of the 2X Gd-sensor solution

2.1) Prepare the assay buffer (20 mM HEPES, 2 mM $MgCl_2$, 150 mM NaCl, 5 mM KCl). Adjust the pH to ~7.4 with NaOH and HCl, and filter through sterile disposable bottle top filters with 0.2 μm PES membrane. Store in sterile bottles. If filtered and stored properly (at room temperature), the buffer is stable thus far, for 2 years.

2.2) Dissolve 1 μL of the Gd-aptamer stock solution (from step 1.2) and 2 μL of the QS stock solution (from step 1.2) in 497 μL of the assay buffer. Mix well using a vortex. Their concentrations in the 2X Gd-sensor solution are 200 nM and 400 nM, respectively.

Note: The volume of the 2X Gd-sensor solution prepared in this step is 500 μL, which is sufficient to test 6 – 7 varying concentrations of Gd^{3+} for the calibration curve and the contrast agent solutions (step 3). Each sample will give duplicate wells in a 384-well plate.

2.2.1) Adjust the volume of the 2X Gd-sensor solution according to the number of Gd^{3+} solutions that needs to be tested.

2.3) Transfer the 2X Gd-sensor solution into 9 PCR tubes, with 50 μL into each tube. Place the tubes in a thermal cycler.

2.4) Set the program in the thermal cycler to heat the solution in the tubes to 95 ^oC, hold for 5 min, and then slowly cool the solutions to 25 ^oC over ~15 min (at the rate of ~0.05 – 0.1 $^oC/s$). The heating and cooling cycle is to ensure optimal hybridization between the Gd-aptamer and the QS. Partial hybridization results in incomplete quenching and a higher background fluorescence of the sensor. If a thermal cycler is not available, carry out this process using a hot water bath instead.

2.5) Once cooled to 25 ^oC, immediately use the solution, or keep in the thermal cycler (up to about 2 h) until ready to be used. When a water bath is used for the heating, leave the tubes in the bath as the water slowly cools to room temperature.

3. Constructing the fluorescence calibration curve and detecting the presence of unchelated Gd^{3+} in a solution of Gd contrast agent.

3.1) Dissolve $GdCl_3$ solid in the assay buffer (the same buffer as in step 2.1).

3.2) Through serial dilution, prepare 100 μL each of 6 different Gd^{3+} solutions in microcentrifuge tubes at double of the final desired concentrations for the calibration curve (2X solutions).

3.2.1) For example, to construct a calibration for 0 (buffer only with no $GdCl_3$), 50, 100, 200, 400, and 800 nM of Gd^{3+}, prepare solutions containing 0, 100, 200, 400, 800, and 1600 nM of the ion. Make sure to always include the 'blank' with 0 nM Gd^{3+} as the negative control.

3.3) Dissolve the contrast agent to be tested in the assay buffer. Prepare 2 or 3 different concentrations of the contrast agent solutions via serial dilution.

Note: Testing 3 different concentrations of the contrast agent solution is recommended. This is to ensure that these concentrations are within the linear range. If the samples tested do not display a linear relationship, reduce the concentrations of the contrast agent used.

3.4) Take the PCR tubes containing the 2X Gd-sensor solution from step 2.5 out of the thermal cycler.

3.5) Add 50 μL of each Gd^{3+} solution from step 3.2 into 6 of the 9 PCR tubes containing the 2X Gd-sensor solution. Mix by pipetting up and down. Each PCR tube now contains the desired concentration of Gd^{3+} to be tested, 100 nM Gd-aptamer, and 200 nM QS.

3.6) To the remaining PCR tubes containing the 2X Gd-sensor solution, add 50 μL of the contrast agent solutions from step 3.3. Mix well by pipetting up and down a few times.

3.7) Incubate the solutions in the PCR tubes for around 5 minutes at room temperature. They may be left to stand for up to 30 minutes.

3.8) Transfer 45 μL of each tube into a 384-well plate. Each PCR tube will give duplicate wells.

3.9) Record the fluorescence of each well on a plate-reader. The fluorophore (FAM) used in the Gd-sensor design has excitation and emission maxima of 495 and 520 nm respectively, as listed on the supplier's website. Choose appropriate excitation and emission wavelengths or filters depending on whether the plate-reader is monochromator- or filter-based.

