Aptamer-antibody sandwich lateral flow test for rapid visual detection of tetrodotoxin in pufferfish

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Abstract

Tetrodotoxin (TTX) is a highly potent marine toxin which can cause severe poisoning after the consumption of contaminated fish and seafood. Thus, a sensitive, reliable and simple test is required for rapid screening of samples and prevention of intoxication. In our previous work we demonstrated the first aptamer-antibody sandwich assay for TTX detection in microtiter plates. Herein, we translated this assay into a rapid lateral flow assay (LFA) test with a signal-on format. The test relies on the aptamer immobilized on the membrane and the antibody conjugated with gold nanoparticles to provide a visual result as a red coloured line when TTX is present in the sample. The optimized test is simple (one-step), rapid (less than 20 min), highly sensitive (visual limit of detection of 0.3 ng/mL TTX in buffer), specific, reproducible and with long storage life. It was validated by analysing contaminated pufferfish tissue extracts and it successfully detected TTX way below the current limits set by official bodies. The analysis performed with this device in combination with a simple LFA reader for quantification was in excellent agreement with other established methods, further demonstrating the value of this test as a simple, low-cost and reliable analytical tool to ensure food safety and broaden the knowledge on the correlation between biological parameters and environmental data.

Keywords

tetrodotoxin, aptamer, antibody-aptamer sandwich, lateral flow assay, gold nanoparticles, pufferfish.

Synopsis

Competitive immunoassays are typically used to detect tetrodotoxin, but they are challenging to optimize, and results can be difficult to interpret as they are based on an inverse relationship between signal and target concentration. Here we demonstrate the first signal-on rapid test based on an aptamer-antibody sandwich for fast visual detection of tetrodotoxin in pufferfish.

Introduction

Tetrodotoxin (TTX) is a highly potent alkaloid marine toxin, and it mainly accumulates in pufferfish and other marine species. It blocks sodium voltage-gated channels, thus impairing nerve function, and can cause paralysis, respiratory failure, and even death.[1-3] Although TTX was assumed to occur only in tropical waters, in the last two decades it has emerged in temperate areas around the world, including the Mediterranean Sea. Since 2003, the toxic pufferfish species *Lagocephalus sceleratus* has been increasingly recorded in Mediterranean coasts, due to the Lessepsian migration through the Suez Canal, reaching not only eastern Mediterranean countries, such as Israel, Lebanon, Turkey, Cyprus and Greece, but also central Mediterranean countries, such as Italy, Croatia, Malta, Libya, Algeria and Tunisia, and even Spain in the western Mediterranean coast.[2] Additionally, in 2007, the first human TTX-poisoning in Europe was reported, attributed to the consumption of a trumpet shell *Charonia lampas lampas* from the south of Portugal but purchased in Spain. Since then, TTX has also been increasingly found in bivalve molluscs of European countries, such as United Kingdom, Greece, Netherlands, Portugal, Spain, Italy and France.[2]

A regulatory limit of 2 mg TTX equivalents (equiv.)/kg pufferfish has been established in Japan where pufferfish is traditionally consumed. In Europe, products deriving from fish of the Tetraodontidae family (including different pufferfish species) cannot be sold, and the European Food Safety Authority (EFSA) has concluded that an amount lower than 44 µg TTX equiv./kg shellfish meat is not anticipated to pose a risk to human health.[2,3] As TTX is thermostable, cooking does not destroy or eliminate it and intoxication cases after ingestion of contaminated food have been reported in the last two decades around the world.[3] Considering that doses of 4 - 42 µg TTX per kg body weight can cause acute poisoning in humans and that no antidote is available,[4] intoxications can be prevented mainly by limiting exposure to contaminated food. Therefore, sensitive, accurate and specific assays or tests which can be performed rapidly and in a user-friendly manner are required for the early detection of TTX.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is currently the most common and powerful technique for the detection and quantification of TTX and its analogues.[5-7] However, the complexity of operation, matrix interferences requiring extensive sample preparation, long analysis time, low throughput and high costs limit the wide practical application of this technique. Enzyme Linked Immunosorbent Assays (ELISA) and immunosensors are alternative tools which can provide accurate and sensitive quantification of TTX in samples at reduced cost and analysis time compared to LC-MS/MS.[3-13] However, these

multi-step assays are still dependent on equipment and trained professionals. Other methods used for TTX analysis include the mouse bioassay, cell-based assays (CBA), and receptor binding assays among others.[6,14,15] Overall, even though sensitive and accurate methods have been developed for TTX detection in fish and are widely exploited, several hurdles including cost, analysis time and complexity limit their application for in field screening.

Lateral flow assays (LFA) on the other hand can provide rapid and sensitive detection exploiting simple, user-friendly, and low-cost devices.[16-19] These devices are typically based on two biomolecule probes for target capture and detection: one of the probes is immobilized on the membrane, usually nitrocellulose, while the other one is conjugated with a reporter (e.g., gold nanoparticles) and deposited on the conjugate pad. The membrane is laminated with the sample, conjugate and absorbent pad on an adhesive backing card and cased within a plastic cassette. As the liquid sample containing the target analyte is applied to the device, it flows through the strip by capillary force. The target is recognized by the probe-reporter conjugate and the complex formed is detected by the probe at the test line where signal is generated (signal-on). A competitive format is adopted when only one probe is available and, in this case, the presence of target in the sample is demonstrated by a signal decrease at the test line (signal-off). This is the format employed most often for small molecules since their size hinders the binding of two probes simultaneously.[20,21] With regards to TTX, only a handful of LFAs have been reported to date. All of them employ a competitive format based on antibody conjugate with gold nanoparticles (AuNPs) or gold nanoflower (AuNFs) in combination with a TTX-protein conjugate at the test line. The presence of TTX in the sample is indicated by a signal decrease at the test line visible by naked eye.[22-27] However, there are two reports describing competitive fluorescent LFAs based on fluorescence quenching and signal generation in the presence of the target (signal-on format),[28,29] and this is the preferred approach in rapid tests, allowing easier interpretation of results. These assays unfortunately require a dedicated fluorescence lateral flow reader since they cannot be evaluated by visual inspection. To date, there are only four commercially available rapid tests for TTX (Table S1), and these are all based on competitive formats. In summary, while the development of all these tests is a step forward towards achieving rapid and facile detection of TTX, competitive immunoassays are more difficult to optimize, and the preparation of toxin-conjugates and result interpretation can also be challenging. Sandwich assays are more robust, with all reagents in excess, and due to this have longer shelf-lives, and often are more sensitive and specific.

