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EVALUATION OF U.S. COMMERCIAL-OFF-THE-SHELF HAND-HELD ASSAYS TO DETECT OPIATE PAIN RELIEVER COMPOUNDS IN MULTIPLE BIOFLUIDS

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Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

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14. ABSTRACT:
Law enforcement, first responders, and the DOD community require fast, reliable, and inexpensive screening technologies for drugs and other compounds that can pose a threat to safety and health. However, there are gaps in the ability to detect many compounds in the field. For some compounds, the problem may be poor screening tests that can include false positives or impractical limits of detection, and others may have no field tests at all. In this study, we evaluated the potential for several U.S. commercial-off-the-shelf (COTS) hand-held assays (HHAs) to detect members of the opiate pain reliever class of compounds. Each HHA was developed for urine testing, and each came with its own stated claims of sensitivity and specificity. We evaluated the performance of three HHAs and tested for sensitivity and specificity to compounds in buffer conditions and in exposed animal biofluids. All HHAs successfully detected Compounds 1 and 2 in urine to varying degrees, some showed some detection of Compound 1 in saliva, and none detected Compound 3. In collaboration with the leading HHA company from this study, future work will functionalize and improve a COTS HHA platform for enhanced sensitivity of detection of Compound 1 in saliva and plasma.

15. SUBJECT TERMS

Hand-held assay (HHA)	Blood	Plasma	Serum
Compounds 1–3	Saliva	Urine	Opiate pain reliever (OPR)

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EXECUTIVE SUMMARY

Law enforcement, first responders, and the DOD community require fast, reliable, and inexpensive screening technologies for drugs and other compounds that potentially pose a threat to safety and health. However, gaps in screening abilities remain between low-cost, low-technology products and the field-ready detection of many of the compounds of interest.

One class of compounds that many in the First Responder community are encountering and treating with greater frequency, due to abuse and overdose, is the opiate pain reliever (OPR) class. To accurately diagnose and treat individuals as quickly as possible, a rapid, low-technology, and low-cost field test is required. However, to date there has been no head-to-head testing of the commercial-off-the-shelf (COTS) lateral flow, hand-held assays (HHAs) produced in the United States.

To address this gap, we evaluated the ability of several current COTS HHAs, manufactured in the United States, to specifically identify several members of the OPR class of compounds. Each of the HHAs evaluated were developed for urine testing, and each came with its own stated claims of sensitivity and specificity. None of the HHAs were FDA approved for diagnostic use, and none were available for laboratory use only. We evaluated the performance of three HHAs, testing for sensitivity and specificity to compounds in buffer conditions and in exposed animal biofluids. All HHAs successfully detected two main compounds (Compounds 1 and 2) in urine to varying degrees, a few HHAs showed some detection of Compound 1 in saliva, and none detected a less frequently encountered compound (Compound 3) at all.

This work successfully identified the most-sensitive HHA available in the United States for this class of compounds and created an opportunity to further optimize and develop a second, and possibly, a third field-deployable detection HHA using saliva or plasma. Working with the company responsible for the production of the leading HHA, we plan to develop COTS HHA detection platform(s) for the enhanced sensitivity of detection of Compound 1 in saliva and plasma.

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PREFACE

The work described in this report was authorized under grant number R.0010565.75.1 by the Department of Homeland Security (DHS). This work was started in June 2014 and completed in May 2015.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

Conclusions and opinions presented here are those of the authors and are not the official policy of the U.S. Army, U.S. Army Edgewood Chemical Biological Center (ECBC), or the U.S. Government.

Acknowledgments

The authors would like to thank the DHS Chemical Detection Division, specifically Dr. Kevin Anderson and Dr. George Famini for funding this work. The biofluid investigation aspect of this work was made possible by leveraging an ongoing project funded by the Defense Threat Reduction Agency, Joint Science and Technology Office. Additional thanks are extended to Michael Feasel, Daniel Carmany, Theodore Moran, Bernardina Gaviola, and Ruth Ann Moretz (ECBC) for their assistance in the animal work and biofluid collection. The authors are especially grateful to the technical writing and editorial assistance of Matthew Caples (Booz Allen Hamilton; Belcamp, MD), without whose work the polished market survey, product sheets, and clear graphical delivery of our testing results would not have been possible.