3.10) Plot the graph of fluorescence in arbitrary fluorescence units (AFU) against concentration of Gd^{3+}.

3.11) Plot the graph as fluorescence fold change against concentration of Gd^{3+}. Calculate the fluorescence fold change by dividing the AFU of each concentration by the AFU of the 'blank' solution (with 0 nM of Gd^{3+}). The fluorescence fold change will allow for the normalization of the results, should the AFU display some periodical (different days, *etc.*) variations.

3.12) Compare the fluorescence emission of the solution containing the contrast agent and the 'blank', which is the solution containing 0 nM $GdCl_3$ (buffer only).

Note: A higher fluorescence of the GBCA solution implies the presence of unchelated Gd^{3+}, which may necessitate further purification of the contrast agent. The amount of unchelated Gd^{3+} present may be estimated using the calibration curve constructed in step 3.10 or 3.11.

REPRESENTATIVE RESULTS:

A typical fluorescence change of the Gd-sensor solution in the presence of unchelated Gd^{3+} is shown in Figure 2. The emission may be plotted as the fluorescence fold change (Figure 2A) or the raw fluorescence reading (Figure 2B) in arbitrary units (AFU). Both plots yield very similar calibration curves with a linear range for concentrations of Gd^{3+} below 1 μM and saturation of the signal at > 3 μM. The limit of detection is ~100 nM with a signal-to-noise ratio of 3.

In solutions containing the GBCA of interest, the presence of unchelated Gd^{3+} will be translated into a fluorescence increase of the sensor when compared to the 'blank' solution. The fluorescence changes in solutions of 2 different batches of Gd-DOTA complex, one of higher purity than the other, are shown as examples of representative results (Figure 3). Gd-DOTA (gadoteric acid) is a gadolinium complex of Gd^{3+} surrounded by an organic ligand DOTA that is found in a commercial contrast agent. The batch of higher purity does not display a significant increase in emission up to 20 mM of Gd-DOTA. When unchelated Gd^{3+} is present, a change that is noticeable even at Gd-DOTA concentrations below 5 mM is observed. In this example where the data points are plotted as fluorescence fold change of the sensor, quantification of the amount of unchelated Gd^{3+} may be estimated using the calibration curve in Figure 2A.

Figure 1. The sensor (Gd-sensor) that consists of the 44-base long aptamer (Gd-aptamer) tagged with fluorescein (a fluorophore) and the 13-base long quenching strand (QS) tagged with dabcyl (a dark quencher).
In the absence of unchelated Gd^{3+}, the fluorescence of the sensor is minimal. With addition of Gd^{3+}, displacement of the QS occurs and an increase in fluorescence emission is observed.

Figure 2. Representative Gd-sensor fluorescence calibration curve plots.
All data points were performed at least in duplicates and the average values plotted with standard deviation as the error bars. (A) A calibration curve obtained using 100 nM Gd-aptamer and 200 nM QS. The graph is plotted with fluorescence fold change as the y axis. (B) The same calibration curve as in (A) with raw fluorescence in arbitrary units (AFU) as the y axis.

Figure 3. Representative Gd-sensor fluorescence change when testing the presence of unchelated Gd^{3+} in samples of Gd-DOTA molecule.
2 different batches of Gd-DOTA complex solutions are shown in this plot, one of higher purity (circle marker) and the other containing unchelated Gd^{3+} (blue triangle marker). Each data point is an average of two readings with standard deviation as the error bars.

DISCUSSION:
Using the aptamer-based Gd-sensor, an increase in fluorescence emission that is proportional to the concentration of unchelated Gd^{3+} is observed. To minimize the amount of sample used, the assay may be run in a 384-well microplate with a total sample volume of 45 μL per well. In this design, the choice of fluorescein (FAM) and dabcyl (Dab) was primarily based on the cost of the reagents; to modify the emission wavelength, a different pairing of fluorophore and quencher may be used[11].

