TECHNICAL NOTE

Development of an ultrasensitive immunochromatography test to detect nicotine metabolites in oral fluids

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Abstract Passive exposure to tobacco smoke causes a variety of illnesses ranging from allergic responses to cancer. Assessment of exposure to second-hand tobacco smoke (SHS), particularly among vulnerable populations enables intervention and prevention of future disease. A minimally invasive oral fluids-based onsite test to detect such exposure would create a valuable tool for researchers and clinicians. Here we describe the development of a test that uses an inexpensive reader that utilizes a CMOS image sensor to reliably quantify a reporter signal and determine nicotine exposure. The rapid lateral flow test consists of a nitrocellulose strip with a control line containing goat anti-rabbit IgG, used as an internal standard, and a test line containing BSA–cotinine conjugate. To run the test, diluted sample containing antibodies against cotinine, the major metabolite of nicotine, is mixed with protein A–gold nanoparticles and placed on the sample pad. As the sample runs up to the nitrocellulose pad, antibodies in the running buffer bind to available cotinine. If cotinine is absent, the antibodies will bind to the BSA–cotinine derivative immobilized on the test line, resulting in an intense purple–red band. The concentration of cotinine equivalents in the sample can be estimated from interpretation of the test line. In this article we describe the effect of different cotinine derivatives, oral fluid pretreatment, and application and running buffers on assay sensitivity. The test can reliably detect as little as 2 ng mL^{-1} cotinine equivalents. The assay is sensitive, simple, rapid, inexpensive, and easily implementable in point-of-care facilities to detect second-hand smoke exposure.

Introduction

The deleterious effects of active tobacco smoke have long been established and are routinely reported [1]. Numerous scientific studies also show that passive exposure can be as dangerous as active smoking to health [2–6] this is especially true for children, who often cannot choose to remove themselves from the source of the smoke. In recent years laws and regulations have been put into effect trying to limit exposure of all ages to passive tobacco smoke [7–11].

Also important have been scientific efforts to document and measure tobacco smoke exposure [12–15]. The purpose of this article is to report on the design and testing of an onsite method to measure the major metabolites of nicotine in oral fluids at concentrations that reflect passive exposure. Although there is no consensus on what constitutes a passive exposure cutoff concentration in saliva or oral fluids, the literature suggests concentrations between 3 and 10 ng mL^{-1} [16–18].

Nicotine is a natural component of tobacco leaves comprising approximately 95% of the total alkaloid content. In most tobacco strains, nornicotine and anatabine are the most abundant of the minor alkaloids followed by anabasine [19]. Bacterial action can lead to the formation of traces of other compounds, for example cotinine, nicotine *N'*-oxide, 3-pyridyl propyl ketone, 3-pyridyl methyl ketone, nicotinamide, *N*-methylnicotinamide, and nicotinic acid [20].

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Nicotine can be ingested through a variety of vehicles including smoke inhalation, chewing of tobacco, or through pesticides. Nicotine has also been found in the food supply as a result of its use in agriculture. Nicotine has a relatively short half-life (24–84 min) [21] and is rapidly metabolized to a number of compounds [19], making it an unreliable indicator of smoking status. In humans approximately 70 to 80% of nicotine is converted to cotinine. The cotinine is further metabolized to *trans*-3′-hydroxycotinine, cotinine glucuronide, *trans*-3′-hydroxycotinine glucuronide, cotinine *N*-oxide, norcotinine, and others [19]. After inhalation of tobacco nicotine is rapidly absorbed, metabolized, and redistributed in the oral cavity as cotinine. Similar to other drugs with comparable pK_a values, cotinine seems to accumulate in the mouth, enabling longer detection than in blood but shorter than in urine [22].

Various in-vitro devices are available to test oral fluids or urine for the presence of nicotine metabolites. An example is the NicAlert test which can be used to quickly distinguish between users and non-users of tobacco, but is not sufficiently sensitive to detect and quantify lower concentrations of cotinine among non-smokers with second-hand exposure [12]. In addition, many laboratory-based ELISAs are available to test oral fluids. One example from OraSure Technologies involves collecting the sample remotely and sending it back to the laboratory for analysis. The product does not claim to detect passive exposure and is used only to identify active smokers.

Oral fluid is a combination of gingival crevicular exudates released from the major salivary glands of which the parotid, submandibular, and sublingual are the main sources. The fluids found within the mouth are indicative of much of what is in the bloodstream, although it is not infallible. The physiological functions of the oral cavity are involved with digestion and protection from infection. The submandibular glands produce a mucous material whereas the parotid glands produce a low-viscosity fluid. Submandibular fluid comprises approximately 75% of the fluid in the mouth. This means that the amount of fluid available for diagnostic testing is limited by the dynamic production of oral fluid. It also means that any diagnostic test must expect interferences from the oral fluid components and establish pretreatment strategies to eliminate such interferences without hindering release of the target analytes.