Aptamers are single-stranded, synthetic oligonucleotides whose target biorecognition properties have rendered them popular for the detection of virtually any type of target.[30,31] Their increased stability, easier chemical synthesis, no batch-to-batch variation and lower cost compared to antibodies have encouraged their broad use in bioanalysis and particularly for small molecule detection.[32-36]

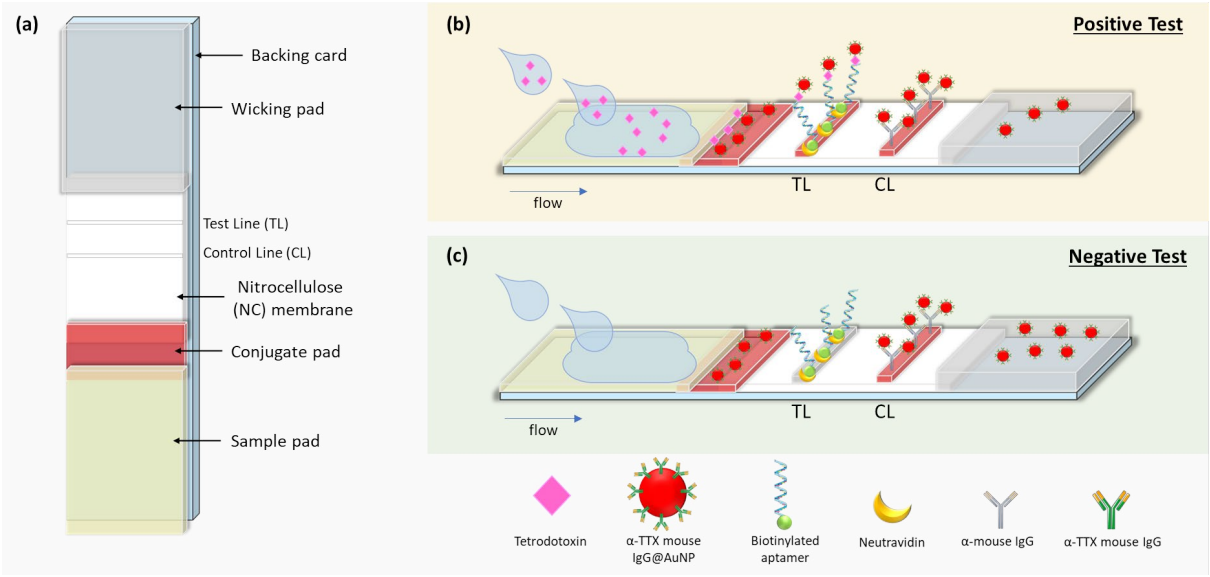


Figure 1. Schematic illustration of the TTX LFA tests. Design of the strips (a) and representation of the detection process in the presence (b) and absence (c) of TTX.

We recently selected high affinity aptamers against TTX and demonstrated that the unique cage-like structure of TTX facilitated the formation of an antibody-TTX-aptamer complex enabling the detection of TTX with a sandwich assay.[38] Here we report the implementation of the developed sandwich assay in a LFA format for the detection of TTX in pufferfish. The test is based on an α-TTX IgG@AuNPs conjugate and the TTX aptamer immobilized on the membrane as illustrated in Figure 1. In the presence of TTX in the sample, a sandwich is formed at the test line between the antibody, TTX and the aptamer and signal is generated. The test was optimized and characterized in terms of sensitivity, reproducibility, specificity, and stability. Finally, the optimized LFA was applied to the detection of TTX in extracts from contaminated pufferfish and compared to other techniques such as CBA and LC-MS/MS. To the best of our knowledge, this is the first example of a sandwich, signal-on rapid LFA test for TTX detection, as well as the first one to use an aptamer, and furthermore in a hybrid aptamer-antibody sandwich format.

Materials and Methods

Material and apparatus

Tetrodotoxin (TTX) of 98.8% purity was obtained from Latoxan (France), and 1 mg/mL TTX standard solutions were prepared in 0.1 M sodium acetate buffer pH 4.8. Saxitoxin (SXT) and domoic acid (DA) were obtained from the National Research Council of Canada (NRC, Halifax, Canada). Okadaic acid potassium salt (OA) from *Prorocentrum concavum*, goat α-mouse IgG antibody, skimmed milk powder, Empigen BB and streptavidin-HRP (SA-HRP) were from Merck (Spain). Mouse monoclonal α-TTX IgG antibody (CABT-L3089, CD Creative Diagnostics) was purchased from Deltaclon S.L. (Spain). The D3 TTX-binding aptamer[38] with a 5'-biotin was synthesized by Biomers.net (Germany). Neutravidin (NeuA), phosphate buffered saline tablets (PBS; 10 mM phosphate buffer pH 7.4, 137 mM NaCl, 2.7 mM KCl), assay buffer (PBS with 1.5 mM $MgCl_2$), PBST (PBS with 0.05% *v/v* Tween-20), carbonate-bicarbonate buffer (0.2 M, pH 9.4), bovine serum albumin (BSA) and sucrose were provided by Fisher Scientific (Spain). Gold nanoparticle colloid suspension (AuNPs; 40 nm diameter) at optical density (OD) 1 in water was from BBI Solutions (United Kingdom). Nitrocellulose (NC) membranes (grades FF80HP, FF120HP and FF170HP), Whatman CF4 sample pads and CF7 wicking pads were purchased from Cytiva (Spain). Fiberglass sample/conjugate pads (grades 8951 and 8964) were from Ahlström (Finland), backing cards from DCN (USA) and the cassettes from TV Plastics (India). All other reagents were provided by Fisher Scientific (Spain) and Scharlau (Spain). Ultra-pure double deionized water (18.2 MΩ.cm) was used for all experiments. The Varian Cary 100 Bio UV-Visible spectrophotometer, the JEOL JEM-1011 transmission electron microscope (TEM) operated at 100K, the Malvern Zetasizer Nano ZS, the Molecular Devices SPECTRAmax 340PC-384 microplate spectrophotometer and the Branson ultrasonic water bath M2800-E were used for the preparation and characterization of the gold nanoparticle conjugate. The ClaremontBio Automated Lateral Flow Reagent Dispenser (ALFRD) set at 6 V coupled with the Chemyx Fusion Classic 200 automated syringe pumping system set at 0.25 mL/min were utilized for reagent dispensing on the nitrocellulose membranes and the AUTOKUN HGS220 guillotine cutter for cutting the strips. The ChemBio Diagnostics CubePlus lateral flow reader was used to measure the intensity of the lines of the LFA tests.

Preparation of the α-TTX IgG@AuNPs conjugate

Initially, the minimum concentration of the IgG antibody required for conjugation with the AuNPs to achieve colloidal stability was determined by titration using a gold aggregation test as described in the Supplementary Information. For the final preparation of the conjugate, the AuNPs were incubated with the chosen concentration of the α-TTX IgG for 30 min at 22 ºC under tilt rotation, and then BSA was added to a final concentration of 1% (*w/v*) for blocking. After another incubation for 30 min under the same conditions, the conjugate was washed three times by resuspending with conjugate buffer (5 mM sodium borate buffer pH 9, 1% (*w/v*) BSA, 10% (*w/v*) sucrose) and separating it by centrifugation for 30 min at 14000 rpm and 10 °C. The final conjugate was resuspended with the same conjugate buffer and stored at 4 °C until further use. For characterization, UV-Vis spectroscopy, transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential were used with particle suspensions prepared in water.

Construction of the LFA tests

A schematic illustration of the test strips is shown in Figure 1a. They were prepared by assembling (in the given order) the NC membrane, the wicking (absorbent) pad, the conjugate pad containing the α-TTX IgG@AuNP conjugate and the sample pad on an adhesive backing card with a 3 mm overlap between each part. For the construction of the test (TL) and control (CL) lines on the NC membrane, NeuA (0.3 mg/mL in PBS) and goat α-mouse IgG (1.2 mg/mL in PBS) were automatically dispensed, respectively, at a 7 mm distance between them and then the membrane was dried for 2 h at 37 °C. The biotinylated D3 TTX aptamer (38 µM in PBS) was then dispensed on top of the previously dispensed NeuA at the TL and incubated for 1 h at 4 °C as described previously.[39] The NC membranes were blocked with membrane blocking solution (10 mM carbonate-bicarbonate buffer pH 9.4, 5% (*w/v*) skimmed milk powder, 0.5% (*v/v*) Empigen BB) for 30 min under mild shaking at 22 ºC, washed twice with PBS for 10 min and finally dried for 2 h at 37 °C. For the conjugate pad preparation, this was first briefly soaked in pre-treatment solution (5 mM sodium borate buffer pH 8.8, 1% (*w/v*) BSA, 0.05% (*v/v*) Tween 20), followed by drying for 1 h at 37 °C. The α-TTX IgG@AuNPs conjugate suspension (OD 5 - 10 in conjugate buffer) was then manually dispensed onto the pre-treated conjugate pad with a micropipette ensuring uniform spreading and then dried for 1 h at 37 °C. Following assembly of the material in the order described earlier, the strips were cut at 4 mm, inserted into the plastic housing cassettes and stored in vacuum-sealed bags with desiccants at 4 °C.

