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Enzyme-linked oligonucleotide hybridization assay for direct oligo measurement in blood

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Abstract

Small oligonucleotides (oligos) are increasingly being utilized as diagnostics or treatments for disease. An impediment to broader use is the ability to readily measure oligos in biological fluids. Here, we describe a very straightforward assay with detection in the sub-picomole range that does not require extraction from serum/plasma or polymerization chain reaction amplification. As a result, there are no losses or errors due to sample handling, and the assay can be used to measure oligos modified in a variety of ways that increase therapeutic efficacy. The enzyme-linked oligonucleotide hybridization assay (ELOHA) is based on competition with a detection oligo for hybridization to a capture oligo covalently linked to a solid substrate. The versatility of ELOHAs is demonstrated by application to the measurement of three oligos, including two morpholino-oligos with 3′-octaguanidine derivatization for efficient cell uptake. The third oligo is unmodified and has a DNA sequence equivalent to miR93. The assays have sensitivity as low as 0.28 pmol/sample reaction at 50% hybridization. Adding to clinical utility is the need for only a simple 96-well absorbance plate reader and the finding that neither EDTA nor heparin interferes with detection.

Keywords: oligonucleotide therapy; assay in serum; sub-picomole; assay with blocked 3′

Introduction

Oligo therapeutics, including splice-modulating oligonucleotides (oligos), short interfering RNAs (siRNAs), oligos antisense to microRNAs (anti-miRs) and short hairpin RNAs, are increasingly being used or proposed to treat a variety of diseases [1–3]. In addition, miRNA signatures are being employed as biomarkers in the diagnosis of cardiovascular diseases [4, 5], Alzheimer's disease [6] and ovarian, breast, prostate, lung and other types of cancers [7, 8]. Thus, the need for efficient methods to detect these oligos is of utmost importance in both research and clinical applications. Current methods such as capillary electrophoresis [9], electrospray mass spectrometry [10] or quantitative PCR [11] involve extraction from the biological fluid and often cumbersome, complicated detection methods with inherent quality control and reproducibility issues. In addition, PCR-based methods cannot be used if the 3′-OH is blocked such as is the case with morpholino-oligos [12]. Taking advantage of the specificity of oligo hybridization, others have developed hybridization assays. Each has specific advantages and disadvantages in terms of sample manipulation, cost, the need for specialized equipment and sensitivity [13–21]. By way of example, Wei *et al.* [20] describe an assay that has good sensitivity and specificity for pharmacokinetic analyses, but require incubation of each sample in S1 nuclease. This increases the potential for some loss of sample since S1 nuclease can cause double-strand breaks, and adds to sample manipulation and cost. As another example, Vargas *et al.* [21] have developed an extremely

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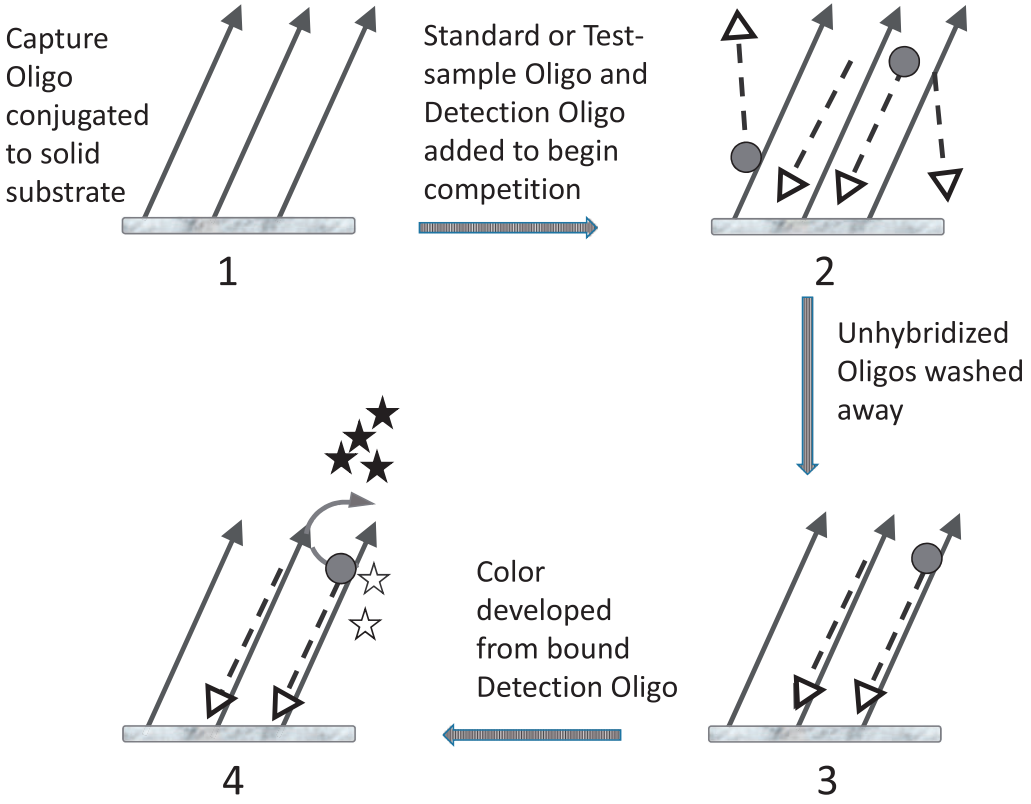