New developments in oral diagnostics have resulted in the development of on-site non-invasive tests for a variety of biological markers of interest [23–26]. For many years on-site testing was primarily limited to two analytes, glucose and HCG for pregnancy. Glucose testing has evolved to use simple electronic measuring devices that use a small drop of blood. Tests for pregnancy utilize urine to identify hormones using immunoassays that result in a visual or instrumental interpretation. This article focuses on tests similar to those that measure HCG for pregnancy, using new techniques to develop a rapid on-site test to detect passive exposure to tobacco smoke by targeting cotinine and other metabolites in oral fluids. Similar to other on-site tests, it is based on lateral flow immunochromatography using antibodies specific for cotinine as the target. The specimen is sampled by the device by capillary action and transferred to a nitrocellulose membrane where it encounters the target capture line. Any free cotinine antibodies will bind to the line and then become labeled with protein A–gold nanoparticles. If the sample contains cotinine or other cross-reacting nicotine metabolites, the antibody-active sites will be blocked thus preventing binding and yielding a lighter line; consequently, the intensity of the line is inversely proportional to the concentration of cotinine equivalents.

Detection of low levels of the analyte by visual means is unreliable, because interpretation of the results may vary depending on the operator; we therefore report the use of an inexpensive reader from Alverix that uses a CMOS image sensor to quantify reporter signal. A control line is included in each test as an internal standard and used to help estimate the concentration of cotinine and its metabolites in the sample, hence identifying passively exposed subjects. Stable readings are obtained after 15 min and concentrations as low as 2 ng mL^{-1} cotinine equivalents are reliably measured.

Materials and methods

All experiments were conducted at room temperature (RT; 21–23 °C) and environmental relative humidity. Test materials were stored at room temperature and 20% relative humidity. Reagents were stored at 4 °C.

Chemicals and materials

Protein A–gold conjugate was obtained from Arista Biologicals (Allentown PA, USA), goat anti-rabbit and goat anti-human IgGs were supplied by Affinity Bioreagents (Rockford IL, USA), bovine serum albumin (BSA), tryptone, and other buffer reagents were supplied by Sigma–Aldrich (St Louis MO, USA).

Nitrocellulose (15 μm pore size), glass fiber sample pads, and absorbent pads were products of Arista Biologicals. Cotinine antibodies were obtained from OraSure Technologies (Bethlehem, PA , USA).

Equipment

Immunoreagents were applied to nitrocellulose strips by use of a Camag Linomat 5 semiautomatic reagent application

device (Camag Scientific, Wilmington, NC, USA); the prepared membranes were dried in a Thermo/Precision Scientific Model 3EG Economy Incubator (Fisher Scientific, Pittsburgh, PA, USA), and stored in a Dry-Keeper Desiccator Cabinet with a dial hygrometer (Bel-Art products, Pequannock, NJ, USA). The Kinematic Matrix 2210 Universal Card Laminator (Kinematic automation, Twain Harte, CA, USA) was used for placement of card stocks (nitrocellulose membrane with control and test lines, sample pad, and absorbent pad) on to a laminate backing in the manufacture of assay cards. The Azco Sur-Size automatic guillotine cutter model SS-4 (Azco, Elmwood Park, NJ, USA), is a motor feed, precise-cutting machine used to cut assembled assay cards into assay strips. Alverix Rapid Diagnostics RDx reader and software (Alverix, San Jose, CA, USA), was used to measure signal intensity of control and test lines in accordance with the manufacturer's instructions. Anti-sera were purified using a BioLogic LP system equipped with a Bio-Scale Mini Affi-Prep Protein A cartridge (Bio-Rad, Hercules, CA, USA).

Preparation of cotinine derivatives

Several derivatives of cotinine were synthesized. The objective was to vary the length of the spacer and to conjugate the derivatives to BSA. The structures are shown in Table 1.

The CCM derivative was synthesized by reacting cotinine with *tert*-butyl 6-bromohexanoate which was produced by reaction of 6-bromohexanoic acid and *tert*-butanol; the ester group was subsequently removed with trifluoroacetic acid. The resulting derivative was conjugated to BSA by use of the EDC/NHS method [27].

To prepare the CTBE conjugate, cotinine was reacted with 3-bromo-1-propanol; the resulting alcohol reacted with 4-isothiocyanatobenzoyl chloride, which was produced by reaction of 4-isothyocyanatobenzoic acid with thionyl chloride. The resulting isothiocyanate cotinine derivative reacted with BSA to produce the corresponding conjugate. Cotinine carboxylic acid glycine derivative (CCAG) was synthesized by activating *trans*-4-cotinine carboxylic acid (Aldrich 34,757-4) with EDC/NHS, the activated compound then reacted with glycine *tert*-butyl ester hydrochloride (Aldrich 347957). The ester group was then removed with trifluoroacetic acid. The resulting cotinine carboxylic acid glycine derivative was conjugated with BSA by use of the EDC/NHS method.