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EVALUATION OF U.S. COMMERCIAL-OFF-THE-SHELF HAND-HELD ASSAYS TO DETECT OPIATE PAIN RELIEVER COMPOUNDS IN MULTIPLE BIOFLUIDS

1. INTRODUCTION

Opioid pain relievers (OPRs) make up a class of compounds that are chemically related and interact with opioid receptors on nerve cells in the brain and nervous system to produce pleasurable effects and relieve pain.[1] These OPRs are abused for their intense euphoric effects and unfortunately, result in frequent cases of overdose, respiratory depression, and death. Based on research conducted by the World Health Organization, more than 2 million Americans are addicted to OPRs.[1] In 2008, prescription drug overdoses in the United States caused 36,450 deaths and OPRs were involved in 14,800 of those deaths (73.8%).[2]

There is currently no fielded, validated hand-held assay (HHA) that tests for a specific class of OPRs in the United States; although the European test market does have several options available. There are currently multiple HHAs that test for one or more members of a class of these compounds in the United States. However, none of the available tests have been officially tested and evaluated for the purposes of use with law enforcement, first responders, or military applications. In addition, the majority of these specific-compound HHAs can report testing only for one or two compounds in a urine matrix but not in any other biofluid. Therefore, they have not been tested for some of the other closely related compounds or their metabolites. The current gap in OPR HHA detection in the United States contributes to the problem of delayed diagnosis of the type of overdose in the field, which can prevent the timely and proper administration of treatment.

This project is a direct evaluation of HHAs that target a specific class of OPR compounds. We performed a market survey to down select HHA products to identify the top three that are based in the United States. These three HHAs were then analyzed for the primary compound that they were designed to detect, Compound 1. We evaluated detection performance of each HHA for Compound 1 in five different sample sources: blood plasma, serum, urine, saliva, and environmental samples. The limit of detection (LOD) and specificity were also determined for these products. The final results delineate the detection characteristics of each product and are the first step toward identifying the leading Compound 1-specific HHA that should be used in the field by law enforcement, first responders, and other members of the civilian and Department of Homeland Security community.

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[1] National Institute on Drug Abuse. *Drugs of Abuse: Opioids*. National Institute on Drug Abuse: Bethesda, MD, 2015. http://www.drugabuse.gov/drugs-abuse/opioids (accessed 7/1/16).

[2] Centers for Disease Control and Prevention (CDC). *Morbidity and Mortality Weekly Report (MMWR), Vital Signs: Overdoses of Prescription Opioid Pain Relievers – United States, 1999–2008*; CDC: Atlanta, GA, 2011. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6043a4.htm (accessed 7/1/16).

2. MATERIALS AND METHODS

2.1 Reagents

Reagents: HHAs were purchased from American Screening Corporation (Shreveport, LA; ONEScreen ASC-DFE-114), Creative Diagnostics (Shirley, NY; part no. DTS237), Express Diagnostics International, Inc. (Blue Earth, MN; Drugcheck 30102), and Alfa Scientific Designs, Inc. (Poway, CA; part no. 03-4872). Glass plates were purchased from R.G. Collins Glass Company, Inc. (Dundalk, MD), and environmental sampling swabs were purchased from Fisher Scientific (Pittsburgh, PA; part no. 14960202). Compounds 1 and 2 were purchased from Sigma-Aldrich Company, LLC (St. Louis, MO). Compound 3 was synthesized by U.S. Army Edgewood Chemical Biological Center (ECBC) personnel. The metabolite of Compound 3 was also purchased from Sigma-Aldrich.

HHAs: Each HHA was used in accordance with the manufacturer's instructions but with a few changes. Dipstick assays were dipped into 200 µL fluid in a polymerase chain reaction plate well. Either 120 µL (Creative Diagnostics) or 150 µL (Alfa Scientific Designs) samples were pipetted into each cassette assay, instead of using the provided droppers.

2.2 Animal Inoculation

Young adult male New Zealand White rabbits, weighing 2.5–2.7 kg, were procured from Covance, Inc. (Princeton, NJ) and pair-housed for 3 days before testing. Husbandry, feed and water provisions, and sanitation schedules were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals*.[3] Rabbits were on a cycle of 12 h of light and 12 h of dark. Individual rabbit rooms were maintained at 70 ± 2 °F with 30–70% relative humidity. Rabbits were housed in a facility that was fully accredited by the Association for Assessment and Accreditation of Laboratory Care International. Rabbits were pair-housed in plastic cages on racks and provided with certified laboratory chow and reverse-osmosis water ad libitum, except during testing. Animal care and use for these experiments was approved by the Institutional Animal Care and Use Committee (IACUC) for ECBC (Aberdeen Proving Ground, MD).

Animal OPR exposures were conducted in accordance with a protocol approved by the ECBC IACUC. Exposures took place for each group (n = 4) while the animals were mated to a nose-only exposure chamber, 20 cm^3 inner volume, with a flow rate of 19 L/min and under pressure of –0.5 in. H_2O. Chamber concentration was measured in real time using a TSI, Inc. Dust-Trak II, model 8530 (Shoreview, MN). Two glass fiber filter pads and a 7-stage cascade impactor were used for chamber concentration and particle sizing, respectively. Filter pads and

[3] Committee for the Update of the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research; Division on Earth and Life Studies; National Research Council. *Guide for the Care and Use of Laboratory Animals.* 8th ed.; The National Academies Press: Washington, DC, 2011.

stages were analyzed using liquid chromatography (LC)–tandem mass spectrometry (MS-MS) with an Agilent LC triple quadrupole 6490 system (Agilent Technologies, Inc.; Santa Clara, CA). Animal exposure durations were 5 min, with 50% effective concentration–time (ECT_{50}) doses of 0.2 ECT_{50}, 1 ECT_{50}, and 3 ECT_{50}. ECT_{50} was measured as the collapse of the rabbit.