Optimization of the LFA tests

Several parameters were optimized to enhance the performance of the LFA tests. For the immobilization of the aptamer on the NC membrane, two strategies were evaluated: direct dispensing of a pre-incubated mixture of NeuA (0.3 mg/mL) with the biotinylated aptamer (10 - 75 μM) or stepwise immobilization of the NeuA first and then of the aptamer. For the latter approach, NeuA was first spotted (0.5 μL of 0.3 mg/mL in PBS) at the test line of the NC membrane, dried for 2 h at 37 ºC, followed by spotting the aptamer (0.5 μL of 10 - 75 μM in PBS) on top of the previously dispensed NeuA and incubation for 2 h at 4 ºC. After immobilization, the membranes were blocked and dried in both cases as detailed earlier. The strips were dipped in 20 μL of solution containing the α-TTX IgG@AuNPs (OD 5) and TTX in assay buffer and let to run for approximately 30 min. Other parameters optimized were the type of NC membrane (FF80HP, FF120HP or FF170HP), the amount of the α-TTX IgG@AuNPs conjugate used per test (OD 5, 7 or 10) and the type of sample pad (Ahlstrom 8951 or 8964 fiberglass or Cytiva CF4 cellulose). Different concentrations of TTX were used when evaluating the different material (0 - 5 ng/mL).

Sensitivity and reproducibility

To evaluate the sensitivity and reproducibility of the test, a series of TTX standard solutions were analyzed with the tests on different days. To this end, TTX was prepared at 40 ng/mL in assay buffer and then serially diluted two-fold down to 0.04 ng/mL using the same buffer. Then, 150 μL of each solution were added to the sample window of the tests and let to run for 20 min. Blank samples containing only assay buffer were analyzed in parallel. The cassettes were imaged with a smartphone, or with the Cube lateral flow reader to measure the intensity of the test lines. To construct the calibration curve, the intensity of the test lines was plotted against the logarithm of the concentration of TTX and the data were fitted into a four-parameter sigmoidal curve (4PL) using GraphPad Prism 8. The limit of detection (LOD) was interpolated from the curve as $bottom+3SE_{bottom}$, where bottom is the bottom of the fitted curve and SE its standard error. Duplicate measurements were acquired in three independent experiments on different days to evaluate the reproducibility of the tests.

Specificity of the LFA test

This was studied by analysing samples containing TTX or other marine toxins and recording the signals of the test lines. TTX was tested at 1 ng/mL, while saxitoxin (STX), domoic acid

(DA) and okadaic acid (OA) were used at 20 ng/mL, individually or in combination with TTX. Each sample (150 µL in assay buffer) was applied to the sample window of the cassette and after 20 min, the cassettes were imaged and the test line intensities were recorded using the lateral flow reader. Each sample was analyzed in duplicate with two independent experiments.

Stability of the LFA test

The LFA tests were prepared as described earlier, packaged in vacuum-sealed plastic bags with desiccants and stored at either 4 °C or 37 °C for up to 6 weeks to perform an Arrhenius accelerated stability study. To this end, a TTX standard (1 ng/mL in assay buffer) and a blank control (only assay buffer) were analyzed with the LFA tests stored at the two temperatures at different time intervals and imaged with a smartphone. The test line intensities were estimated and used to predict the long-term storage of the tests under refrigerated conditions as described previously.[40]

Pufferfish samples

Extracts of three *L. sceleratus* pufferfish captured on Crete coasts of the Libyan Sea in 2019 (PF1, PF2, PF3) were obtained from a previous study.[14] For the preparation of the extracts, the fish were dissected, and the different tissues (gonads, intestine, liver, muscle, and skin) were retrieved. TTX was extracted from each of these tissues using 0.1% (*v/v*) acetic acid. Extracts from a *Lagocephalus lagocephalus* TTX-free pufferfish were used as controls. All the extracts (200 mg tissue/mL) were stored at -20 ºC until use.

Analysis of the pufferfish extracts using ELAA on microtiter plates

The extracts were analyzed with an Enzyme Linked Aptamer Assay (ELAA) using microtiter plates as detailed in our previous work.[38] Briefly, 50 µL of 5 µg/mL of the α-TTX IgG in 50 mM carbonate-bicarbonate buffer pH 9.4 were added to the wells of a MaxiSorp immuno plate for overnight coating at 4 °C. The wells were washed with PBST (3 x 200 µL) and then blocked for 30 min with 200 µL of 1% (*w/v*) BSA in PBST. After another washing step, 50 µL of TTX standards (starting from 40 ng/mL and performing 1/2 serial dilutions to 0.04 ng/mL with PBS) or pufferfish extracts (diluted 1/500, 1/1000, 1/3000 or 1/5000 with PBS) were added to the wells and let to incubate for 1 h at 22 ºC under mild shaking. The wells were washed again and

50 µL of 500 nM biotinylated D3 TTX aptamer in assay buffer were added for incubation during 30 min at 22 ºC under mild shaking. Following washing, streptavidin-HRP was added (50 µL of 0.1 µg/mL in PBST) for another 30 min incubation, followed by a final wash and addition of the TMB chromogenic substrate. After 6 min, colour development was stopped with the addition of 50 µL of 1 M of H_2SO_4 and the absorbance at 450 nm was measured. A TTX standard curve was constructed by fitting the data into a four-parameter logistic model (4PL) using the GraphPad Prism 8 software and used to interpolate the concentration of TTX in the samples. All samples were analyzed in triplicate.

Analysis of the pufferfish extracts with the LFA tests

For the analysis of the pufferfish tissue extracts with the LFA tests, different sample dilution factors were considered to ensure correct quantification regardless of the TTX content in each tissue. Specifically, 1 µL of each extract was serially diluted with assay buffer to achieve final dilution factors of 500 to 5000. Subsequently, 150 µL of diluted sample was applied to the sample window of the cassette and let to run for 20 min. The cassettes were imaged, and the intensity of the test lines was measured with the Cube LFA reader. The concentration of TTX in each sample was calculated after interpolation of the intensity of the test line into a TTX calibration curve constructed in parallel. All samples and standards were analyzed in duplicate.

Results and Discussion

Design of the LFA test

In our previous work we reported the selection of novel TTX aptamers and combined them with an α-TTX IgG antibody to develop a sandwich assay to detect TTX in contaminated pufferfish extracts using microtiter plates.[38] Herein, we show the implementation of this system into an LFA test with a classical sandwich-type design as illustrated in Figures 1a and S1. The aptamer with a 5'-biotin modification was immobilized on the NC membrane through affinity interactions with NeuA to construct the test line, while the α-TTX mouse IgG antibody was adsorbed on gold nanoparticles and dried on a conjugate pad. For the control line, an α-mouse IgG antibody was dispensed. For analysis, the sample is diluted with assay buffer and added to the sample window of the device. As it flows along the strip by capillary forces, it rehydrates the conjugate pad containing the α-TTX IgG@AuNPs conjugate, which in turn binds TTX present in the sample. The IgG/TTX complex migrates towards the test line where it is captured by the aptamer. Excess gold conjugate is captured at the control line by the α-mouse IgG, thus

confirming the correct performance of the test, while remaining liquid flows towards the wicking (absorbent) pad. The presence of TTX in the sample is indicated by the development of red colour at the test and control lines (Figure 1b), while only the control line is visible when there is no TTX in the sample (Figures 1c and S1).