Immobilization of reagents

The control line solution was prepared by diluting goat anti-rabbit IgG to 0.1 mg mL^{-1} in various application buffer

Table 1 Cotinine derivatives that were tested as capture lines in the cotinine assay device developed

Derivative name	Structure
CCA Trans-4-cotinine carboxylic acid	
CCAG Trans-4-cotinine carboxylic acid glycine	
CCM Cotinine (5-carboxymethyl) ion	
CTBE Cotinine (2-((4-isothiocyanatobenzoyl)oxyl) ethyl)ion	

mixtures described later. The test line solution was prepared by diluting the different cotinine–BSA derivatives to 0.1 mg mL^{-1} in application buffer. The control and test line solutions were applied to the nitrocellulose membrane (190 mm×25 mm) at 2 µL cm^{-1} by use of the Camag Linomat 5 reagent application device.

The membrane was dried in an incubator at 37 °C overnight, and then transferred to a desiccated chamber at ambient temperature and 20% relative humidity.

Strip assembly

After the membranes had been dried, they were assembled into assay cards (laminate backing, nitrocellulose membrane with control and test lines, absorbent pad, and sample pad) using the Kinematic Matrix 2210 Universal Card Laminator and cut into 3.8-mm strips using the Azco Sur-Size model SS-4 automatic guillotine cutter. The assay strips were then placed into lateral flow assay cassettes provided by Arista Biologicals.

Oral fluids collection

No specific collection device was used for oral fluids. Volunteers provided small specimens in 12 mm×75 mm tubes. The samples were not centrifuged and were used fresh to mimic typical on-site testing procedures.

Test procedure/optimized procedure

Purified antibodies were diluted in buffer (running buffer described below). A total of 2 μL protein A–gold conjugate (30 nm diameter, OD 50 @ 530 nm) was added to a test tube with 50 μL running buffer/antibody solution and 50 μL oral fluid sample filtered through a 0.45-μm Nylon membrane. This solution (100 μL) was dispensed on the sample pad of the lateral flow device. Test strips were interpreted using the Alverix reader between 15 and 25 min after dispensing of all solutions. The reader completed each analysis in 6 s.

Test optimization

Application buffer

The effect of salts and methanol present in the application buffer was studied. The objective was to determine the application buffer composition resulting in the lowest non-specific binding (NSB). Buffers tested included 5 mmol L^{-1} phosphate buffer, pH 7.2, with 3% methanol, 10 mmol L^{-1} phosphate buffer, pH 7.2, 10 mmol L^{-1} phosphate buffer, pH 7.2, with 3% methanol, and HEPES buffer, pH 7.0, with 0.24 mg mL^{-1} BSA and 3% methanol.

Running buffer

The effect of the running buffer on NSB was also studied. Buffers tested included 10 mmol L^{-1} phosphate buffer, pH 7.2, containing 1% BSA, 2% BSA, 1% PVP, or 1% Tween 20 and 10 mmol L^{-1} HEPES, pH 7.0, containing 1% BSA and 1% Tween 20+1% tryptone.

Sample pretreatment

As described in the "*Introduction*", oral fluid is a complex mixture of proteins, mucus, and other substances that can interfere with immunoassays. That interference is evident in lateral flow assays and can be seen as aggregation of reporter gold particles. Often this is solved by diluting the sample; however, for second-hand smoke detection, the concentration of target analyte is low and dilution further reduces the analytical sensitivity of the assay. Our work therefore focused on developing a method to pretreat the sample with minimum dilution. Different strategies for

pretreatment were evaluated, including oral fluid clarification by centrifugation, immunoglobulin removal by affinity chromatography, centrifugal filtration through devices with 3 kDa, 30 kDa, or 100 kDa molecular weight cut off (Millipore), and simple filtration through active adsorbent materials, for example nitrocellulose and Nylon.

Results and discussion

Alverix reader method development

The Alverix reader is a small, portable reader designed to interpret on-site lateral flow devices. It uses a CMOS detector with proprietary software that enables calibration and background correction of assays. In our experiments the software was calibrated with a device containing an unused test strip. As fluid travels up a strip, the reader takes sequential readings and plots the intensity to create a curve. The resulting curve shows the development of the test readout at both the test and control lines. Alternatively, the reader may determine a fixed end point or take multiple readings that may be plotted.

Figure 1 shows results for a negative cotinine sample read in kinetic mode using the Alverix reader. After 15 min the test-to-control line ratio reaches a relatively stable value; however, the individual values of the test and control lines increased steadily during the time studied and did not reach steady state. This finding indicates that the ratio is a better indicator for the system than the individual line values and under the conditions tested provides a reliable result after 15 min.