2.3 Animal Sample Collection

Euthanasia was performed in accordance with the *American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition*.[4] Rabbits did not have visual or auditory access to the euthanasia of other rabbits. The method of euthanasia was cervical dislocation of the C1 vertebra using a stainless steel RP-3000 rabbit and poultry wringer (MHS, LLC; West Grove, PA). The death of a rabbit was verified by three methods: loss of pupillary light response, retrobulbar reflex, and loss of respiration or cardiac arrest. Terminal blood and tissue harvest immediately followed the cervical dislocation procedure.

Samples were collected from three inoculated rabbits, as shown in Figure 1. Blood, plasma, urine and saliva were collected at 30 min and at 4 and 24 h time points.

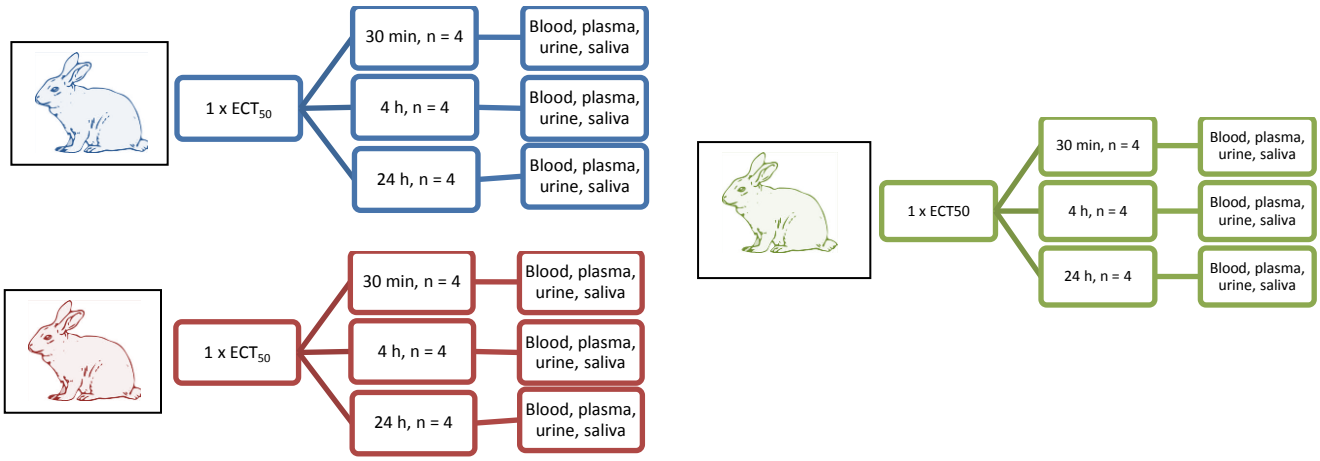


Figure 1. Schematic of sample collection from rabbits exposed to three compounds of interest.

A total of 36 samples each of blood, plasma, urine, and saliva were obtained for the three compounds of interest (n = 144).

Immediately after confirmation of euthanasia, blood was removed via a heart stick. The blood sample was directly drawn from the heart chamber with a single stick. The BD P100 Blood Collection System (Becton, Dickinson, and Company [BD]; Franklin Lakes, NJ)

[4] American Veterinary Medical Association (AVMA). *American Veterinary Medical Association Guidelines for the Euthanasia of Animals*: 2013 Edition; AVMA: Schaumburg, IL, 2013.

was used to remove the blood from the syringe. The tube was inverted five times to ensure distribution of the anticoagulant, and it was placed on ice. In accordance with the manufacturer's instructions, the tubes were then spun at 2500 × *g* for 20 min at 4 °C to separate the plasma. The plasma was carefully removed from the tubes and aliquoted before storage at –80 °C.

Urine samples were collected using syringes during the necropsies. Saliva samples were collected by buccal lavage using 1 mL cold saline and suction to remove as much saliva as possible (~1.2–1.4 mL).

2.4 Environmental Sample Collection

Samples were collected in triplicate over a range of approximately 10 operationally relevant concentrations for two collection scenarios involving three compounds of interest. In total, 180 environmental samples were collected and tested. As illustrated in Figure 2, the operational scenarios tested were as follows: (1) A dried, adherent compound (or blank control) on a smooth surface (e.g., glass) was swabbed with a standard, foam-tipped applicator wetted with either deionized water (diH_2O), phosphate-buffered saline (PBS, pH 8), or ethanol (ETOH, 95%). (2) The liquid compound of interest was diluted in diH_2O, PBS, or ETOH and spotted directly on the HHA (volume was adjusted to equate to that of swabbed sample). In preliminary experiments, the optimal collection and sample diluents(s) were determined on the basis of their ability to avoid interference with the HHA assay performance. PBS was chosen because it was shown to be compatible with the assay, to be an effective diluent of these OPRs, and was a commonly available environmental sample collection medium. Concentrations of each condition tested are shown in Tables 1 and 2.