Preparation and characterization of the α-TTX IgG@AuNPs conjugate

The α-TTX IgG antibody was conjugated to 40 nm AuNPs by passive adsorption. Prior to conjugate preparation, the amount of antibody and pH conditions required for assuring conjugate stability were determined through a gold aggregation test. As demonstrated in Figure S3, the minimum antibody concentration providing colloidal stability was 6 µg/mL at pH 8. For more efficient bioreceptor spacing and considering that the particles would be blocked with BSA,[41] the final concentration of the antibody used was 4 µg/mL. The conjugate was first characterized by UV-Vis spectroscopy. A characteristic peak at 526 nm was observed for the bare AuNPs (40 nm), while a red-shift to 533 nm was produced after conjugation with the α-TTX IgG, verifying the presence of protein on the surface of the particles, while the peak maintained its width evidencing the narrow size distribution of the conjugate (Figure 2a). Moreover, TEM images revealed spherical and homogenous particles after conjugation (Figure 2b) with an increased average diameter of 39.1 ± 3.2 nm as compared to the bare AuNPs (37.5 ± 4.0 nm). DLS analysis showed a diameter of 110.6 ± 0.5 nm for the α-TTX IgG@AuNP conjugate and 42.2 ± 0.1 nm for the bare AuNPs (Figure 2c). The discrepancy in the measurements obtained with the two methods, TEM and DLS, is probably due to the difference in the technique: TEM measures physical diameter of the particles as compared to the hydrodynamic diameter provided by DLS which are typically larger.[42] Finally, the zeta potential was -46.7 ± 1.1 mV for the bare AuNPs and -26.3 ± 2.3 mV for the α-TTX IgG@AuNPs conjugate, demonstrating the stability of the particles before and after conjugation with the antibody (Figure 2d). On the other hand, the increased zeta potential of the conjugate, which is a measurement of surface charge, attests to the successful immobilization of protein on the surface of the particles which leads to decreased repulsive charges between the particles, also observed in the TEM images. Overall, these studies confirmed the preparation of a stable gold conjugate.

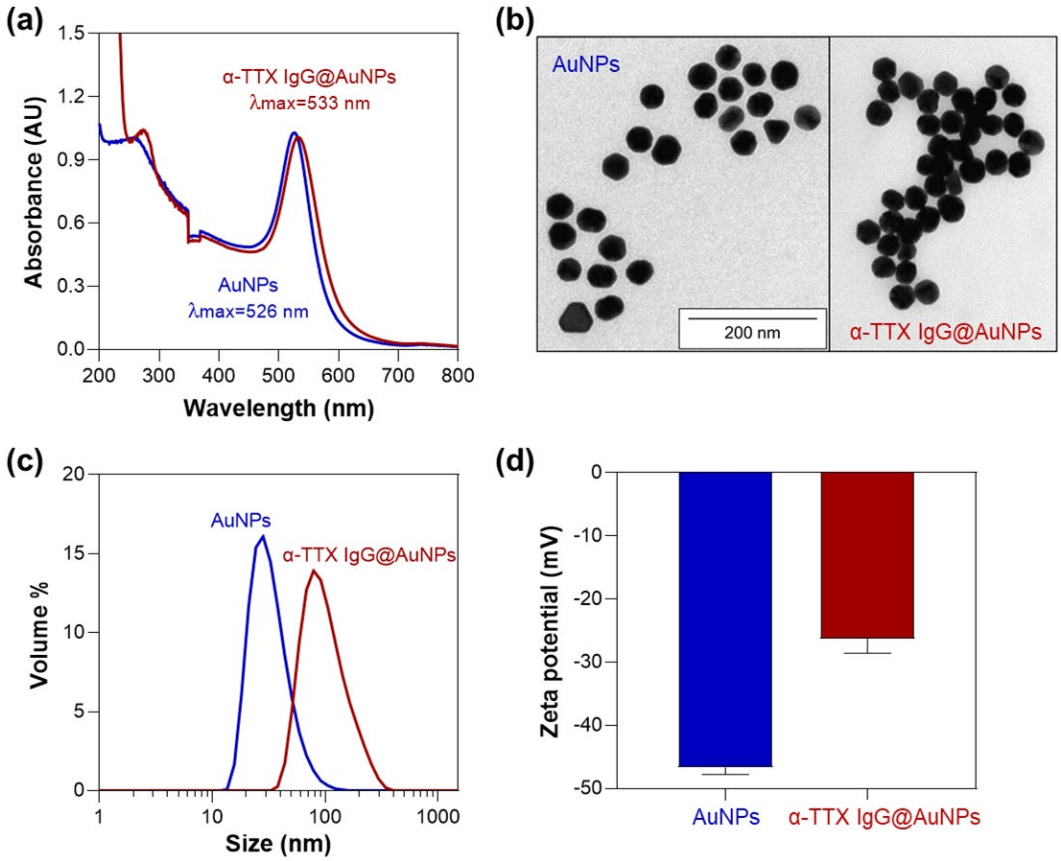


Figure 2. Characterization of the α-TTX IgG@AuNPs conjugate by (a) UV-Vis spectroscopy, (b) TEM, (c) DLS and (d) Zeta potential measurements.

Optimization of the LFA tests and sample analysis conditions

With the aim of developing an accurate and robust TTX LFA test, several experimental parameters were optimized. Initially, the immobilization of the aptamer on the NC membrane was studied. Its concentration should be balanced for optimal analyte detection, since excess could lead to non-specific signals or generate steric hindrance, whilst low concentration could result in rapid saturation of the signal at the test line and consequent decreased sensitivity of the test.[41] Therefore, a series of concentrations of the aptamer with a 5'-biotin label were tested in combination with NeuA, to facilitate immobilization through affinity interactions. Two different strategies were evaluated: direct absorption of a NeuA/biotinylated aptamer pre-incubated mixture[32] and a two-step approach of first dispensing NeuA on the NC membrane followed by the aptamer as a second layer on top.[39] The FF120HP membrane was used for this study and the test was performed in dipstick format. As can be seen in Figure S4, highest specific signals in the presence of TTX were observed when 25 μM of the aptamer were used to immobilize employing the two-step strategy. These conditions were thus chosen for

following experiments which were performed in an integrated lateral flow format. The type of NC membrane was evaluated next. Membranes FF80HP, FF120HP and FF170HP with large, medium and small pore size, respectively were used to prepare the detection strips. Faint and diffused test line bands were observed on the strips prepared with the FF80HP membrane (largest pore size and fastest capillary flow rate), while clear and sharp lines were obtained with the other two membranes (Figure 3a). The FF170HP membrane was finally chosen because it was hypothesized that the slower capillary flow rate would increase the contact time between the antibody-TTX complex and the aptamer at the test line and potentially increase the sensitivity of the test. With regards to the amount of the α-TTX IgG@AuNPs conjugate, an OD 5 was considered optimum since higher amounts of the conjugate did not improve the sensitivity of the test, while at the same time no signal was observed for the blank control (Figure 3b). Finally, three types of sample pads were compared: two were made of fiberglass (Ahlström grades 8951 and 8964) and one of cellulose (CF4). Both materials are widely exploited in LFAs with the cellulose sample pads having more filtration capacity. As can be seen in Figure 3c, the intensity of the test lines in the presence of TTX was similar for all three sample pads, with slightly less intensity observed with the Cytiva pad. Considering the general characteristics of wicking rate and water absorption, the Ahlström 8964 was chosen as more appropriate for high sample volumes (≥ 150 μL).