Application conditions

Lines were applied to a nitrocellulose membrane using a CAMAG Linomat 5 semiautomatic reagent application device, at a rate of 0.2 μg protein cm^{-1}. Two lines were created, the control line formed by goat-anti rabbit IgG and a test line with BSA–cotinine derivative conjugate. Tests were performed to determine the effect of salt content in the application solution on the quality of the lines formed and NSB. The results showed that addition of sodium chloride to the application solution promoted the formation of artifacts and reduced the quality of the lines.

The use of alcohols to improve protein binding to nitrocellulose has been suggested in the literature [28]. Our studies focused on the use of methanol. It has been suggested that the presence of methanol helps rewet the membrane, reduces static charge, and destabilizes proteins helping them bind. To study these effects, a series of experiments was performed in which the quality of the applied lines was evaluated for NSB by examining

Fig. 1 Test and control line
kinetic monitoring. This figure
demonstrates typical develop-
ment curves for the test and
control lines, with their calcu-
lated ratios. Although individual
signals increase continuously,
the ratio is relatively consistent
after 15 min; therefore any
reading based on the absolute
value of the test line signal, will
change with time whereas a
reading based on the ratio will
be relatively constant within
these time intervals
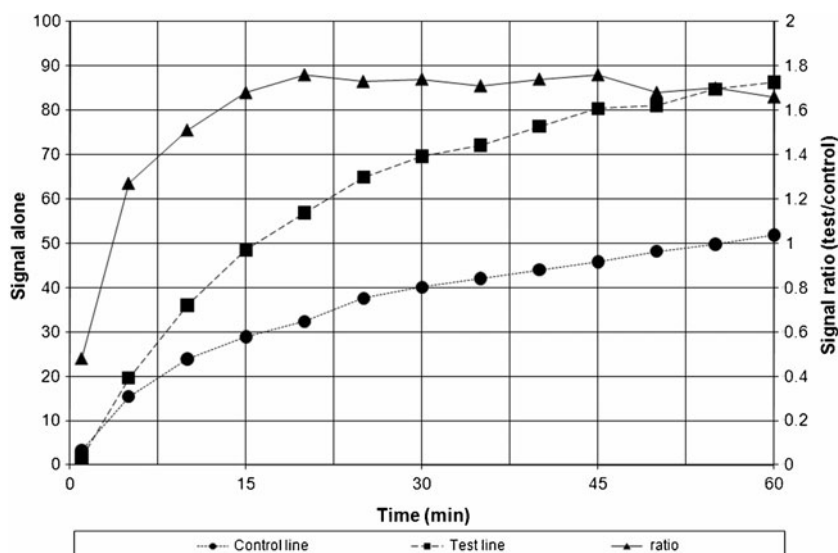
intensity, area, and consistency in the presence and absence of methanol in the application buffer. Also displacement values, defined as the percentage difference between the signals with no cotinine and those with cotinine, were measured between 0 and 2 ng mL^{-1} and 0 and 20 ng mL^{-1}. The experiments were conducted in triplicate and showed that despite the beneficial effects reported in the literature, the presence of methanol in the application solution does not seem to increase the quality of either the control or the test line in our system. Similarly, when the displacement values were calculated from the readings taken by the Alverix device, no significant improvement was observed in experiments in which methanol was used; in fact, when the sample was spiked with 2 ng mL^{-1} cotinine, the displacement was higher in the strips where methanol was not used.

The effect of the application buffer on non-specific binding was studied by applying three lines with solutions containing 5 mmol L^{-1} phosphate buffer, pH 7.2, with 3% methanol, 10 mmol L^{-1} HEPES buffer, pH 7.0 with 3% methanol, and 10 mmol L^{-1} HEPES buffer, pH 7.0 with 3% methanol+0.24 mg mL^{-1} BSA. None of these lines contained BSA–cotinine conjugate. Running buffer containing 1% BSA and 1% Tween 20 in 10 mmol L^{-1} HEPES buffer, pH 7.0, was prepared and used to evaluate two reporting solutions, one containing protein A–gold conjugate and another containing uncon-jugated gold nanoparticles. No cotinine antibody was present in the running solution. As a result, any signal observed, was the consequence of the non-specific binding of gold nanoparticles to the applied lines. On the basis of the results from these experiments the optimum applica-tion buffer was 5 mmol L^{-1} phosphate buffer, pH 7.2.

Running buffer

In order to test the effect of the running buffer composition on line quality and NSB, six different solutions containing no cotinine antibodies were formulated and tested. The running buffer was mixed with protein A–gold and run on strips with test and control lines prepared with 5 mmol L^{-1} phosphate buffer, pH 7.2, containing cotinine conjugate and goat anti rabbit IgG on each line. On the basis of the results, the buffers PBS+1% BSA, PBS+2% BSA, PBS+1% PVP and PBS+1% Tween 20 were rejected because of high NSB in the test line and thick and not well defined control lines. Use of the buffer HEPES+1% BSA, did not result in NSB but the control line was of low intensity. The buffer 10 mmol L^{-1} HEPES, pH 7.0, + 1% (v/v) Tween 20+1% (w/v) tryptone resulted in a very well defined control line and no NSB; therefore it was selected as the running buffer for the assay.