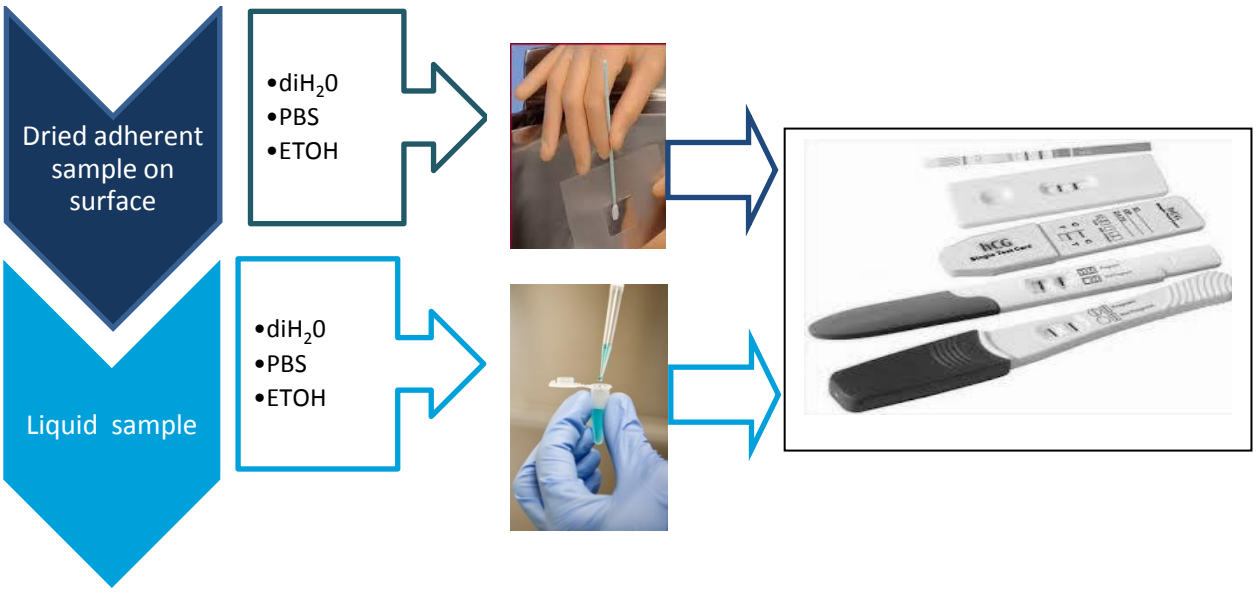


Figure 2. Environmental sample optimization.

Table 1. Liquid LOD Testing

HHA	Concentration (ng/mL)											
	5	10	20	25	30	40	50	75	100	125	150	200
Express Diagnostics	✓	✓	✓	✓	✓	✓	✓					
Alfa Scientific							✓	✓	✓	✓	✓	✓
American Screening							✓	✓	✓	✓	✓	✓

Note: Each of the three HHAs was tested at the indicated (✓) concentrations of Compound 1.

Liquid samples: Serum and plasma samples were diluted 1:400 in PBS for analysis. Saliva was diluted 1:3 in PBS, if the neat sample had an invalid result. Urine samples were not diluted.

Table 2. Solid LOD Testing

HHA	Concentration (ng/mL)				
	100	200	400	700	1000
Express Diagnostics	✓	✓	✓	✓	✓
Alfa Scientific	✓	✓	✓	✓	✓
American Screening	✓	✓	✓	✓	✓

Note: Each of the three HHAs was tested at the indicated (✓) concentrations of Compound 1.

Solid samples: For each 12 in. square glass plate, five 4 in. squares were delineated for samples. Samples were diluted in water, and 500 µL of sample was spotted onto each square and allowed to dry. For sampling, 5 mL of the total diluent was added to environmental swab, and the excess was squeezed into a collection tube. The swabs were used in accordance with the Centers for Disease Control and Prevention (CDC; Atlanta, GA) sampling protocol (http://www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthracis.html). Each swab was placed in a collection tube and gently mixed with the remaining diluent. Excess liquid was squeezed from the swab, and the diluent was used for the HHAs.

3. RESULTS

3.1 Market Survey

The execution of a market survey was required to determine the proper HHAs to use in the Compound 1 analysis. The market survey used a number of characteristics to narrow down the HHA selection, including the following questions:

- **U.S.-based company:** Are the HHAs produced by companies that have their primary address in the United States, or are they manufactured in the United States?
- **U.S.–based antibody production:** Are the antibodies used in the HHAs produced in the United States?
- **Intended matrices:** What matrices are the HHAs intended for?
- **HHA type:** Is the HHA a dipstick or a cassette type?
- **Performance:** What are the sensitivities and specificities of the HHAs?
- **Multiplex:** Are the HHAs used for a single analyte, or do they detect multiple analytes?