Figure 1: Diagram of the assay. (1) The capture oligos (arrows to indicate 5′–3′ directionality) are covalently linked to a DNA-bind plate, followed by standard blocking. (2) Competition between the test sample or standard oligo (dashed arrows with open heads) and detection oligo (dashed arrows with open heads plus gray ovoids to indicate HRP or other detection moiety). Note the random orientation before hybridization. If increased sensitivity is desired, then addition of the detection oligo can be delayed to provide a hybridization advantage to the test sample or standard oligo. (3) After removal of unhybridized oligos. (4) Color development and amplification of the detection moiety by conversion of the enzyme substrate (open star) to product (black star).

sensitive assay capable of measuring 5 fmol in 25 µl of sample, but one that requires both antibody amplification and specialized equipment to detect amperometric transduction at screen-printed carbon electrodes.

Although we are accustomed to thinking about specificity of oligo hybridization under the conditions developed for PCR, the breakthrough in our thinking about development of the current assay occurred with the realization that small oligos function specifically *in vivo* and thus, physiological conditions should support a specific hybridization assay. Here, we describe a competitive enzyme-linked oligonucleotide hybridization assay (ELOHA) for the quantification of sub-picomole amounts of oligos directly in biological fluids without prior extraction and at physiologic temperatures. The simple, sensitive assay is applicable to monitoring drug delivery, pharmacokinetics and diagnostic, as well as therapeutic responses. The basic principles are shown in the graphical abstract and Fig. 1.

Methods

To show versatility of the method, ELOHAs were developed for three different oligos. Two were morpholino oligos with octaguanidine derivatization. Morpholino oligos are often used to enhance half-life in serum [22], and octaguanidine derivatization results in tissue and cell penetration [22]. This combination is called a vivo-morpholino™ (Gene Tools, Philomath, OR, USA). The third oligo was a 23-deoxynucleotide sequence equivalent to miR93 (BioSynthesis, Lewisville, TX, USA). Following is the stepwise procedure developed:

Preparation of the solid-state capture system

Step 1, Fig. 1: capture oligos are antisense to the Test Oligos being measured. Capture oligos were synthesized with a 12-carbon (–CH_2–) aliphatic spacer at the 5′-amino terminus, followed by a 3-base poly T extension, then the anti-sense oligo and then a final 5-base poly T sequence (BioSynthesis, Lewisville, TX, USA). Immobilization of the capture oligo on 96-well polystyrene DNA-binding plates (Corning, Corning, NY, USA) occurred by an amination reaction of the 5′-amino group and the N-oxysuccinimide groups on the plate to produce: Plate-aliphatic spacer–C(=O)–NH–$(CH_2)_{12}$–TTT–Oligo(antisense to Test)–TTTTT. The aliphatic carbon spacer and poly T extensions minimized steric hindrance of hybridization. For the sensitivity described, we used capture oligos at 2 pmol/well, unless otherwise noted, incubated at 4°C overnight or longer in 100 µl 0.05 M phosphate (pH 8.5)—1 mM EDTA. The covalent binding reaction was stopped by washing three times with 200 µl/well 0.05 M Tris–HCl (pH 8)—1 mM EDTA (Wash Buffer), each with shaking for 5 min prior to buffer removal.

Following covalent binding of the capture oligo to the plate, nonspecific binding was minimized in a standard fashion by incubation at 37°C for 2–4 h with 3% bovine serum albumin (BSA)—0.05 M Tris–HCl (pH 8)—1 mM EDTA (200 µl/well). Excess BSA was removed by washing three times with wash buffer.

Competition and hybridization to capture oligos

Steps 2 and 3, Fig. 1: The test sample or standard concentrations of the oligo being assayed and constant concentrations of

competing detection oligos were incubated with the now solid-phase capture oligos. The detection oligos were the same oligo sequences (without morpholino or octaguanidine derivatization) as the oligos to be measured, but with the sequence extended to include five base poly As at the 5′-end, three base poly As at the 3′-end plus horseradish peroxidase (HRP) or biotin conjugated to the poly A 5′-end (i.e. HRP- or Biotin–AAAAA–Oligo–AAA) (BioSynthesis, Lewisville, TX, USA). Competition and hybridization were carried out overnight in 0.05 M Tris–HCl (pH 7.5)—1 mM EDTA at 37°C in a dark incubator with or without serum or plasma. The poly As were incorporated to reduce unwanted tertiary structures of the oligos, to provide some steric freedom from the plate and/or the detection moiety and to stabilize binding of the detection oligo. The binding of the poly As to the poly Ts of the capture oligo did not result in nonspecific hybridization, as will be demonstrated. All components were prepared in buffer and added together unless the effect of delayed addition of detection oligo was being studied. Although the volume of each component can be varied depending on the needs of the experiment, the total incubation volume for all data presented was 100 µl. Routinely, incubations included: (i) 50 µl test sample Oligo at variable concentrations up to 2 pmol/50 µl competing with (ii) a constant concentration of detection oligo (0.2–1 pmol in different assays/25 µl); and (iii) buffer and/or serum or plasma to a final volume of 100 µl. If test samples in serum or plasma were assayed, then serum (Atlanta Biologicals, Flowery Branch, GA, USA) or plasma (prepared in the laboratory) was added to a final constant concentration in all standards and samples; the amount ranged from 10 to 25 µl/100 µl.