Cotinine derivatives

The three cotinine derivatives used to develop the assay are shown in Table 1. All derivatives were conjugated to BSA using the NHS/EDC method at a cotinine derivative-to-BSA molar ratio of 35:1 [29]. Additionally the CCA cotinine derivative was conjugated at a cotinine-to-BSA molar ratio of 2:1, also using the NHS/EDC.

All the conjugates were tested for binding and displace-ment in lateral flow devices using 0, 2, and 20 ng mL^{-1} cotinine. The strips were read between 15 and 30 min using the Alverix reader, and the ratio of the intensity of the control line to the test line was calculated. The results are shown in Fig. 2.

Fig. 2 Comparison of displacement performance of several conjugates. CCA 35:1 conjugate gave displacements higher than 20% with 2 ng mL^{-1} cotinine. CCAG 35:1 and CTBE 35:1 gave displacements of approximately 15% at 2 ng mL^{-1} cotinine. With cotinine concentrations of 20 ng mL^{-1}, the displacement increased to approximately 45% with CCA 35:1, 35% with CCAG 35:1, and only 20% with CTBE 35:1. CCA 2:1 resulted in poor performance and CCM 35:1 gave no displacement
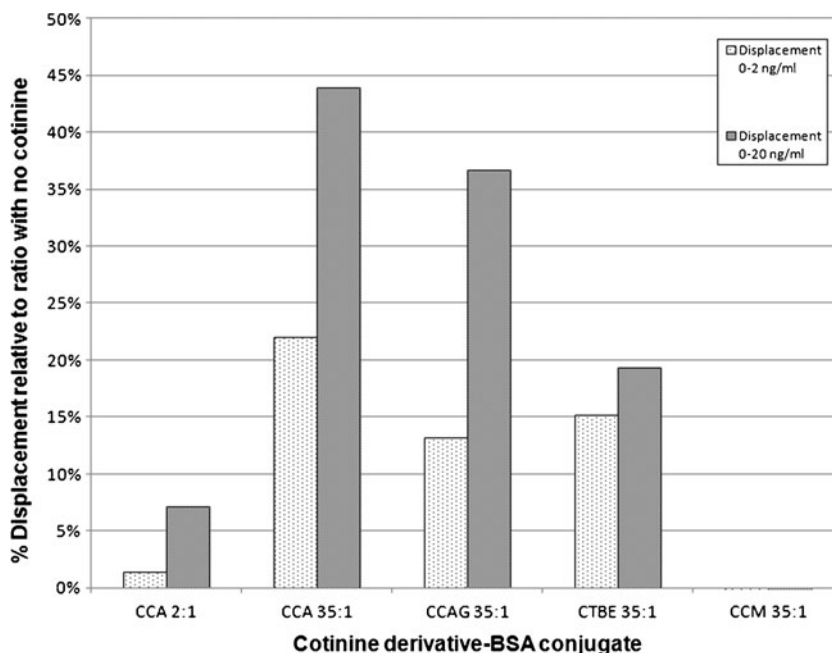

As seen in Fig. 2, the CCA 35:1 derivative offered the highest displacement thus performing best, followed by the CCAG, the CTBE, and the CCA 2:1 which gave very low signals (data not shown). There was no displacement of the CCM derivative. Subsequent experiments were, consequently, performed with the CCA derivative conjugated using a cotinine derivative-to-BSA ratio of 35:1.

Optimized reagent demonstration

Figure 3 shows the results obtained from standards (0–20 μg mL^{-1}) tested with the combination of optimized application buffer, running buffer, and cotinine conjugate. The test-to-control signal ratio was calculated and compared with the respective ratio with zero cotinine. NSB was negligible.

The signal ratio becomes significantly smaller as the concentration of cotinine increases in the sample, being 74% of the ratio at zero with 2 ng cotinine mL^{-1}, 48% with 20 ng cotinine mL^{-1}, 39% with 200 ng cotinine mL^{-1}, and 4% with 20 μg cotinine mL^{-1}. These results show that nonspecific binding in the system is minimal, and that the system is suitable for estimation of cotinine equivalents between 0 and 20 ng mL^{-1}.
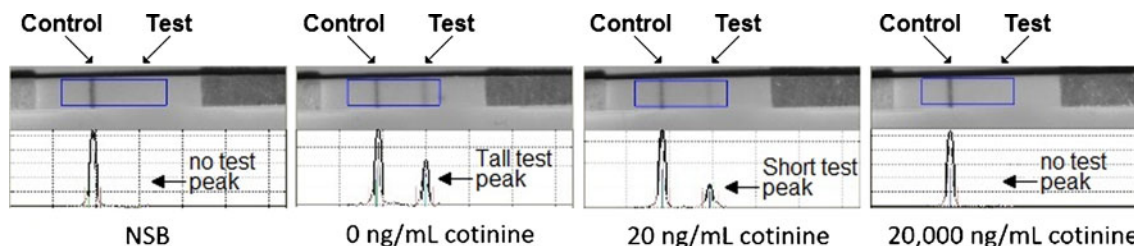
Cotinine concentration (ng/mL)	NSB	0	2	20	200	20,000
Ratio (test/control)	0.002	0.75	0.55	0.36	0.29	0.03
% displacement relative to zero	—	0	26	52	61	96