The results of the market survey are presented in the appendix. Eleven HHAs were identified on the basis of the criteria listed above, and of these products, 10 HHAs come from U.S.-based companies. After analyzing the survey data and determining availability, the field of HHAs was narrowed to the final three products:

- **Express Diagnostics International, Inc.**: DrugCheck was chosen for this study because it was listed as the most sensitive (10 ng/mL) HHA, and it can be purchased directly from the manufacturer.
- **Alfa Scientific Designs, Inc.**: Insta-View cassette was chosen because it had a stated sensitivity (200 ng/mL) that was roughly equivalent to the remaining products. In the market survey data, this HHA appeared roughly equivalent to the Creative Diagnostics product. However, in preliminary laboratory trials, the Creative Diagnostics product failed to yield a consistent positive signal in repeated positive-control tests, whereas the Alfa product performed consistently well.
- **American Screening Corporation**: Instant-View dipstick was chosen because it was a dipstick with a reported sensitivity comparable to the other HHA products (300 ng/mL).

3.2 Product Sheets

The three HHAs identified in the market survey and preliminary testing were moved forward into the final analysis to determine the best candidate for Compound 1 detection. The HHAs were tested using four different biofluids (urine, saliva, serum, and plasma) from rabbits inoculated at three different concentration levels of Compound 1. In addition, the LOD for each HHA was investigated using liquid and solid samples. All data for each HHA are presented in the following product sheets.

3.2.1 Alfa Scientific Designs, Inc. Product Sheet

alfa SCIENTIFIC DESIGNS

Product Sheet

Alfa Scientific Designs, Inc.

SYSTEM DESCRIPTION:

Alfa Scientific Designs, Inc., is a biotechnology company based in Poway, CA. This company is cGMP, as well as ISO13485 and ISO9001 registered. They produce in vitro diagnostic immunoassay products for the point of care market. The antibody used in their compound 1 cassette test is provided by a U.S. distributor. It is an IVD product, but not FDA-cleared, and therefore not available in the U.S. However, they were willing to sell the product to use with the label "For Forensic/Research Use Only". They also have cross-reactivity data for the nor metabolite of compound 1, with a cutoff of 500ng/mL. The cassettes are made to order, so require a lead time of 2-3 weeks. A dipstick version of the compound 1 test is listed in their product brochure, however, that version has been discontinued.

Related Compounds Analysis

Compound	[ ] ng/mL	Alfa
Compound 2	2000	⊖
Compound 2	1400	⊖
Compound 3	2000	⊖
Compound 3	1400	⊖
metabolite of Compound 3	2000	⊖
metabolite of Compound 3	1400	⊖

Key:

⊕ - *Positive*

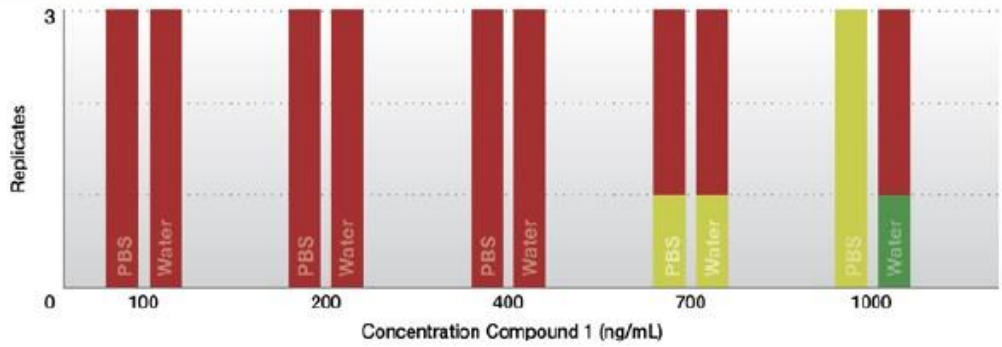
⊖ - *Negative*

! - *Negative, but very faint (almost positive)*

⚠ - *Invalid Assay*

ND - *Not Determined (no sample)*

Level of Detection Analysis: Solid Sample



BioFluid Sample Analysis

Compound 1

ANIMAL DOSE

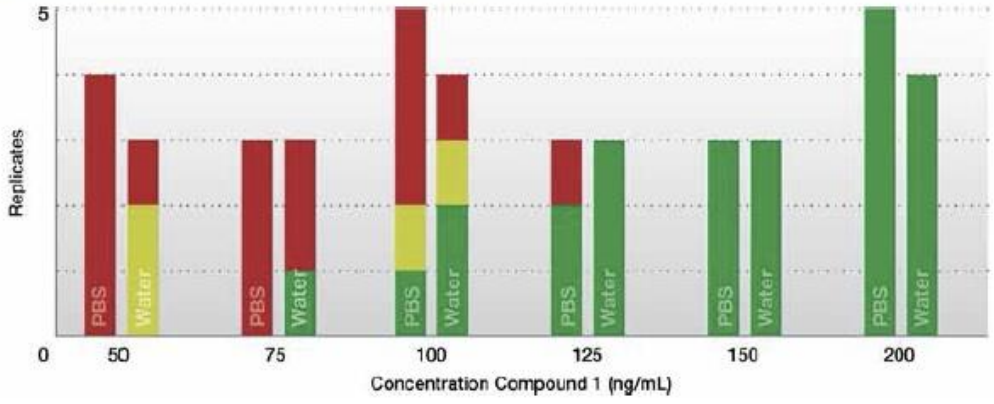
	High			Medium			Low			Negative Control		
Plasma	−	−	−	−	−	−	−	−	−	−	−	−
Serum	−	−	−	−	−	−	−	−	−	−	−	−
Saliva	+	−	−	f	−	ND	ND	ND	ND	−	−	−
Urine	+	+	+	+	+	+	+	+	−	−	−	−
Hours	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**

Compound 2

ANIMAL DOSE

	High			Medium			Low			Negative Control		
Plasma	−	−	−	−	−	−	−	−	−	−	−	−
Serum	−	−	−	−	−	−	−	−	−	−	−	−
Saliva	−	−	−	−	−	−	−	−	−	−	−	−
Urine	−	−	−	−	−	−	−	−	−	−	−	−
Hours	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**

Level of Detection Analysis: Liquid Sample



3.2.2 American Screening Corporation Product Sheet

AmericanScreening

Product Sheet

American Screening Corporation

SYSTEM DESCRIPTION:

American Screening Corporation is a biotechnology company based in Shreveport, LA. They are ISO13485 registered and CE marked. Their tests are manufactured in-house, as are the antibodies used for their compound 1 tests. The dip card (ONESCREEN), is assembled in China. The company was unclear about the difference between manufacturing and assembling a product. American Screening offers two different compound 1 immunoassays: one dipstick, and one dip card. They are both Forensic Use Only, and the dipstick is 510k. Both assays have a 24 month shelf life, with cutoffs of 200ng/mL and 300 ng/mL, respectively. Both tests cross-react with the nor metabolite of compound 1 with a cutoff of 50µg/mL.

Related Compounds Analysis

Compound	[ ] ng/mL	American
Compound 2	2000	⊖
Compound 2	1400	⊖
Compound 3	2000	⊖
Compound 3	1400	⊖
metabolite of Compound 3	2000	⊖
metabolite of Compound 3	1400	⊖

Key:

⊕ - *Positive*

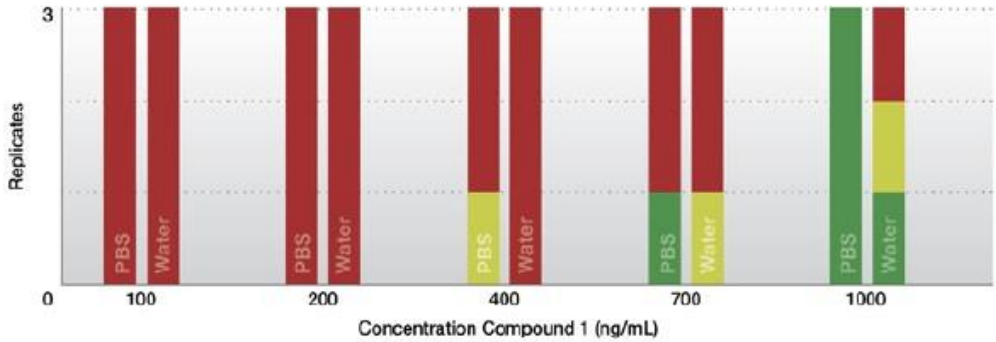
⊖ - *Negative*

! - *Negative, but very faint (almost positive)*

⚠ - *Invalid Assay*

ND - *Not Determined (no sample)*

Level of Detection Analysis: Solid Sample



BioFluid Sample Analysis

Compound 1

ANIMAL DOSE

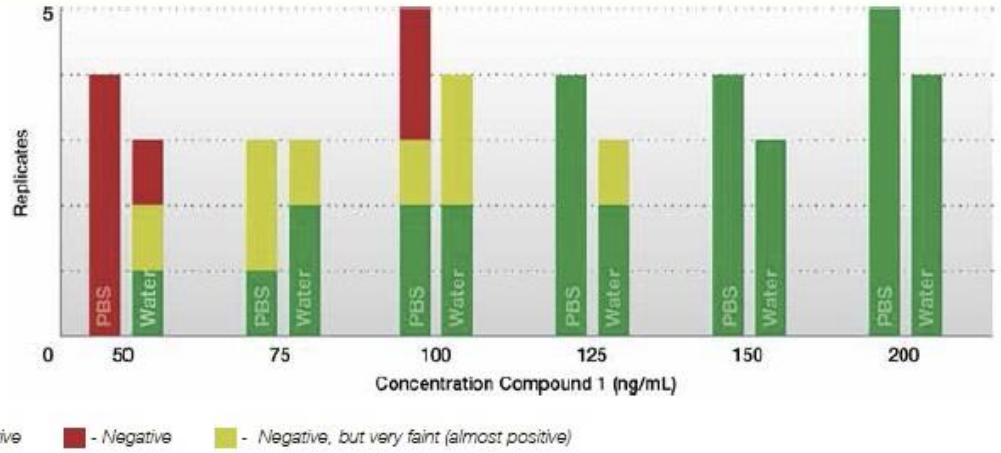
	High			Medium			Low			Negative Control		
Plasma	−	−	−	−	−	−	−	−	−	−	−	−
Serum	−	−	−	−	−	−	−	−	−	−	−	−
Saliva	f	−	−	−	−	ND	ND	ND	ND	−	−	−
Urine	−	f	−	−	−	−	−	−	−	−	−	−
Hours	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**