If HRP detection oligos were used, then the plate was washed three times with 200 µl 0.01 M Tris–HCl (pH 7.5)—0.1 M NaCl—0.1% Tween (TBST) to remove unbound test sample oligos and detection oligos. An additional step was required if biotin-detection oligos were utilized. With the latter, the plate was washed three times with TBST, incubated for 30 min at room temperature with 100 µl 10 nM streptavidin-conjugated HRP in TBST/well (AAT Bioquest, Sunnyvale, CA, USA), followed by a series of three more washes with TBST.

Detection of hybridization

Step 4, Fig. 1: The bound Detection Oligo was quantified using freshly prepared 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma Aldrich, St Louis, MO, USA)—1% stable peroxide (100 µl/well; Thermoscientific, Rockford, IL, USA). After color development, reactions were stopped with 50 µl 1 N H_2SO_4/well and the yellow product quantified after 5 min by absorbance at 450 nm (Finstruments 347 Plate Reader).

Serum enhancement

In experiments studying serum enhancement of sensitivity, the potential effect of two helicases found in serum, Mre11 and RAD52 [23] was examined by preincubation of mouse serum (2 h at 37°C) with antibodies against Mre11 (GTX70212; Gene Tex, Irvine, CA, USA) and/or RAD52 (B1119; Assay BioTechnology, Sunnyvale, CA, USA). A second approach utilized preloaded antibodies on Protein A/G beads (5 µl antibody/20 µl Protein A/G beads incubated for 2 h 4°C) and immunoprecipitation of the enzymes from serum. Additionally, the possible influence of serum cyclophilins on hybridization was tested by preincubation of serum for 30 min at 37°C with the cyclophilin inhibitor, cyclosporin A [24] (0–66 µg/ml serum; Sigma Aldrich, St Louis, MO, USA).

In vivo application

Three-month-old Balb/c, male mice were anesthetized with halothane, and 50 nmol test sample oligo in 100 µl phosphate-buffered saline was injected via the tail vein. Approximately, 50 µl blood was removed from the tail and collected in heparin-treated tubes at 30 and 150 min. All animal procedures were approved by the University of California, Riverside, Institutional Animal Care and Use Committee.

Presentation of results

Most results are presented as the mean absorbance at 450 nm (A450) ± SD after subtraction of the reagent blank, with a minimum of triplicate samples. Multiple conditions were explored, with approximately 50 assays performed. Specific experimental conditions were repeated at least twice if the conclusion was that there was no effect. All other experimental conditions, including results presented, were repeated on at least three separate occasions. Note that for many of the graphs, the errors were so small that they are obscured by the size of the symbols. In each assay, all standards and samples were in triplicate and a minimum of four concentrations was used for the standard curve. For the mouse antimaia assay, the intra-assay coefficient of variation was 8.4% and the coefficient of interassay variation was 13.6%. For the DNA sequence equivalent to miR93, the coefficient of intra-assay variation was 12.9% and that of interassay variation was 16.4%. For the control SMO, the coefficient of intra-assay variation was 9.1% and that of interassay variation was 15.1%. Data on serum spiking and other measures of assay characterization are presented under Results section.

Data availability

All supporting data, including negative data not presented, are available from the authors.

Results

The first ELOHA designed was an assay for a 25-nucleotide splice-modulating oligomer that specifically knocks down the long form of the prolactin receptor. This splice-modulating oligomer has potent anti-metastatic activity [25] in two mouse models of breast cancer and is being evaluated for its clinical potential. We have named it antimaia because of its anti-cancer activity and used that name herein. Antimaia is a morpholino oligo with 3′-octaguanidine derivatization: it is a vivo-morpholino™. An ELOHA was developed for mouse antimaia (i.e., targeted against the mouse long form PRL receptor) so that we could examine potential toxicities in an homologous system; the sequence of mouse antimaia is GCCCTTCTATTGAA ACACAGATACA. The second ELOHA was also designed for a vivo-morpholino™. Because it has no predicted biological activity per BLAST analysis, this oligo was constructed as the control for antimaia in the *in vivo* breast cancer experiments, and therefore is referred to herein as control oligo. In demonstrating specificity of the ELOHAs, we test the Control Oligo in the antimaia assay and vice versa. Therefore, it is important to stress that the word Control in this instance is not synonymous with nonspecificity in any of the three ELOHAs. The Control Oligo has the sequence AGACGAGATTCGATCGGAGTA. The third ELOHA was designed to measure an unmodified oligo with a DNA sequence equivalent to human miR93, CAAAGTGCTG TTCGTGCAGGTAG. In order not to be repetitive, we do not present all data for each ELOHA, but using the three ELOHAs