It is important to note that to obtain the best result with the sensor, one of the critical steps is the heating to 95 ^{o}C and slow cooling (step 2.4) in the assay buffer to achieve optimal hybridization between the Gd-aptamer and the QS strands. As previously mentioned in the protocol, if a thermal cycler is not available, the incubation at 9 5 ^{o}C may be carried out in a water bath. Another key parameter to control is the compositions of the buffer solutions; the use of the assay buffer listed in step 2.1 to dissolve the contrast agent is recommended, or deionized water may also be used. However, solutions that contain potential interferents should be avoided. An example of such a buffer is one that contains phosphate anions, which can coordinate to

unchelated Gd^{3+} to form insoluble gadolinium phosphate[12]. The precipitate will not react with the sensor, resulting in a false negative result.

A few steps in the experiments may be modified without affecting the outcome. First, to simplify the assay and calculation, prepare both the Gd-sensor solution and the aqueous $GdCl_3$ for the calibration curve at 2X concentrations. If desired, other dilution factors may be used (for example, 10X solutions), provided that the final assay concentrations of the Gd-aptamer and the QS are maintained at 100 nM and 200 nM, respectively. Second, the assay buffer does not have to be exactly pH 7.4. Any value between 7 – 7.4 will produce the desired fluorescence increase, as long as the same buffer is used throughout the experiment. Third, once the fluorescence emission reading is obtained, the data points may be plotted either as raw fluorescence in arbitrary unit (AFU) or as fluorescence fold change. To calculate the fluorescence fold change, the raw fluorescence reading of each concentration is normalized to (divided by) the reading of the negative control (0 nM Gd^{3+}). As shown in Figures 2A and B, the fluorescence emission trends in both plots are almost identical. The fold change may be a more convenient way to analyze the data if the plate reader displays some variations in the raw readings recorded at different times. Finally, if the laboratory is equipped with a fluorometer, but not a plate reader, each data point may be measured using a cuvette, instead of a microplate. Depending on the size of the available cuvettes, the volumes of the solutions prepared in the assay may need to be adjusted.

The method reported herein provides an alternative to chromatographic- and/or spectrometric-based techniques for detecting aqueous Gd^{3+}. Compared to the latter, the Gd-sensor assay is more limited in terms of sensitivity, accuracy, and ability for simultaneous detection of multiple species. On the other hand, the spectroscopic-based sensor requires an instrumentation that may possibly be more readily available, may be performed within a shorter time period, and the sample preparation is minimal. The contrast agent may be simply dissolved in the buffer solution, mixed with the Gd-sensor solution, and the fluorescence emission directly measured. Furthermore, the sensor is able to detect a much lower concentration of unchelated Gd^{3+} than the xylenol orange indicator (about 2 orders of magnitude difference between the two methods) and has a higher selectivity for Gd^{3+} over several other biologically important and transition metal ions[9].

There are two drawbacks of this assay that may restrict its use under some experimental conditions. One limitation is that the sensor is not specific for Gd^{3+}; it displays a response to other lanthanide ions (such as Eu^{3+} and Tb^{3+})[9]. However, these are not ions commonly found in contrast agents or the biological systems and therefore, their interference are minimal. The second point to note is that at higher concentrations (above ~10 μM) of Gd^{3+}, a gradual decrease in the Gd-sensor fluorescence emission is observed. The effect of quenching by lanthanide ions is a well-documented phenomenon[13] that has also been used as a technique for detecting and quantifying them[14]. While this limits the utility of the sensor for measuring high concentrations of Gd^{3+}, the goal of this design is to detect small amounts of the free ion in the solution to ensure a higher purity of the contrast agent.

In this work, the use of a convenient fluorescence-based technique for detecting toxic unchelated Gd^{3+} in aqueous solution has been described. This assay is meant for the early-stage evaluation