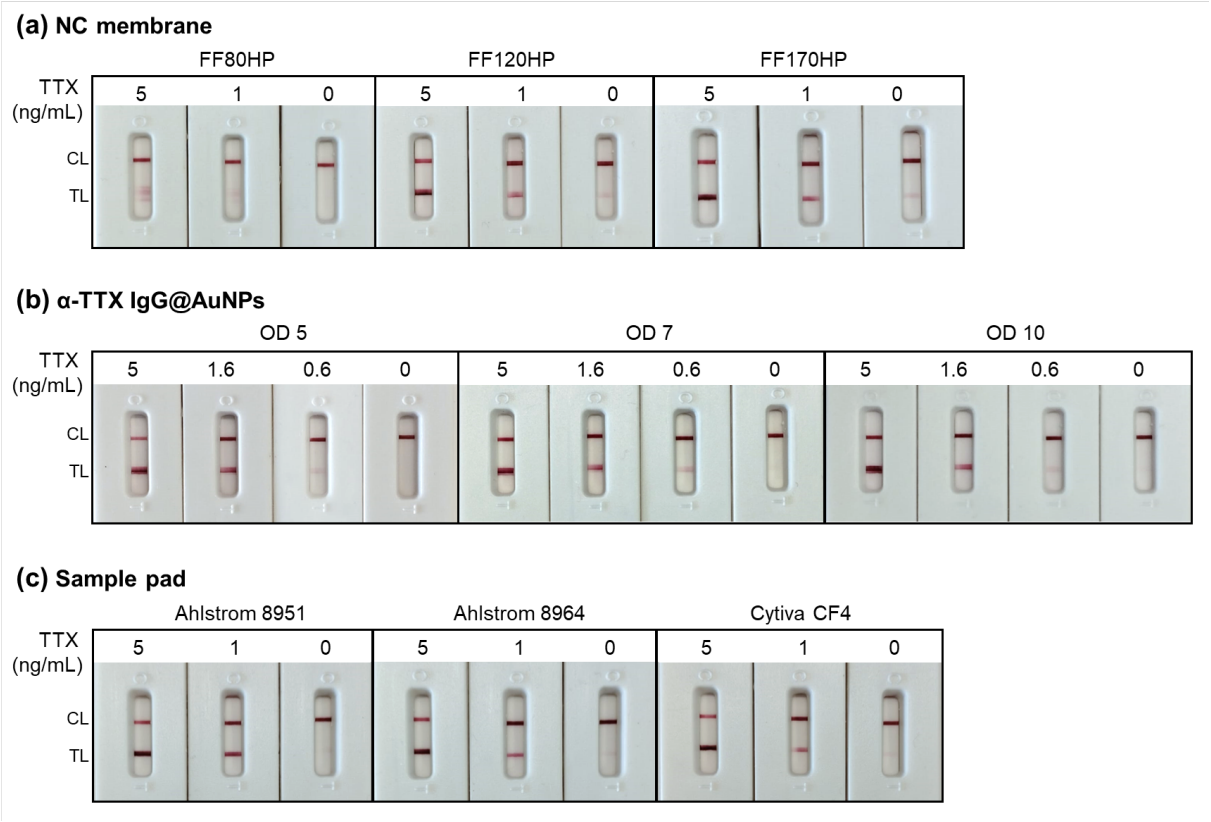
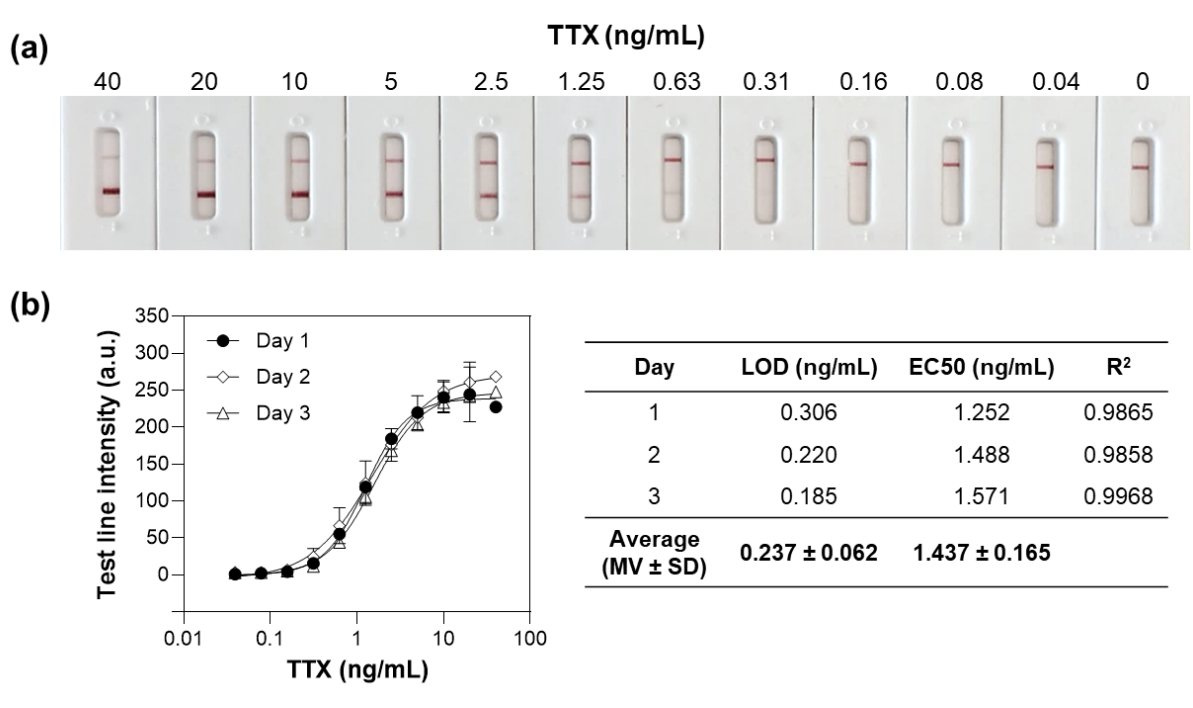


Figure 3. Optimization of LFA conditions. (a) NC membrane, (b) OD of the α-TTX IgG@AuNPs conjugate and (c) type of sample pad. The tests were evaluated with different concentrations of TTX (0 - 5 ng/mL).

Analytical performance of the LFA test

Test strips were prepared using the optimum conditions and materials described above and were used to evaluate the analytical performance of the assay. This was done by analysing TTX standard solutions in the range of 0.04 - 40 ng/mL (starting from 40 ng/mL and performing serial 1/2 dilutions with assay buffer). Representative photos of the tests are shown in Figure 4a, where a gradual increase in the intensity of the test line is observed for TTX concentrations of ≥ 0.3 ng/mL and up to 10 ng/mL at which point the signal saturates. Based on these results, the visual LOD was defined at approximately 0.3 ng/mL TTX. The sensitivity of the test was evaluated on three different days and after quantifying the intensity of the test lines with the Cube Reader (Figure S2), sigmoidal calibration curves were constructed with very high reproducibility (Figure 4b). The calculated LODs were in the range of 0.185 - 0.306 ng/mL, with an average of 0.237 ± 0.062 ng/mL TTX and an average half-maximal effective concentration (EC50) value of 1.437 ± 0.165 ng/mL TTX. The test achieved a visual LOD of 0.31 ng/mL and a dynamic range of 0.31 - 10 ng/mL TTX, spanning approximately 1.5 orders

of magnitude. Moreover, based on the analysis of different TTX standard solutions on different days, both the intra-assay and inter-assay coefficients of variation (CV) were less than 13% (Table S2). These results demonstrate that the developed test was not only very sensitive, but it also exhibited high precision and reproducibility, and all these are desired characteristics of reliable rapid tests.