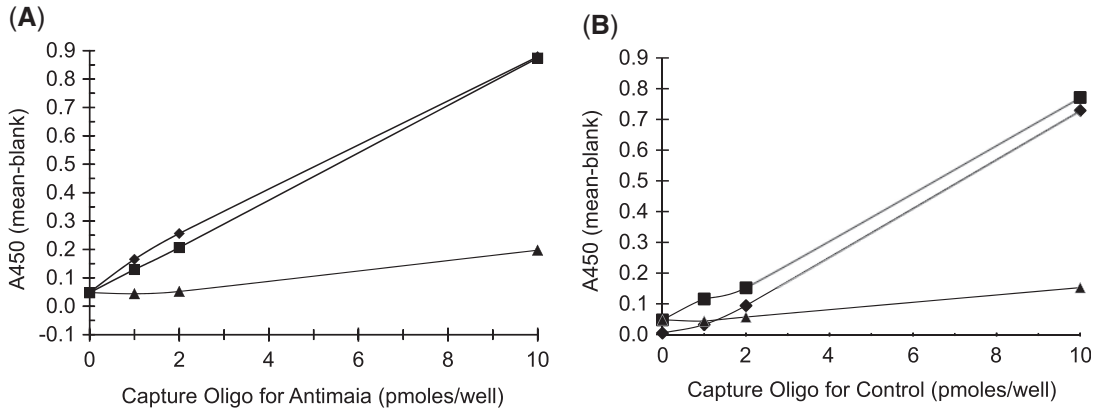

Figure 2: Hybridization detection is proportional to the amount of capture oligo per well, and specificity is dictated by the capture oligo. **(A)** Increased antimaia capture oligo results in a linear increase in absorbance due to hybridization of detection oligo, and the antimaia capture oligo is specific for detection of antimaia. Results are shown for hybridization of antimaia capture oligo (0–10 pmol/well) with antimaia HRP-detection oligo (0.2 pmol/well) alone (filled diamond), with addition of 5 pmol antimaia (filled triangle) or with 5 pmol control SMO (filled square). **(B)** Similar results are seen for detection of control oligo under similar conditions. Shown are results of hybridization of control capture oligo (0–10 pmol/well) with control HRP-detection oligo (0.2 pmol/well) alone (filled diamond), with the addition of 5 pmol control (filled triangle), or with 5 pmol antimaia (filled square). Results are expressed as the color development measured at A450 (mean − blank) ± SD; $n=3$. Note that the error bars are obscured beneath the symbols, as is true for Fig. 3, parts of Figs 4, 5A, 5B, 6 and 7.

generalize the results to demonstrate broad application of the method.

Preliminary experiments were carried out to confirm the prediction that there is a linear relationship between the amount of capture oligo on the plate and color development from the Detection Oligo. It was also important to establish that hybridization could be carried out under mild conditions (i.e., 37°C). The HRP-antimaia detection oligo has the same nucleotide sequence as antimaia, but, unlike antimaia, it is not a vivo-morpholino-modified oligo. Likewise, the HRP-control detection oligo has the same nucleotide sequence as the Control Oligo but is not a modified oligo. Figure 2A shows the linear relationship for the antimaia detection oligo and Fig. 2B for the control detection oligo (diamonds on both parts of the figure). The capture oligo was bound to the plate using 0, 1, 2 and 10 pmol/well followed by incubation for 12 h at 37°C with 0.2 pmol HRP-detection oligo/100 µl reaction. Figure 2A also shows the ability of antimaia to compete with the HRP-antimaia detection oligo for hybridization to the cognate antisense capture oligo for antimaia (triangles). In contrast, the control oligo had no ability to compete with the antimaia detection oligo (squares). Also, if control oligo was added together with antimaia and the antimaia detection oligo, it had no influence on the ability to measure antimaia (data not shown). Similarly, Fig. 2B shows the ability of control oligo to compete with the HRP-control detection oligo (triangles) and that antimaia does not compete in this assay (squares). In other words, the presence of a second oligo does not interfere with assay. Data in both Fig. 2A and B also confirm that the poly A sequences in detection oligos coincident with the poly T sequences in the capture oligos were not sufficient to result in nonspecific hybridization. Thus, these assays are specific to the antisense capture oligo used.

Assay optimization is crucial to development of the required sensitivity and appropriate range. For example, Fig. 3 shows the importance of titrating the detection oligo by illustrating the effect of increasing the concentration of HRP-detection oligo in the control oligo studies to 5-fold that used for Fig. 2B (i.e. to 1 pmol/reaction) holding all other conditions the same. With increased HRP-detection oligo, maximal absorbance is achieved too rapidly (diamonds) for the development of an effective assay. Nevertheless, specificity was maintained since competition

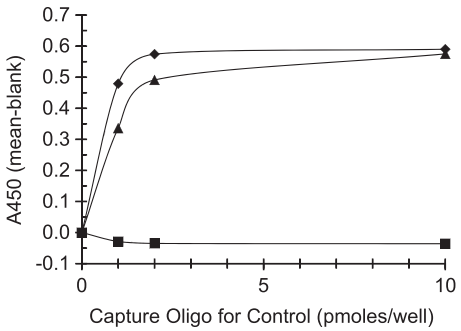

Figure 3: The sensitivity of the hybridization detection is dependent on the concentration of detection oligo. In contrast to Fig. 2B, hybridization was carried out with control HRP-detection oligo at 1 pmol/well alone (filled diamond) or together with the addition of 5 pmol control oligo (filled square) or 5 pmol antimaia (filled triangle). Results are depicted as the A450 (mean − blank) ± SD; $n=3$.

was only achieved with control oligo (squares) and not antimaia (triangles).