of gadolinium-based contrast agent purity, specifically during the synthesis and formulation for *in vitro* experiments. With the current growth of magnetic resonance imaging in diagnosis, an increasing number of novel contrast agents are continually being designed and tested. The aptamer-based Gd-sensor will facilitate this development by providing a means for rapidly detecting the presence of sub-micromolar concentrations of unreacted or dissociated Gd^{3+} in aqueous solution at ambient pH. Furthermore, since the sensor displays cross-reactivity with other trivalent lanthanide ions, its application may be extended to these areas of research.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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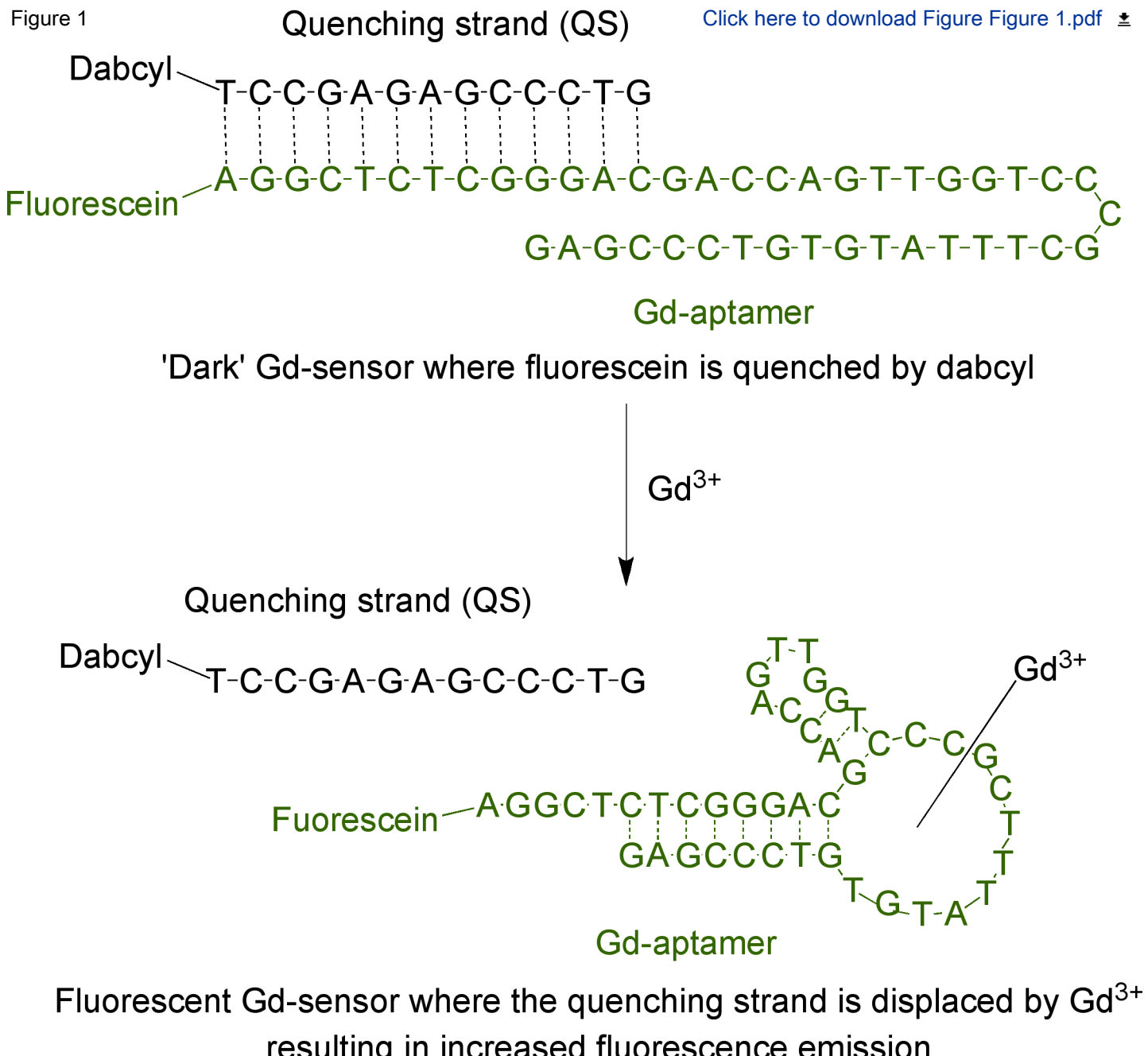
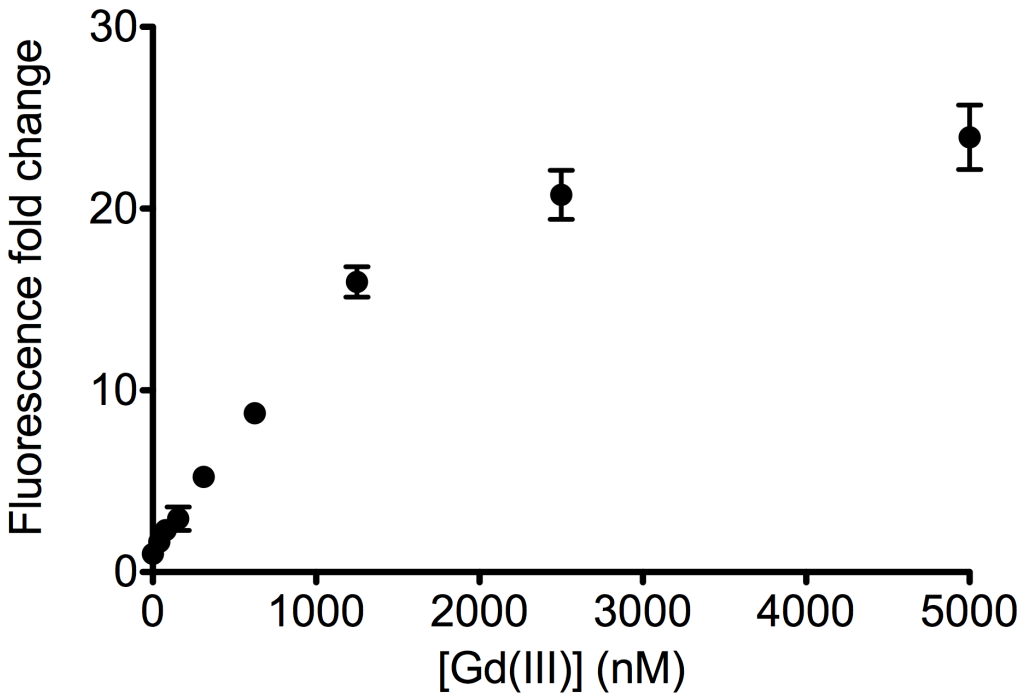
Figure 1