Day	LOD (ng/mL)	EC50 (ng/mL)	R^2
1	0.306	1.252	0.9865
2	0.220	1.488	0.9858
3	0.185	1.571	0.9968
Average (MV ± SD)	**0.237 ± 0.062**	**1.437 ± 0.165**	

Figure 4. Sensitivity and reproducibility of the LFA tests. (a) Representative images of the tests where TTX standard solutions of 0.04 – 40 ng/mL were analyzed. (b) Calibration curves constructed after measuring the test line intensities with the LFA reader. Three independent experiments were performed with duplicate measurements.

There are very few works in the literature detailing the development of LFAs for TTX and they are summarized in Table 1. The majority of the tests employ a TTX-BSA conjugate at the test line to compete with TTX in the sample for binding to an α-TTX IgG immobilized on AuNPs,[22,24,26] AuNFs[23,25] or coloured latex microspheres.[26] There is one report in which the same strategy was used but, in this case, TTX was detected simultaneously with OA by combining two gold nanoparticle immunoconjugates and constructing two test lines, one for each toxin.[24] The visual LODs of all the above-mentioned tests were in the range of 1 - 40 ng/mL TTX. Higher sensitivity was demonstrated in two other reports where fluorescence detection was employed instead of colorimetric. A competitive signal-off test was demonstrated

by Sun *et al*. who used TTX-BSA at the test line and a TTX IgG antibody conjugated to fluorescent microspheres for detection, reporting a sensitivity of 0.05 ng/mL.[27] On the other hand, a competitive signal-on LFA was designed by using AuNFs[29] conjugate with IgG antibody as quenchers for the fluorescently labelled TTX-BSA conjugates at the test lines in the absence of TTX. In this way, lower LOD was achieved, 0.78 and 0.2 ng/mL, respectively. Although these tests exhibit improved sensitivity compared to the ones based on colorimetric detection, fluorescence systems are generally expensive as they require expensive light sources to illuminate the fluorescent reporters, as well as needing interference filters and detection systems to process and capture the emitted light, and finally the data processing required to produce the result.[43] The sandwich-type colorimetric test developed in this work employs lower-cost material (ssDNA aptamer, antibody and AuNPs), provides fast analysis (less than 20 min) and is more sensitive than all the other colorimetric tests reported previously (LOD of 0.31 ng/mL TTX).

Table 1. LFAs reported in the literature to date for TTX detection. The LOD refers to the minimum concentration of TTX in buffer required to produce a change in the intensity of the test line, by visual inspection or fluorescence imaging.

Assay type	Test Line	Conjugate	Assay time	Range	Reported LOD	Sample	Reference
Competitive, visual, signal-off	TTX-BSA[a]	α-TTX IgG/AuNP[f]	10 min	40 - 8000 ng/mL	40 ng/mL	Pufferfish (spiked)	22
Competitive, visual, signal-off	TTX-BSA	α-TTX IgG/AuNP	5 min	n/a	2 mg/kg	Pufferfish (naturally contaminated)	23
Competitive, visual, signal-off	TTX-BSA	α-TTX IgG/AuNP	10 min	20 - 80 ng/mL	20 ng/mL	Clam (spiked)	24
Competitive, visual, signal-off	TTX-BSA and OA-BSA[b]	α-TTX IgG/AuNP and α-OA IgG/AuNP	10 min	1 - 20 ng/mL	15 ng/mL	Clam (spiked and naturally contaminated)	44
Competitive, fluorescence, signal-on	Fluorescent TTX-BSA[c]	α-TTX IgG/AuNP	12 min	0.78 - 50 ng/mL	0.78 ng/mL	Pufferfish (spiked)	28
Competitive, fluorescence, signal-on	QDNB-BSA[d] and TTX-BSA	α-TTX IgG/AuNF[g]	8 min	1.56 - 100 ng/mL	0.2 ng/mL	Pufferfish (spiked)	29
Competitive, visual, signal-off	TTX-BSA	α-TTX IgG/AuNP	5 min	1 - 50 ng/mL	10 ng/mL	Crucian and clam (spiked)	25

Competitive, fluorescence, signal-off	TTX-BSA	Eu^{3+}/α-TTX IgG[h]	20 min	0.5 - 40 ng/mL	0.05 ng/mL	Sea flower beetle and clam (spiked)	27
Competitive, visual, signal-off	TTX-OVA[e]	α-TTX IgG/AuNF	10 min	9.5 - 331 ng/mL	9.49 ng/mL	Yellow croaker, grass carp, perch, pufferfish (spiked)	26
		α-TTX IgG/LMs[i]	10 min	5.4 - 443 ng/mL	5.40 ng/mL		
Sandwich, visual, signal-on	TTX ssDNA aptamer	α-TTX IgG/AuNP	20 min	0.31 - 10 ng/mL	0.31ng/mL	Pufferfish (naturally contaminated)	This work

[a] BSA-conjugated TTX, [b] BSA-conjugated okadaic acid; [c] TTX-BSA conjugated with fluorescent microspheres, [d] quantum dots-BSA conjugate, [e] ovalbumin-conjugated TTX, [f] gold nanoparticles, [g] gold nanoflowers, [h] fluorescent europium microspheres, [i] coloured latex microspheres, n/a: not available.

Specificity of the LFA test

The cross-reactivity of the developed LFA with other marine toxins was evaluated using saxitoxin (STX), okadaic acid (OA) and domoic acid (DA). In this study STX is the most relevant as this toxin can be found with TTX in pufferfish as well as in shellfish, and as it is hydrophilic, is often co-extracted with TTX. STX is also a paralytic neurotoxin and intoxication after ingestion could produce similar effects to TTX. DA, another hydrophilic neurotoxin, is not found in pufferfish but it does accumulate in shellfish like TTX, STX and OA. OA is a diarrhetic toxin commonly found in shellfish. Since it is lipophilic, it is unlikely to be extracted using the method of extraction for TTX and is thus not expected to be as potentially problematic in terms of false positives, as STX. As can be seen in Figure 5, due to the high specificity of the hybrid aptamer-antibody sandwich system, no cross-reactivity or non-specific binding with the other marine toxins studied was observed when STX, OA or DA were analyzed individually or in mixtures with TTX. Additionally, as can be observed in the results with the mixtures, the detection of TTX was not hampered by the presence of other toxins present in the sample. These results demonstrate that the test is specific for TTX, it exhibits no cross-reactivity with the other marine toxins studied and their concurrence with TTX in the same samples is not expected to interfere with the accurate detection of TTX.