Having thus established that specificity is dictated by the antisense capture oligos, and that an appropriate concentration of capture and detection oligos needs to be established, ELOHAs were developed in which detection and capture oligos were constant. The concentration of a Test Oligo could then be quantified by comparison to an established Standard Oligo curve. This is first illustrated with the ELOHA for the third oligo tested, the DNA sequence mimicking miR93 (Fig. 4). Since the concentrations of both Capture (2 pmol/well) and Detection (0.2 pmol) Oligos were constant, increasing concentrations of miR93 Oligo resulted in less hybridization of Detection Oligo to the capture oligo and therefore less color development. A clear concentration relatedness is apparent in the 0.1–1.0 pmol range. A biotin-tagged Detection Oligo was used in this instance.

To optimize each ELOHA, concentrations of capture oligos and detection oligos were titrated to determine conditions for greatest sensitivity. For example, using 2 pmol/well antimaia capture oligo and 0.5 pmol/well HRP-antimaia detection oligo, the competition assay for antimaia was linear, and extremely sensitive ($y=0.37\ \mathrm{Ln}(x)+503$, $R=0.997$) (Fig. 5A). Competition was readily apparent with antimaia amounts >0.2 pmol, and

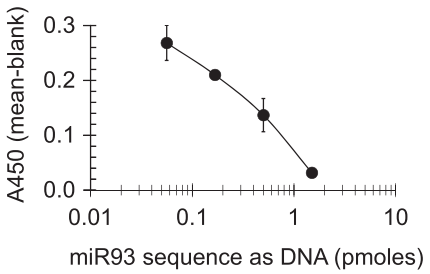

Figure 4: An ELOHA for a deoxynucleotide with a sequence equivalent to miR93 was developed. The miR93 capture oligo was constant at 2 pmol/well, and a biotin-detection oligo at 0.2 pmol/well was utilized. Color development results are given as the A450 (mean − blank) ± SD, $n=3$.

the concentration of antimaia resulting in 50% maximal color development ([Antimaia]$_{50}$) was 0.62 pmol.

Sensitivity of the assay could be increased further if addition of antimaia detection oligo was postponed until after antimaia addition. For example, using 2 pmol/well antimaia capture oligo and adding 0.3 pmol HRP-antimaia detection oligo at the same time as antimaia (0 time) or with a delay of 5 or 15.5 h (total reaction time held constant at 20 h), dose response curves were shifted to the left as delays in addition of detection oligo increased (Fig. 5B). For example, the [Antimaia]$_{50}$ was 0.72, 0.44 and 0.28 pmol for delays of 0, 5 and 15.5 h, respectively. Results are presented as a percent of hybridization in the absence of antimaia to allow a direct comparison since delays produced different color development ranges.

Although antimaia did not cross react in the control oligo ELOHA and vice versa, these two oligos maximally have 3 nucleotide stretches in common. We therefore tested the antimaia sequence specific to the human long form prolactin receptor in the mouse antimaia ELOHA. In a mouse xenograft model, the human antimaia is specific to the human receptor [25]. Human antimaia only differs from mouse antimaia by two bases (A for G and C for T) that are nine bases apart. It has the following sequence and the two different bases are emphasized: GCCCTTCTATT**A**AAACACAGA**C**ACA. The mouse antimaia ELOHA did recognize human Antimaia at high concentrations, but not within the desired working range (Fig. 6).

Since EDTA was in the hybridization buffer, we knew that plasma samples could be assayed if they were prepared with EDTA. However, we questioned whether heparin-prepared samples could also be utilized. No effect on the antimaia standard curve was observed when 1.0 µl heparin (1000 mU/µl) was included in the wells (data not shown). This amount approximates a 50-fold excess compared with what would likely be present in a clinical sample.