Quenching strand (QS)
Click here to download Figure Figure 1.pdf
Dabcyl
T-C-C-G-A-G-A-G-C-C-C-T-G
Fluorescein
A-G-G-C-T-C-T-C-G-G-G-A-C-G-A-C-C-A-G-T-T-G-G-T-C-C
C
G-A-G-C-C-C-T-G-T-G-T-A-T-T-T-C-G
Gd-aptamer
'Dark' Gd-sensor where fluorescein is quenched by dabcyl
Gd3+
Quenching strand (QS)
Dabcyl
T-C-C-G-A-G-A-G-C-C-C-T-G
Gd3+
Fuorescein
A-G-G-C-T-C-T-C-G-G-G-A-C
G-A-G-C-C-C-T-G
Gd-aptamer
Fluorescent Gd-sensor where the quenching strand is displaced by Gd3+
resulting in increased fluorescence emission

A.



B.

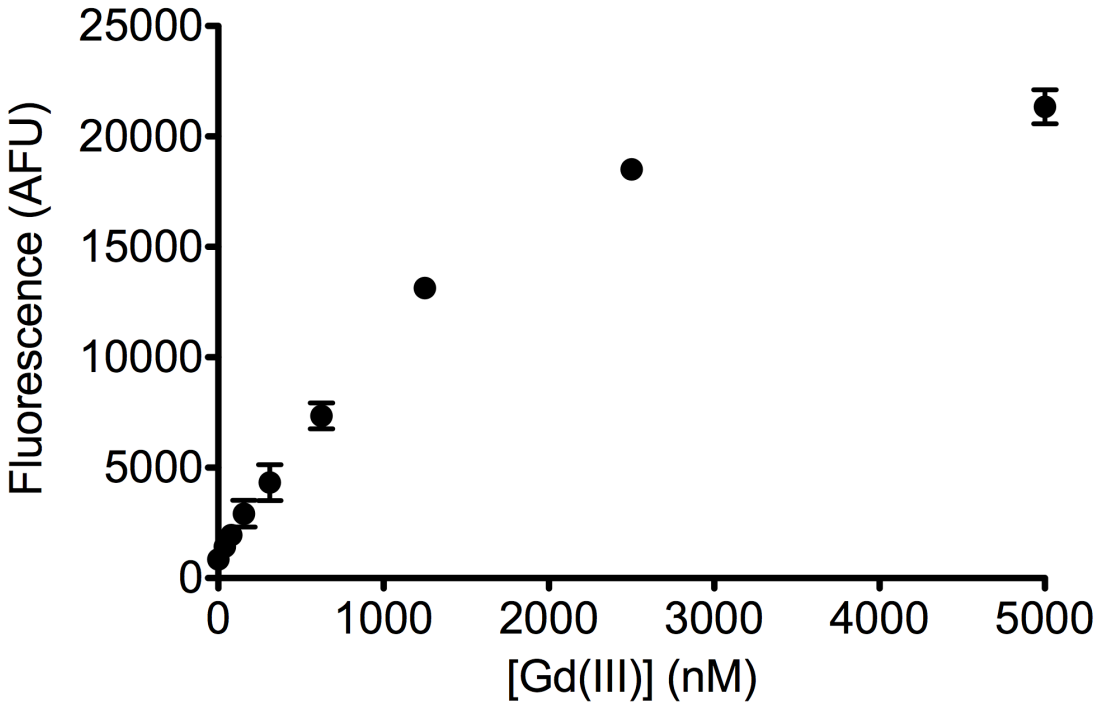


Figure 3

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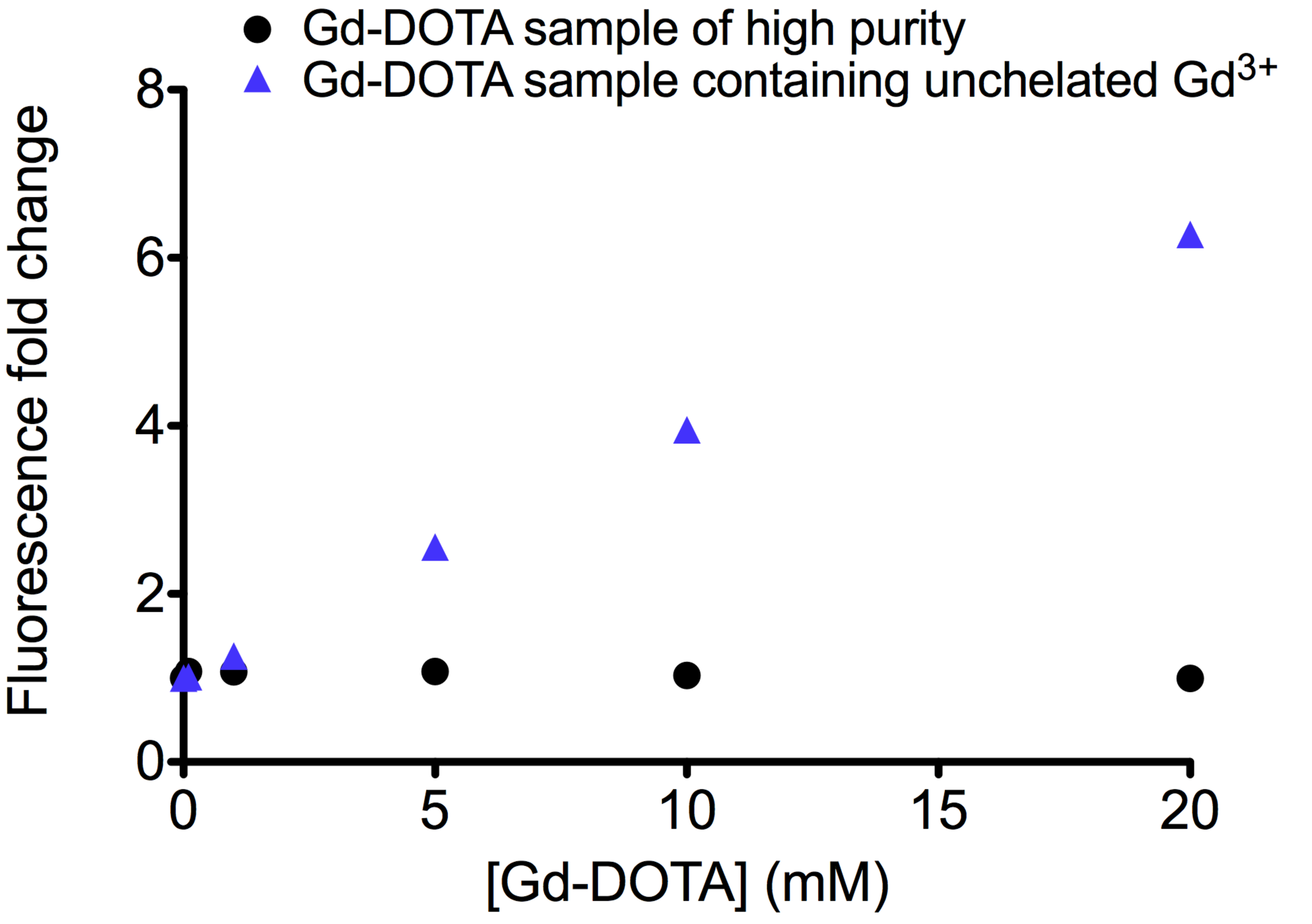