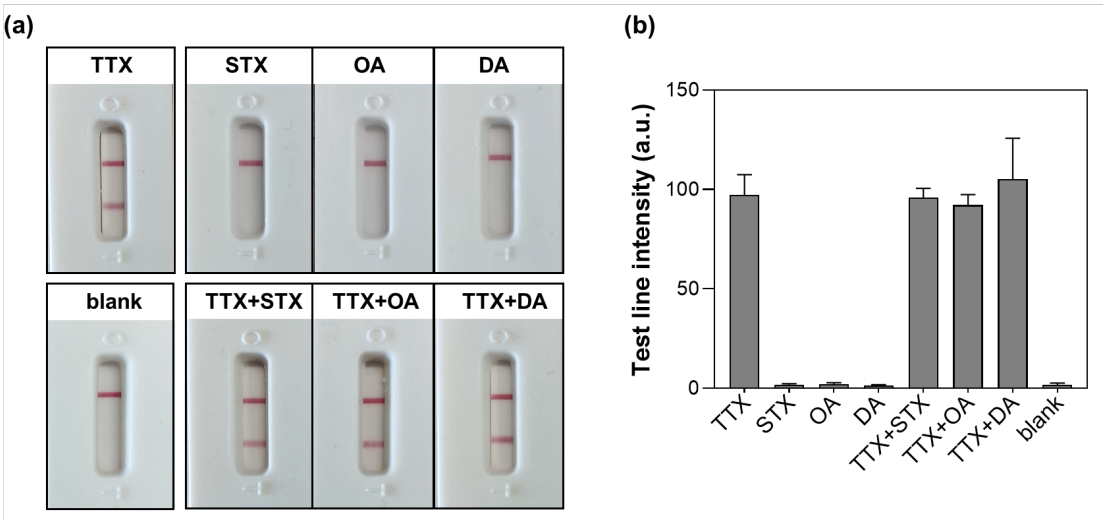


Figure 5. Specificity of the LFA test. TTX (1 ng/mL) or other marine toxins (20 ng/mL) were analyzed with the tests, individually or in mixtures. (a) Representative images of the LFA tests. (b) Quantification of the tests with the Cube LFA reader. STX: saxitoxin; OA: okadaic acid; DA: domoic acid; *n*=2, error bars: ± SD.

Stability of the LFA test

The LFA tests were stored at 4 ºC and 37 ºC for 6 weeks and their stability was evaluated by analysing a TTX standard solution at different storage time intervals. As illustrated in Figure 6, no remarkable decrease in the intensity of the test lines was observed for the devices stored at 4 ºC and 37 ºC for 6 weeks and no false-positive signals were obtained after 6 weeks of storage at either temperature. Based on the Arrhenius accelerated stability study performed as described previously,[40] the tests can be stored for approximately 4.5 years at 4 ºC. This long shelf life of the tests demonstrates their reliability and suitability for in field implementation.

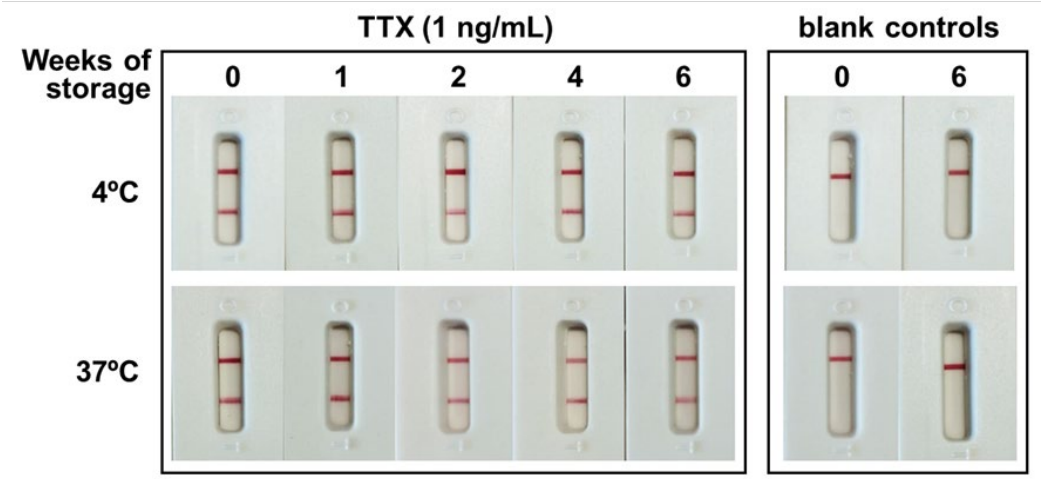


Figure 6. Stability of the LFA tests. The tests were stored at 4 ºC or 37 ºC for up to 6 weeks and were tested at different storage time intervals with TTX (1 ng/mL) or assay buffer alone (blank controls).

Pufferfish sample analysis

The practical applicability of the LFA test for monitoring the TTX content in fish was finally demonstrated by analysing pufferfish samples with known TTX content. Extracts from different tissues (muscle, skin, liver, intestinal tract, and gonads) of three *L. sceleratus* pufferfish specimens caught on the Crete coasts between March and May 2019 were prepared and analyzed by LC-MC/MS and CBA in a previous report.[14] Based on the known TTX content in each extract, different dilutions were performed for analysis with the LFA tests as described in the experimental section. As shown in Figure 7, the test successfully detected TTX in all the tissue extracts from all three contaminated pufferfish, with test lines of different intensities depending on the dilution performed and the TTX content of the sample. These results demonstrate the suitability of the tests to detect TTX in such samples with no interference from

other fish matrix compounds since no signals were obtained when analysing the tissue samples from the TTX-free pufferfish.

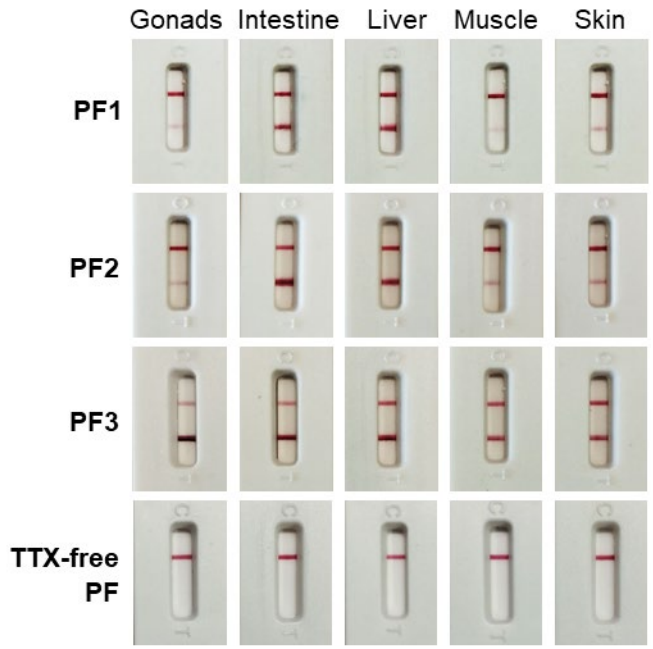


Figure 7. Representative photos from the analysis of contaminated *L. sceleratus* pufferfish tissue extracts and of the TTX-free *L. lagocephalus* pufferfish with the LFA tests. Samples were diluted 1/500 (TTX-free PF), 1/1000 (all extracts from PF1 and liver, muscle, skin from PF3), 1/3000 (PF2) and 1/5000 (gonads and intestine from PF3).

To quantify the TTX content in each extract, TTX standard solutions were analyzed in parallel and the test lines of all the tests were quantified with the Cube lateral flow reader. Based on the calibration curves constructed with the TTX standards, the TTX content in each extract were determined. For comparison, the extracts were also analyzed with the microplate ELAA as detailed in the experimental section, and the results obtained, together with those from the LC-MS/MS and CBA analysis, are shown in Table 2. According to the results obtained with our LFA, all extracts contained TTX at levels higher than the one considered safe for human consumption according to Japanese legislation (2 mg TTX equiv./kg), sometimes even reaching more than 60-fold higher contents. These values have also been found in other works,[14,45,46] and show up the high probability to cause tetrodotoxication in case of consumption of these fish specimens. Although some works evidence that the diet of *L. sceleratus* may be responsible, at least in part, for the TTX contents, the symbiosis with TTX-producing bacteria belonging to the intestinal microflora cannot be ruled out.[45] Additionally, environmental conditions such as

salinity, temperature and water depth have been suggested as possible factors that affect TTX production by bacteria and TTX presence in host organisms.[46] In any case, prediction of TTX contents according to morphological/biological characteristics of pufferfish or to environmental-related data is not straightforward.[46] Therefore, to better understand the trends, it would be beneficial that more data on TTX contents in food and environmental samples are available, and for that, analytical tools such as the one proposed in this work are crucial.