It should be noted that serum was included in the studies shown in Fig. 5 to more closely reflect desired experimental/clinical assay conditions. Since inclusion of serum or plasma often interferes in assays, we examined the effect of serum and plasma on the ELOHAs. Figure 7 shows the effect on the antimaia ELOHA. Conditions included 1.0 pmol/well capture oligo and 0.25 pmol HRP-detection oligo. As shown in Fig. 7, in the absence of serum and using the scale shown, it is hard to discern a decrease in color development with increasing concentrations of antimaia (circles under the crosses). The relatively small amount of color development was due to the low amount of capture oligo used in these experiments in order to fully illustrate the effect of serum. When serum was added, a very dramatic increase in color development occurred. With no competing antimaia (0 antimaia), color development from the detection oligo increased >20-fold with 10 µl mouse serum/100 µl reaction (squares) and as much as 125-fold with 25 µl serum/100 µl reaction (triangles). Even at 1 pmol competing antimaia, 25 µl serum resulted in ~50-fold increase. Results also demonstrate that BSA could not substitute for serum, the data being superimposable on the 'without serum' results (crosses, which overlay the circles). In further investigations, we found: (i) the dose–response for serum indicated little effect <1.0/100 µl reaction and a maximal response at 15/100 µl reaction; (ii) plasma had the same effect as serum; (iii) enhancement was not altered if serum was heated for 15 min at 65°C; and (iv) human, rat, chicken, horse and fetal bovine serum all increased sensitivity of the assay. Furthermore, serum had no influence on color development (i) in the absence of capture oligo; (ii) in the absence of Detection Oligo; (iii) if added after hybridization, but prior to color development; or (iv) as described later, if a biotin-detection oligo was used.

A possible explanation for increased assay sensitivity with serum was that a factor in serum catalyzed a conformational change of components, allowing better hybridization. Thus, for example, annealing helicases [23] could be responsible for dramatically aiding the hybridization reaction. Antibodies against Mre11 or Rad52 had no effect on serum enhancement, whether the serum was preincubated with the antibodies or whether the antibodies were first bound to Protein A/G beads prior to incubation with serum. Secondly, we questioned whether peptidyl prolyl isomerases (e.g., cyclophilins) detected in blood [26–28] altered conformation of a protein that directly or indirectly enhanced hybridization. We found no difference in assay sensitivity when serum was preincubated with cyclosporin A.

In attempting to determine what assay component was being altered by serum, we preincubated each component with serum for 18 h prior to assay. Only preincubation of the HRP-detection oligo with serum resulted in increased absorbance in the assay. To explore this result, further analyses were performed in which a biotin-detection oligo for antimaia was directly compared with the HRP-detection oligo for antimaia in the absence and presence of serum. Figure 8 illustrates that the results with the biotin-detection oligo without serum (open triangles) were similar to those with HRP-detection oligo with serum (closed circles). Additionally, if biotin-Detection Oligo was utilized, serum had no effect on the sensitivity (open circles vs open triangles) in contrast to the dramatic differences without and with serum using the HRP-detection oligos (closed triangles vs closed circles). This experiment also illustrates that there was 100 ± 3% recovery when serum/plasma was 'spiked' with antimaia between 0.25 and 1 pmol/15 µl.

To summarize this section on serum/plasma: (i) serum/plasma was not required for the desired sensitivity unless an HRP conjugate was used and (ii) serum/plasma had no negative effects, including potential interference from the multiple oligos present, on ELOHAs with either detection oligo conjugate.

The antimaia ELOHA was used to determine the rapidity of cellular uptake of antimaia when delivered intravenously. Despite injecting 50 nmol antimaia, we expected values to be low in the assay since we were assaying small volumes of plasma and anticipated rapid uptake from the blood into tissues due to the octaguanidine derivatization of the antimaia. Thus, these studies were also expected to test the detection sensitivity. Plasma was collected at 30 and 150 min, and 1–2.5 µl plasma were assayed using 2 pmol/well capture oligo, 0.25 pmol HRP-detection oligo/100 µl reaction and, using supplementation with a commercial source of serum/plasma, a total of 15 µl mouse plasma/serum/100 µl reaction in all samples and standards. As

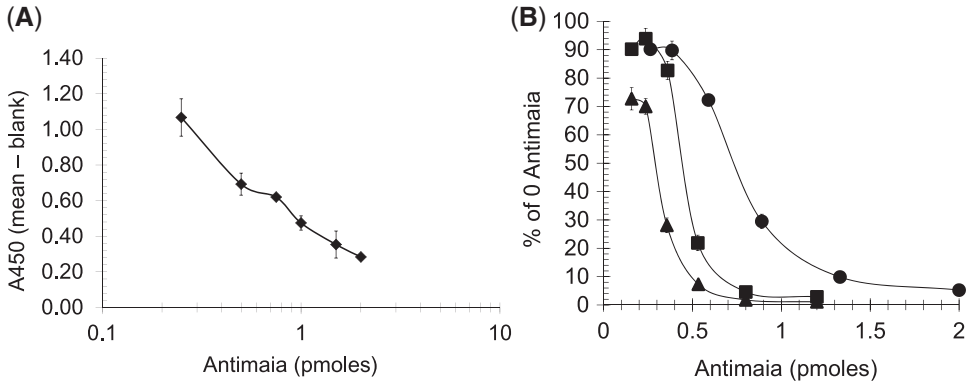

Figure 5: (A) ELOHAs are sensitive below one pmole. With coincident addition of test sample and HRP-detection oligos, the $[Antimaia]_{50}$ was 0.62. Conditions included 25 µl serum/100 µl reaction, and results shown are A450 values (mean − blank) ± SD; $n = 3$. **(B)** With delayed addition of detection oligo, there is increased sensitivity. During a 20 h hybridization time, HRP-detection oligo was added at 0 time (filled circle), after 5 h (filled square) or after 15.5 h (filled triangle). Conditions included 15 µl serum/100 µl reaction, and values are the percentage of no antimaia addition (mean ± SD) for triplicate incubations. The $[Antimaia]_{50}$ was 0.72, 0.44 and 0.28 for delays of 0, 5 and 15.5 h, respectively; $n = 3$.