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagents/Materials	**Company**
Gd-aptamer	IDTDNA
Quenching strand	IDTDNA
Molecular biology grade water	
Gadolinium(III) chloride anhydrous	Strem
HEPES	Fisher Scientific
Magnesium chloride anhydrous	MP Biomedicals
Sodium Chloride	Acros Organics
Potassium chloride	Fisher Scientific
Sodium hydroxide, pellets	Fisher Scientific
Hydrochloric acid	Fisher Scientific
384-well low flange black flat bottom polystyrene NBS plates	Corning
Nalgene Rapid-Flow sterile disposable bottle top filter	Thermo Scientific
Disposable sterile bottles 250 mL	Corning
1.5 mL microcentrifuge tubes	
0.2 mL PCR tubes	
Micropipets	
Pipet tips (non filter) of appropriate sizes	

Name of Equipment	
Plate reader	Biotek Synergy H1

Catalog no.
Input sequence and fluorophore modification in the order form
Input sequence and quencher modification in the order form

936416
BP310-500
0520984480 - 100 g
327300025
P333-500
BP359
SA49
3575
5680020
430281

Comments

A fluorophore with a different emission wavelength may be used. The aptamer may also be ordered fr
A different quencher for optimal energy transfer from the fluorophore may be used. The aptamer may
No specific manufacturer, both DEPC or non-DEPC treated work equally well
Toxic

Corrosive
Toxic and corrosive
Plates which are suitable for fluorescence reading are required.
The bottle top is fitted with 0.2 micron PES membrane
A larger or smaller bottle may be used
No specific manufacturer, as long as they are DNAse and RNAse-free
No specific manufacturer, as long as they are DNAse and RNAse-free
No specific manufacturer
No specific manufacturer, as long as they are DNAse and RNAse-free

Plate readers from other manufacturers would work equally well

ɔm another company.
also be ordered from another company.

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Title of Article: An aptamer-based sensor for aqueous unchelated Gadolinium (III).

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Article Title: An aptamer-based sensor for aqueous unchelated Gadolinium (III)

Signature: Marlin Halim Date: 07/05/16

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August 25th, 2016

Dear Dr. Nguyen,

We are submitting the revised version of our manuscript: 55216_R0_071216.
All the changes made are tracked in the manuscript in red color. We are also submitting a new Figure 2 where we changed the figure part labels in lower case (a and b) to upper case 'A' and 'B', and a new Figure 3 where "GdDOTA" (in the original figure) in the axis title and legend has been changed to "Gd-DOTA" (in the new figure). In addition, please find below our response to the editorial and reviewers' comments and we would be happy to address any additional comments.

Thank you very much for your time.

Editorial comments: The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55216_R0_071216.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please include the DOIs for all references.
3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
4. Please correct the grammar regarding article usage. In particular, "Gd-sensor" should often be "the Gd-sensor". Such editing is required prior to acceptance.
5. Results: Please expand on the results. Two sentences is insufficient. What is the interpretation of the data presented? This can be moved from the figure legends, which should just be brief descriptions of what data is shown.

Our response: we have re-structured a few sentences and made all the necessary changes and corrections to comply with all the suggestions above. We have expanded on the result section. In addition, we have also added the issue numbers for the references.