When comparing the different techniques used in this work, a good correlation of the results obtained between the LFA and ELAA was observed, certainly because they both use the same biorecognition elements, only changing the format and detection technique. This demonstrates the analytical benefits of the LFA assay which can be performed with a single step as opposed to the multi-step ELAA requiring consecutive binding/washing steps and a spectrophotometer for quantification. There are more than 30 known analogues of TTX[3,47] and in the samples analyzed in this work, 13 different analogues were identified including TTX according to previous LC-MS/MS characterization.[14] Table 2 shows the LC-MS/MS results for only TTX contents (which also includes 4-*epi*TTX, which is in chemical equilibrium with TTX) as well as for all TTXs after the application of the toxicity equivalency factors (TEFs) obtained by CBA to the TTX analogues, considering TEF=0 for TTX analogues with unknown TEF. The actual TTX content varies significantly in the samples, from as low as 12% *w/w* (gonads from PF3) to as high as 71% *w/w* (gonads from PF1) within the total TTXs content. The differences observed in the quantification of TTX in the samples with the LFA as compared to LC-MS/MS are certainly due to the different detection principles. The results from the gonads of PF3 contained unusual high 5,6,11-trideoxyTTX and 4-*epi*-5,6,11-trideoxyTTX concentrations and therefore, this datapoint was considered as an outlier and it was excluded from all statistical analyses. The correlation between LFA and only TTX by LC-MS/MS indicates that LFA would be overestimating the TTXs contents (Figure S5a). Nevertheless, when all TTX analogues with their TEFs were included in the correlation, the agreement between LFA and LC-MS/MS was excellent (Figure S5b). This result indicates that not only the LFA is able to properly detect TTX, but it also recognises TTX analogues, probably proportionally to their toxicity. In fact, the correlation between LFA and CBA, which measures toxicity on Neuro-2a cells, was also excellent (Figure S5c). These results indicate that the LFA is a very appropriate tool to guarantee food safety from a toxicological point of view. Future research should be focused on the establishment of cross-reactivity factors (CRFs) of TTX analogues with the LFA to

understand the interactions with the two biorecognition molecules, although the lack of readily available and highly pure TTX analogues hinders this work.

Finally, with regards to the sensitivity of the LFA test for detection of TTX in pufferfish, this can be estimated as follows: the gonads extract from PF1 contains 2.1 mg TTX equiv./kg according to LC-MS/MS, with > 70% being TTX. Considering the 1000-fold dilution performed prior to the analysis of this sample with the LFA, this sample contains 0.42 ng TTX equiv./mL and can be detected with the LFA since a discernible red band could be observed (see Figure 7). This is consistent with the visual LOD of the test in assay buffer (0.31 ng/mL TTX). In fact, taking into account the LOD in buffer and a 500-fold dilution of samples, which has been observed not to cause any matrix effects, an LOD of 0.78 mg TTX/kg tissue for the test in sample extracts can be calculated, way below the Japanese regulatory limit of 2 mg TTX/kg. Moreover, currently there are only four available commercial tests for TTX and they all based on a competitive immunoassay and as shown in Table S1, they all exhibit lower sensitivities than the one we have described here. The LFA rapid test developed in this work is based on an aptamer-antibody sandwich and to the best of our knowledge, is the first signal-on colorimetric test for TTX, which can be assessed by simple visual inspection. The test can be performed in a single step in less than 20 min and the presence of TTX in the sample is easily confirmed by the appearance of a red coloured line. The test is extremely sensitive and specific, reproducible and with long storage stability. Its compatibility with pufferfish analysis for evaluation of potentially toxic samples was confirmed by the excellent correlation between the results from the LFA and ELAA, LC-MS/MS and CBA.

The rising number of poisoning records due to the invasive toxic puffer fish species as well as emerging presence of TTX in shellfish worldwide is of concern. The operational performance of the LFA developed and validated for puffer fish in this work demonstrates the great value of this tool for the rapid screening of this type of samples. Moreover, after proper refinement of the operational parameters, including the integration of a signal amplification strategy to attain even lower limits of detection, and the corresponding evaluation of the matrix effects, the test would be applicable to the analysis of shellfish. The use of this tool for the analysis of seafood samples, together with environmental data, would contribute not only to guarantee food safety, but also to unlock the unknowns regarding the TTX origin, presence and distribution in marine organisms, meanwhile reducing the negative environmental impact on coastal areas.

Table 2. Detection of TTXs in pufferfish tissue extracts with the LFA test and comparison with other techniques. The ELAA on microtiter plates was performed using the same α-TTX IgG/D3 aptamer sandwich system also used in the LFA test. Quantification by LC-MS/MS and CBA were performed in a previous study.[14] TTX equiv. values correspond to MV ± SD, *n*=3.

Puffer fish	Tissue	LFA TTX equiv. (mg/kg)	ELAA TTX equiv. (mg/kg)	LC-MS/MS		CBA TTX equiv. (mg/kg)
				TTX (mg/kg)	TTX equiv. (mg/kg)	
1	Gonads	3.8 ± 0.3	2.8 ± 0.1	1.5	2.1	3.7
	Intestine	11.3 ± 0.4	6.7 ± 0.2	19.2	34.6	19.6
	Liver	12.5 ± 0.7	5.0 ± 0.2	20.6	38.9	21.5
	Muscle	2.5 ± 0.0	1.5 ± 0.1	4.6	7.6	5.6
	Skin	4.3 ± 0.1	1.8 ± 0.2	5.3	14.3	8.0
2	Gonads	9.8 ± 1.7	5.6 ± 0.5	5.4	10.5	6.4
	Intestine	109.6 ± 32.8	25.0 ± 4.3	69.9	205.8	92.4
	Liver	60.1 ± 0.4	19.6 ± 2.7	53.9	188.2	51.4
	Muscle	10.9 ± 0.1	7.0 ± 1.4	15.3	36.5	14.1
	Skin	12.9 ± 0.8	6.2 ± 0.8	13.5	63.2	16.1
3	Gonads	129.1 ± 40.1	91.3 ± 6.5	171.7	1324.4	228.9
	Intestine	95.1 ± 9.6	59.0 ± 2.9	80.4	210.9	113.1
	Liver	21.6 ± 0.6	6.4 ± 0.3	17.0	57.2	26.8
	Muscle	11.6 ± 1.0	3.5 ± 0.6	6.4	11.6	8.5
	Skin	10.4 ± 0.3	2.9 ± 0.2	9.2	34.7	14.7

SUPPORTING INFORMATION

Design and optimization of the LFA tests; performance of the LFA tests and comparison of with other methods.

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Author Contributions

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NOTES

The authors declare no competing financial interest.

ABBREVIATIONS

TTX: Tetrodotoxin; AuNPs: gold nanoparticles; α-TTX IgG@AuNPs: mouse anti-tetrodotoxin IgG antibody conjugated with gold nanoparticles; NeuA: neutravidin; LFA; lateral flow assay; ELISA: enzyme linked immunosorbent assay; ELAA: enzyme linked aptamer assay; STX: saxitoxin; OA: okadaic acid; DA: domoic acid; TEM: transmission electron microscopy; DLS: dynamic light scattering; LC-MS/MS: liquid chromatography with tandem mass spectrometry; CBA: cell-based assay; TEF: toxicity equivalency factor.

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