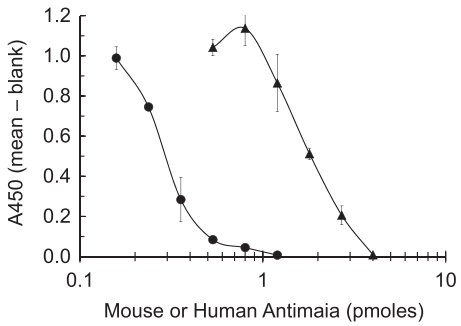

Figure 6: Specificity assessed by comparing mouse and human antimaia. Mouse (filled circle) and human antimaia (filled triangle) vary by 2 nucleotides. When comparing them in the mouse antimaia assay, human antimaia (filled triangle) was recognized, but at much higher concentrations outside of the desired range of the mouse antimaia assay.

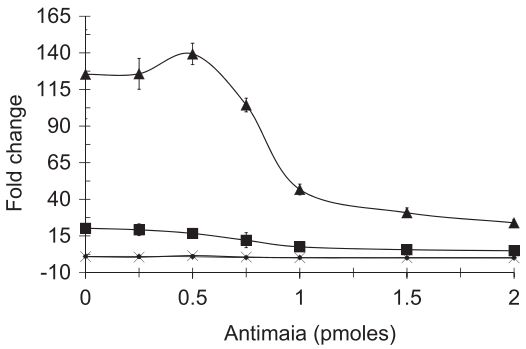

Figure 7: Mouse serum, but not BSA, dramatically increases sensitivity when using HRP detection. Incubations were without mouse serum (filled circle), with 10 µl (filled square) or 25 µl (filled triangle) serum, or with 1% BSA (X) to test whether this would have the same enhancing effect as serum. Results are given as the mean ± SD for triplicate incubations and are expressed as the change in hybridization of HRP oligo in the absence of both serum and the test sample oligo; $n = 3$.

expected, antimaia rapidly disappeared from the circulation, with only 1.3, 1.2 and 0.45 pmol/ml detected after 30 min in the three animals tested. Assuming a mouse blood volume of 2 ml, this would be equivalent to 5.2, 4.8 and 1.8% of the 50 nmol antimaia injected. Antimaia levels continued to fall, and after 150 min, antimaia was only 0.5, 0.71 and 0.3 pmol/ml (2, 2.8 and 1.2%), respectively. Upon repeat of the assay of these samples, using a protocol including a delay of 15 h before addition of the detection oligo, the results from the *in vivo* analysis were within 4.2% of the original determination.

Discussion

To date, methods for measuring oligos in serum involve extraction from serum, PCR amplification, expensive reagents, complex instrumentation or are insufficiently sensitive. ELOHAs have significant advantages over any method that requires plasma/serum extraction since with ELOHAs there are no losses during processing, reduced errors and reduced costs. In addition, since there currently is no standardization of methods for oligo isolation [29], greater uniformity among laboratories would occur if preanalytical processing was unnecessary.

In addition to serum extraction, methods for oligo assay involving PCR also require a free 3′-OH group on the oligo being detected. Many administered oligos are morpholino oligos. Because of their completely unnatural backbones, morpholino oligos have fewer off-target effects, reduced degradation and increased half-life and efficacy [30–32]. Unlike many unmodified oligos, morpholinos also do not activate toll-like receptors and so do not activate innate immune responses [30–32]. They are therefore superior molecules for therapeutic purposes. However, morpholino oligos have a blocked 3′-end and hence cannot be measured by PCR.

Many oligo therapies also require some mechanism to enhance cell penetration in order to be effective at reasonable doses. These include 3′ octaguanidine derivatization as is the case for antimaia and the control oligo [22], or 3′ cell-penetrating peptides [33]. Our results show measurements of morpholino oligos with octaguanidine derivitization by ELOHA and we expect this technique will be effective for measuring a standard morpholino oligo without an octaguanidine moiety. Other 3′ modifications eliminating detection by a PCR-requiring

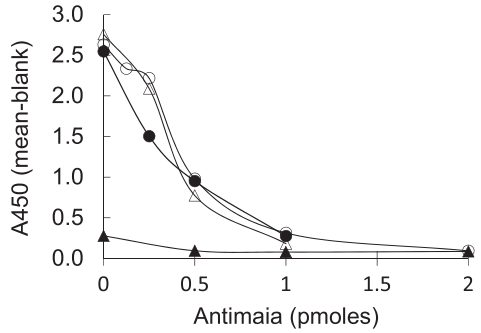

Figure 8: Comparison of antimaia ELOHA using HRP- or biotin-detection oligos in the presence or absence of serum. Hybridization to antimaia capture oligo (2 pmol/well) was carried out either with 0.25 pmol/well HRP-detection oligo with (filled circle) or without (filled triangle) mouse serum (15/100 μl reaction), or with 0.25 pmol/well biotin-Detection Oligo with (open circle) or without (open triangle) mouse serum. Antimaia concentrations tested were 2-fold serial dilutions from 2 to 0.06 pmol/50 μl, and results are given as the A450 (mean − blank) ± SD; $n=3$.

technique include 3′-ddC, 3′-spacer C3 or 3′ inverted end [34]. A final major advantage over methods involving both reverse transcription and PCR is that ELOHAs are not affected by heparin. Heparin interferes with reverse transcriptase and must be cleared with heparinase prior to amplification [29].