Fig. 3 Optimized cotinine reagents: minimum NSB and dynamic range of the rapid assay using optimized reagents are shown

Sample pretreatment

Using the optimized reagents we then addressed the issue of running whole oral fluids. Fresh oral fluid samples were collected from volunteers and spiked with various concentrations of cotinine. The samples were mixed at a 1:1 ratio with running buffer and placed on to test strips. When oral fluids were used, noticeable aggregation of gold nanoparticles at the interface of the sample pad and the nitrocellulose membrane was observed. Additionally, low or no bands were observed at the test and control lines.

Different strategies were tried to eliminate this aggregation: sample clarification by centrifugation, protein A purification of the sample, sample dilution, and sample filtration.

For example; to test clarification of the sample, oral fluid was centrifuged and the pellet discarded, the remaining supernatant was used in the rapid test. To evaluate the effect of immunoglobulin removal, the sample was passed over a Protein A column (Biorad), and the eluent was collected and run in the test. After running these experiments, formation of aggregates was still observed, suggesting that elimination of mucins by centrifugation or removal of IgG/IgA by Protein A chromatography did not remove the interferants. To evaluate the effect of sample dilution, oral fluid samples were mixed with buffer at different ratios before running. Reduction of the aggregation effect was observed only at dilution ratios higher than 1:8. Although this is an easily implemented solution, reduction of analyte concentration resulting from the dilution diminishes the sensitivity of the assay.

Another strategy implemented to eliminate aggregation was centrifugal filtration of whole or clarified oral fluid before analysis. Oral fluid samples were placed in micro centrifuge tubes which had been adapted with a membrane with well-defined molecular weight cut-offs of 3 kDa, 30 kDa, and 100 kDa. They were centrifuged at high speed and the filtrate or retentate was then used in the lateral flow test. In these experiments, elimination of the aggregates was observed for all the filtrates, irrespective of the molecular cut-off of the membrane; furthermore, the sample remain-ing as retentate aggregated the gold nanoparticles, suggesting that the interfering agent is present in oral fluids and eliminated by the filter membranes. This method, although effective, is impractical for point of care; therefore alternative filters not requiring centrifugation were tested.

Nitrocellulose and Nylon filters (0.2 μm pore size) were tested by expressing oral fluids spiked with known cotinine concentrations through the membrane using 3 mL syringes coupled to a 13-mm filter adapter. Both materials gave good results, minimizing the aggregation and resulting in well defined lines and displacement for amounts between 0 and 2 ng mL^{-1}; however, the small pore size required relatively high hand-pressures to express the liquid, increasing the risk of membrane rupture; therefore 0.45 μm pore size Nylon filters were also tried. In this case the larger pore size enabled filtration without high pressure while minimizing the interferant effect.

Test reproducibility

After experimenting with potential approaches to improve performance using whole oral fluids, a series of experiments was conducted to examine the reproducibility of the method with spiked oral fluid samples containing cotinine at concentrations of 2 ng mL^{-1}, tested after filtration to remove interferants. Table 2 shows the results of five days of testing. A different strip preparation was evaluated on each day. Also, fresh samples were collected on each day and evaluated independently of each other. As a result, each testing day reported corresponds to a unique combination of manufacturing conditions and samples collected from volunteers. In general the intra-day results were reproducible with coefficients of variation (CV) in the range 7–17%. Different average ratio values were obtained on different days. This is not unexpected if we consider that manufacturing conditions varied between preparations; however, the average displacement values were reasonably consistent, between 35% and 45%, and the average ratio at 2 ng mL^{-1} plus two standard deviations was never higher than the average ratio at 0 ng mL^{-1} minus two standard deviations; therefore, under the test conditions the assay could

Table 2 Inter-assay reproducibility of the test with oral fluids spiked with cotinine at 2 ng mL^{-1}

Inter assay precision (day)	Average ratio at 0 ng/mL	Number of samples (n)	CV of signal at 0 ng/mL (%)	Average ratio at 2 ng/mL	Number of samples (n)	CV of signal at 2 ng/mL (%)	Average displacement from 0–2 ng/mL (%)
Day 1	0.94	5	7	0.52	5	17	45
Day 2	1.07	3	9	0.64	3	13	40
Day 3	1.29	3	7	0.76	3	5	41
Day 4	1.31	12	11	0.83	5	5	37
Day 5	1.71	11	11	1.11	4	7	35