Compound 2

ANIMAL DOSE

	High			Medium			Low			Negative Control		
Plasma	−	−	−	−	−	−	−	−	−	−	−	−
Serum	−	−	−	−	−	−	−	−	−	−	−	−
Saliva	−	−	−	−	−	−	−	−	−	−	−	−
Urine	−	−	−	−	−	−	−	−	−	−	−	−
Hours	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**

Level of Detection Analysis: Liquid Sample



3.2.3 Express Diagnostics International, Inc. Product Sheet

express diagnostics international

Product Sheet

Express Diagnostics Int'l, Inc.

SYSTEM DESCRIPTION:

Express Diagnostics Int'l, Inc., is a biotechnology company based in Blue Earth, MN. They are ISO 13485:2003 registered and CE marked. They produce their products in-house, but the antibody used in the compound 1 test is provided by an outside vendor, still based in the United States. The company produces both cassette and dipstick compound 1 assays, both listed as Forensic Use Only.

In addition, the company requires potential customers to sign an FUO letter before ordering the tests. These assays have the lowest cutoff in the surveyed companies at 10ng/mL. They also provide cross-reactivity data for similar tests to compound 1. The assays are made to order, and require a lead time of 15 business days, with a shelf-life of 18 months.

Related Compounds Analysis

Compound	[ ] ng/mL	Express
Compound 2	2000	+
Compound 2	1400	f
Compound 3	2000	−
Compound 3	1400	−
metabolite of Compound 3	2000	−
metabolite of Compound 3	1400	−

Key:

+ - *Positive*

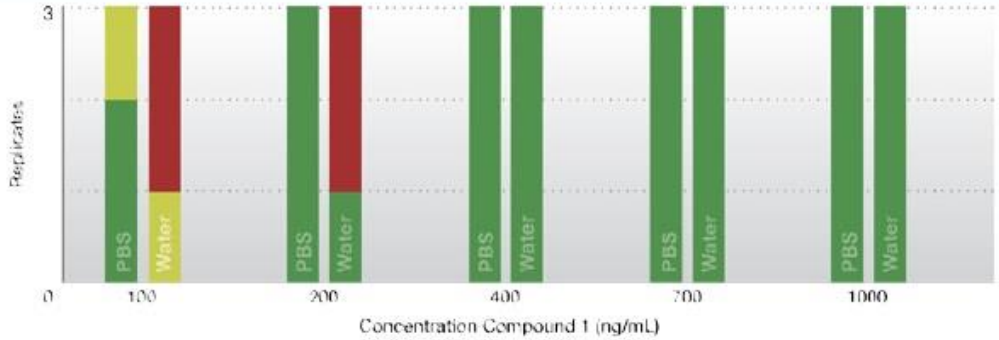
f - *Negative, but very faint (almost positive)*

− - *Negative*

⚠ - *Invalid Assay*

ND - *Not Determined (no sample)*

Level of Detection Analysis: Solid Sample



BioFluid Sample Analysis

Compound 1

ANIMAL DOSE

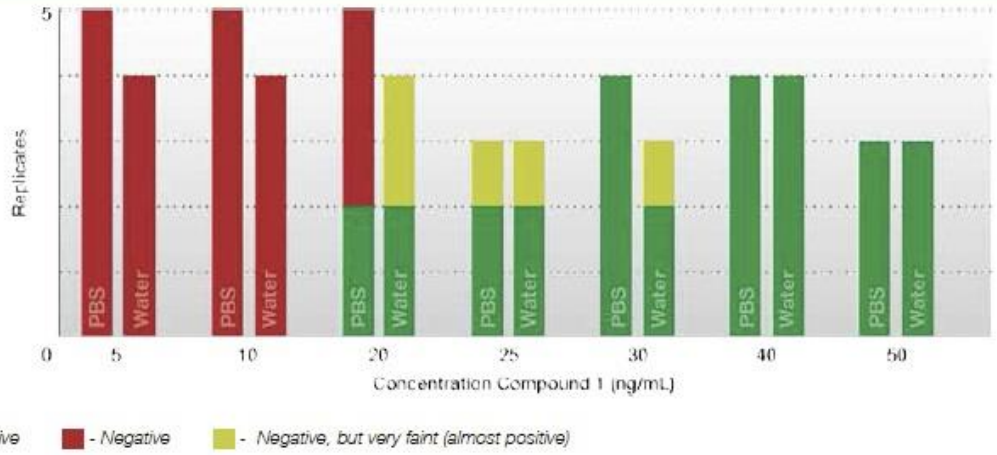
	High			Medium			Low			Negative Control		
Plasma	−	−	−	−	−	−	−	−	−	−	−	−
Serum	−	−	−	−	−	−	−	−	−	−	−	−
Saliva	+	f	−	!	−	ND	ND	ND	ND	−	−	f
Urine	+	+	+	+	+	+	+	+	+	−	+	−
Hours	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**