While in many oligo assays, the target must be amplified after extraction from serum to reach detectable levels, we found we could determine assay conditions to reach detection in the sub-picomole range without extraction. This included optimization of the amount of capture oligo and competing detection oligo, as well as delaying addition of the detection oligo, in the latter case thereby affording the test sample oligo (or standard oligo) an advantage in the competition. While the sensitivity is already excellent and entirely suitable for measurement of therapeutic oligos, some oligos used for diagnostic purposes are found in very small quantities in serum and may require fluorescent detection of HRP activity. Fluorescent detection of HRP activity on either the directly conjugated oligo or on streptavidin will increase sensitivity further and by as much as two orders of magnitude [35].

When present, serum/plasma either had no effect on ELOHAs, such as when a biotin-detection oligo was used, or amplified the sensitivity as much as 125-fold when HRP-detection oligos were utilized. Thus, ELOHAs can be used to detect oligos directly in experimental or patient blood samples and are suitable for pharmacokinetic analyses, diagnostic procedures and monitoring of treatment responses.

Although the lack of serum effect with biotin-tagged Detection Oligos removes the need to understand the serum effect on HRP-tagged detection oligos, the wide use of HRP-tagged entities in a variety of assays perhaps suggests that some wider benefit may derive from a short discussion here. What we can say is that the serum enhancement using an HRP-detection oligo was not due to a general protein-stabilizing influence since BSA did not have the same effect. Also, that despite our initial consideration, no evidence supports an effect of a serum component on conformation of the hybridizing oligos regions since, if so, one would have expected an effect of serum with biotin-detection oligos. Because serum markedly enhanced detection of the HRP-detection oligos in the absence of competing antimaia or control oligo, the enhancing effect was also not related to octaguanidine derivatization of these two oligos.

What then is the basis of serum enhancement when using HRP-Detection Oligos? Considering that HRP is ∼44 000 Da, whereas the molecular mass of biotin is 244 Da, and the oligos tested were in the 3000 Da range, one can envision the accessibility of the oligo for hybridization being very dependent upon the conformation of the HRP. An effect on conformation of the HRP or HRP–oligo junction is supported by the serum preincubation experiments in which only preincubation of the HRP-detection oligo and preincubation of no other component affected the assay. A variety of protein conformation-altering peptidyl prolyl isomerases [27, 28] exists in serum [26], the most abundant of which are the cyclophilins. Cyclosporin A [24] inhibits most of these but was ineffective at altering serum enhancement in our tests. This does not exclude the possibility that a cyclophilin unaffected by Cyclosporin A [28] or another peptidyl prolyl isomerase is responsible for altering conformation of the HRP to promote hybridization efficiency. While we have not identified the serum factor responsible for the enhancement when using an HRP-detection oligo, the determination that it is related to the HRP moiety will be of use to the scientific community for ELOHA design.

Oligo interactions occur *in vivo* and are specific. Thus, we reasoned that physiological conditions could support specificity *in vitro*. Using incubations at 37°C, we found specificity of the ELOHAs not only in terms of one oligo vs another, but also down to the level of a two-base difference in sequence, even under circumstances when the two bases were not adjacent. This replicated the experimentally observed specificity of human vs mouse antimaia *in vivo* for the human vs mouse long form prolactin receptor [25]. While the desired specificity for each ELOHA developed would require testing, just as one does, for example, for primers or siRNAs, this degree of specificity augurs well for the general utility of ELOHAs.

A final benefit is that ELOHAs require relatively inexpensive equipment, and the needed 96-well plate colorimetric reader is standard in clinical and research laboratories. ELOHAs can therefore be designed, optimized and readily commercialized for multiple research and clinical applications.

The motivation for development of the ELOHA method was the absence of a simple and sensitive assay capable of detecting small quantities of antimaia during development of this very effective anti-metastatic breast cancer therapeutic [25]. This was a particular challenge because of the morpholino backbone, the 3′ octaguanidine residues and the very rapid uptake from the circulation because of the octaguanidine derivatization, that is, the very aspects of the modifications to an oligo that makes an oligo an effective therapy.

In summary, ELOHAs are a simple, sensitive, specific, inexpensive assay method that may revolutionize assays of small oligos in serum and other fluids.

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Author contributions

A.M.W. conceived, and A.M.W. and M.Y.L developed the project; K-H.E.C. carried out all *in vivo* experiments; M.Y.L. designed and ran all other assays; and all authors were involved in the preparation of the manuscript.

Competing financial interests

The assay has been submitted for patent protection: (62/346, 492: Methods for detecting oligos in a sample).

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