Fig. 4 Cross-reactivity of nicotine and different nicotine metabolites. Cross-reactivity greater than 85% was observed for *trans*-3′-hydroxycotinine-d3. Because 33–40% of metabolized cotinine is excreted as *trans*-3′-hydroxycotinine, the sensitivity of the assay to detect second-hand smoke exposure is enhanced
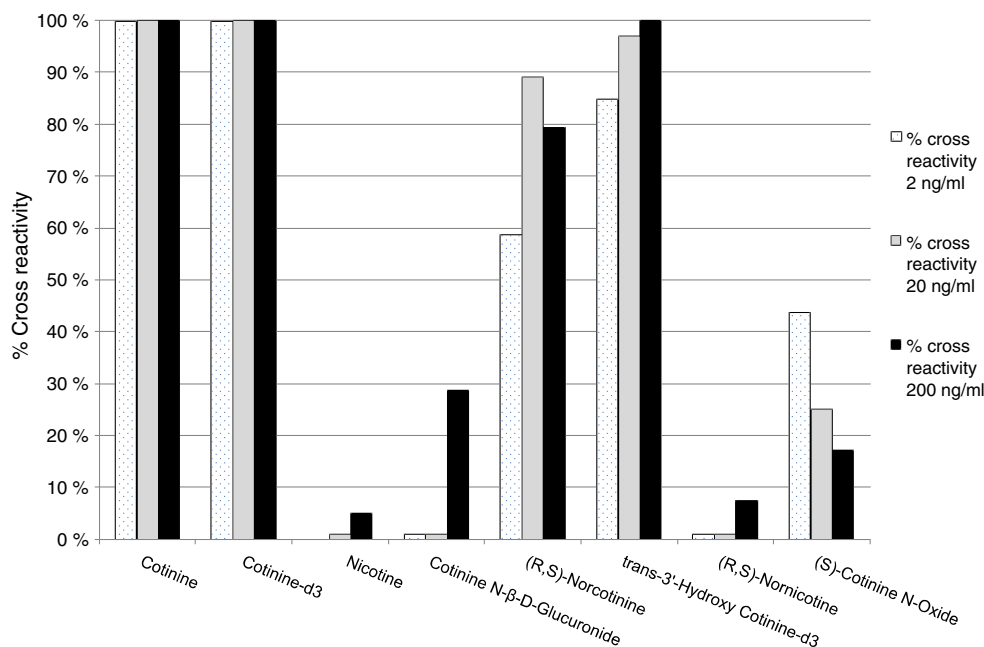
discriminate between 0 and 2 ng mL^{-1} cotinine with 99.99% confidence. This supports the assertion that the assay is capable of detecting relevant concentrations of cotinine.

Device testing with different nicotine metabolites

Small molecule toxicology immunoassays often cross react with metabolites of the parent drug. To determine the precise cross reactivity of the newly developed cotinine assay, Nicotine and six analog compounds or metabolites of nicotine were tested: cotinine *N*-β-D-glucuronide, (*R,S*)-norcotinine, *trans*-3′-hydroxycotinine-d3, (*R,S*)-nornicotine, (*S*)-cotinine *N*-oxide, and cotinine d3. Cross reactivity was determined by comparison with cotinine. Oral fluids were collected from volunteers and spiked with from 0 ng mL^{-1} to 200 ng mL^{-1} of each metabolite. The spiked oral fluid was filtered through a 0.45 μm Nylon membrane and the

Fig. 5 Analysis of oral fluid samples collected from three volunteers after ingestion of 2 mg over-the-counter nicotine. The results suggest cotinine is detectable after approximately 30 min and is resident for at least 5 h
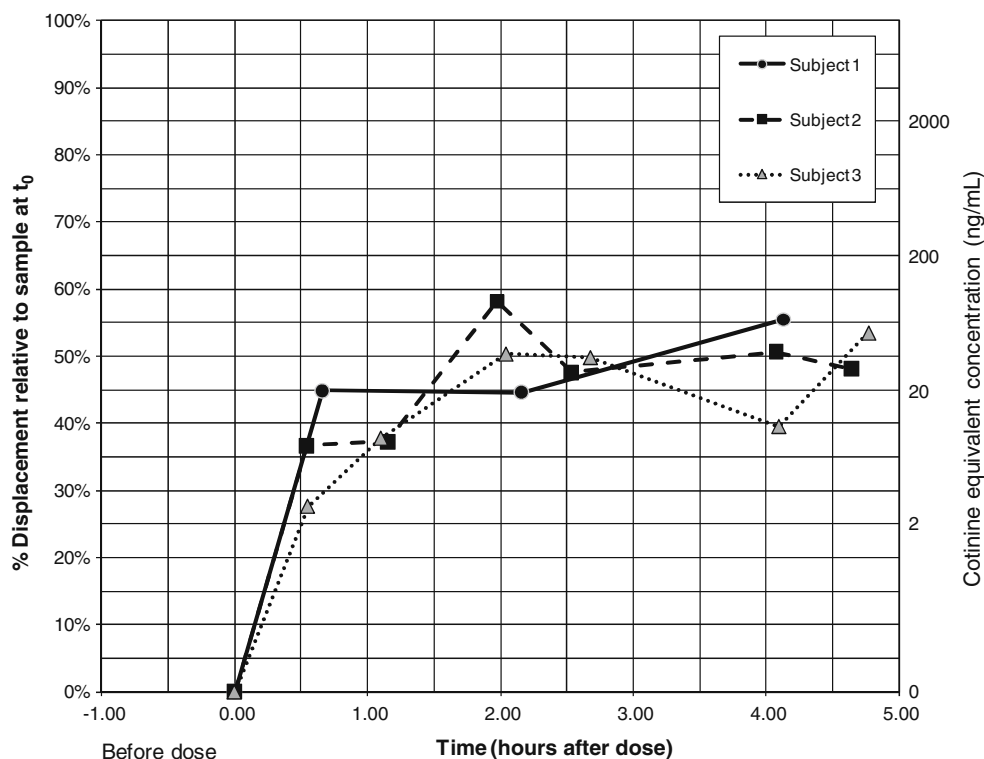
filtrate mixed in a 1:1 ratio with running buffer containing anti-cotinine antibody. The mixture was then applied to the test strip and read after 15 min with the Alverix reader.