Reviewers' comments: Reviewer #1:
Manuscript Summary: The overall manuscript clearly outlined the methodology for using an aptamer previously identified by the PI's laboratory in a fluorescence assay for unchelated Gd(III). While the approach is not overly novel, the method described in sufficient detail for researchers interested in utilizing this approach for Gd ion analysis, or similar analyses with aptamers for other target molecules. The anticipated results are reasonable and consistent with the detailed experimental description. As a whole, the manuscript is appropriate for publication in this journal.

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Major Concerns: None

Minor Concerns:

1. The primary concerns in the manuscript involve a variety of minor grammatical or formatting issues that occur with a high enough frequency to be distracting. These include unnecessary capitalization of element names (e.g. Sodium, Potassium, and Calcium, in lines 60-61) and spelled-out acronyms (Inductively Coupled Plasma-Mass Spectrometry in line 85 and SELEX in line 94), as well as unusual placement of superscripted reference numbers before the periods at the ends of sentences.

Our response: we have made all the necessary corrections on the capitalization of words. However, the unusual placement of superscripted reference numbers is in compliance with JoVE's formatting requirement, and therefore, we have made no changes to the location of the superscripts.

2. In the Protocol section, the details for emission wavelength are unnecessarily confusing (step 3.7 and subsequent references). Presumably, the emission maximum of the fluorophore in 528 nm, but the authors are noting that plate readers with less precise wavelength selection might need to just pick the wavelength rounded to the nearest multiple of 5 nm (although its not clear why they picked 525 nm vs. 530 nm). This issue probably warrants a brief clarification and rationale within the protocol.

Our response: we have clarified this fact in step 3.9 in the new manuscript.

Additional Comments to Authors: N/A

Reviewer #2:

Manuscript Summary: The manuscript describes the detailed procedure for quantitative detection of unchelated gadolinium (III) (Gd) ions in aqueous solution to address a safety issue caused by unchelated ions in GBCAs. The original research paper was published in Analytical and Bioanalytical Chemistry, which described the selection of DNA aptamers targeting Gd. Though the signal-on mode to detect target by combining fluorescently labeled aptamer and a quencher has been reported previously by other groups, the selection of Gd aptamer is novel and the assay described here is of applicable significance. But before the manuscript is considered for publication, I have a few comments below:

Major Concerns:

1. The construction of the fluorescence calibration curve and the detection of unchelated Gd3+ should be carried out simultaneously, not separately, to ensure the comparability of data between the calibration curve assay and detection assay.

Our response: we have re-formatted the protocol section, combining steps 3 and 4 in the original manuscript into one step (step 3 in the new manuscript).

2. As the authors stated, at above ~10 uM of Gd3+, fluorescence emission of the aptamer plateaus and it eventually decreases at even higher concentrations of the ion. The protocol suggested a preparation of 2 or 3 different concentrations of the sample (line 203). What about if the values being detected fall on the decreasing side of the peak? Will this result in a misinterpretation of the result? E.g mistakenly determining the ion at a lower concentration by assuming the dots fall on

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the increasing side of the peak? Should more dilutions of the sample be prepared? Or, is it very unlikely that the concentration of unchelated Gd in GBCAs will fall on the decreasing side of the peak?

Our response: we have included a note to guide the readers on how to avoid preparing solutions that may contain concentrations of Gd^{3+} which may be too high. This note is added under step 3.3 in the new manuscript. We have also made all the necessary changes to the protocol to incorporate this suggestion.

Minor Concerns:

1. Line 121, the company where the aptamer was ordered should be specified.

Our response: we did not make the changes as suggested by the reviewer. The original manuscript submitted to JoVE listed the company in the protocol, the company name was subsequently deleted from the protocol by the JoVE editor. Therefore, we decide to retain the changes made by the JoVE editor and not follow the reviewer's suggestion.

2. Line 130, "….water (molecular biology grade)" is repetitive as it has been mentioned in line 115.
3. Line 184, what is the incubation temperature? I assume it's room temperature.

Our response: we have incorporated the response to suggestions 2 and 3 above in the new manuscript.

Additional Comments to Authors: N/A

Yours truly,

Marlin Halim
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California State University East Bay