The results demonstrated that *trans*-3-hydroxycotinine-d3 and (*R*,*S*)-norcotinine strongly cross-reacted at 20 ng mL^{-1} whereas nicotine and the other metabolites were detected only at higher concentrations (Fig. 4). These cross reactivity results suggest that the assay cutoff should be stated as "equivalents" to reflect the contribution from all potential metabolites.

Analysis of oral fluids after nicotine ingestion

After informed consent, three non-smoking volunteers who self-reported no exposure to SHS in the previous 48 h ingested 2 mg of an over-the-counter nicotine tablet. Whole oral fluid samples were then collected by expectoration into vials at intervals over a period of 5 h. Pre-dose samples were also confirmed to be negative for nicotine metabolites by use of LC–MS–MS and, therefore, in agreement with volunteers' self-report. Collected samples were then analyzed using the lateral flow test and Alverix reader. The results are shown in Fig. 5.

The device was able to detect the presence of nicotine metabolites in as little as 30 min after ingestion of nicotine. After 30 min the immunoassay suggests cotinine/metabolite concentrations greater than 2 ng mL^{-1} but never greater than 65 ng mL^{-1} were present over 5 h. Thus it seems that use of optimized reagents, filtration of the sample, and interrogation using the Alverix reader is a reliable method for measurement of low levels of nicotine metabolites.

Discussion and conclusions

Tobacco smoke is deleterious to health when actively or passively inhaled. Passive exposure is random and most often without consent. Additionally, no tools are available enabling those studying passive exposure to rapidly assess individuals. Oral fluids have recently been used as a medium to detect a variety of drug molecules, particularly drugs of abuse. They are also known to contain the major metabolites of nicotine.

Nicotine is rapidly metabolized to a number of metabolites, and laboratory tests already exist to routinely detect these in a variety of biological fluids, including oral fluids. However a rapid test capable of detecting nicotine metabolites at levels low enough to assess passive exposure is not available. The results presented in this report suggest that it is possible to use available antibodies with optimized reagents to detect cotinine concentrations appropriate for identification of passive tobacco smoke exposure. This can be accomplished using lateral flow methods and an inexpensive reader.

Lateral flow rapid tests are ubiquitous and have been used to detect numerous large and small molecular weight analytes. These tests, however, often suffer from variation because of nitrocellulose or local temperature/light conditions. Therefore lateral flow tests should be interrogated independent of the human eye in order to remove subjective judgment or to normalize test interpretation. This project utilized a new commercial reader from Alverix. The unit was robust and reliable, enabling kinetic measurement during test development. This characteristic, with data analysis, enabled sensitive detection of cotinine and other nicotine metabolites.

Additionally, our study showed that oral fluid tests are negatively affected by components of the glandular matrix. Although we did not identify the precise species responsible for the aggregation, it was shown that centrifugation or immunoglobulin removal could not prevent aggregation. Only extreme dilution or use of filtration through Nylon or nitrocellulose removed the interfering agent. Dilution suggests that the interference is concentration-dependent. However, unlike many laboratory tests that can tolerate such a dilution and still maintain analytical sensitivity, a rapid test is adversely affected by such a dilution. Therefore filtration through Nylon followed by minimal dilution in buffer seems to be a reasonable approach.

In summary, the use of an inexpensive reader combined with appropriate sample pretreatment and carefully selected buffers and reagents, makes it possible to detect cotinine equivalent concentrations of 2 ng mL^{-1} in as little as 15 min. These low-level measurements are suitable for detection of nicotine metabolites in oral fluid samples from individuals suspected of passive exposure to tobacco smoke.

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