Compound 2

ANIMAL DOSE

	High			Medium			Low			Negative Control		
Plasma	−	f	−	−	−	−	−	−	−	−	−	−
Serum	−	f	−	−	−	−	−	−	−	−	−	−
Saliva	+	!	+	−	−	−	−	−	−	−	−	f
Urine	+	−	+	f	f	−	−	f	−	−	−	−
Hours	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**	**0.5**	**4**	**24**

Level of Detection Analysis: Liquid Sample



4. CONCLUSIONS

The three down-selected HHAs were analyzed for the detection of three OPRs, Compounds 1–3. Although the data are not shown in this report, trials using Compound 3 (at 2 µg/mL) were 100% negative. The results for Compounds 1 and 2 were strongly dependent on the biofluid tested. Urine is a typical matrix for OPR detection. Urine from rabbits exposed to Compound 1 was associated with strong detection when using the Express Diagnostics and Alfa Scientific Designs HHA products; however, the American Screening product was unable to detect at any of the available levels of Compound 1. All of the HHAs were less successful when used to detect Compound 1 in the remainder of the tested biofluids (serum, plasma, and saliva). The Express Diagnostics and Alfa Scientific Designs HHAs did exhibit some detection of the highest dose of Compound 1 in saliva at the 0.5 h time point, but none of the HHAs were able to detect Compound 1 in either plasma or serum.

In comparison with Compound 1, which was detected by all three HHAs, Compound 2 was not as easily detected by the HHAs. Only the Express Diagnostics HHA was able to detect Compound 2 in both urine and saliva samples at the highest level of rabbit exposure (3 ECT_{50}). However, the Express Diagnostics HHA was still unable to detect Compound 2 in either serum or plasma from the same rabbits. Neither the Alfa Scientific Designs nor the American Screening HHAs were able to detect Compound 2 across any of the four biofluids at any of the dose levels. The biofluid experiments with Compounds 1 and 2 indicate that the Express Diagnostics HHA was the top performer of the three down-selected HHAs. None of the HHAs were able to detect any of the compounds in blood plasma or serum.

The LOD for each HHA was determined using liquid suspension and solid forms of Compound 1. Tables 1 and 2 in Section 2.4 outline the concentrations of Compound 1 that were tested for each HHA, and the individual results for each HHA can be found on the product sheets (Sections 3.2.1 through 3.2.3). The liquid LOD testing indicated that the Express Diagnostics HHA, with a LOD of approximately 25 ng/mL, was far more sensitive than the American Screening or Alfa Scientific HHAs. The latter two HHAs had approximate LODs of 100 and 125 ng/mL, respectively. For the solid-phase LOD testing, Compound 1 powder was swabbed from a glass surface before testing and provided results similar to the liquid LOD testing. The Express Diagnostics HHA out-performed the other HHAs with an approximate LOD of 100 ng/mL, as compared with the Alfa Scientific (>1000 ng/mL) and American Scientific (approximately 900 ng/mL) HHAs. Although there was a large decrease in sensitivity, these results did indicate that the HHAs could be used for solid-surface sampling.

Analogs of Compound 1 are also used as pain relievers including Compounds 2 and 3 and the metabolite of Compound 3 (meta-Compound 3). These analogs are 2 to 100 times more potent than Compound 1. The three selected HHAs were tested against these three analogs to determine the specificity of the assays. Compound 2 was the only analog detected by any of the HHAs. Once again, the Express Diagnostics HHA was the most sensitive, with a positive response to Compound 2 at 2000 ng/mL and a faint, almost positive response at 1400 ng/mL. Neither Compound 3 nor meta-Compound 3 were detected using any of the three HHAs. These results indicated that the antibodies used in the HHAs were generally very specific

for the main OPR, Compound 1, with the possible exception of the Express Diagnostics HHA, which showed some cross-reactivity with Compound 2.

The collective results of this study indicate that the Express Diagnostics HHA was the most sensitive of the three products. In addition, the Express Diagnostics HHA showed some ability to detect Compound 1 in saliva, and it was the only product to demonstrate detection of Compound 2. While this cross-reactivity could be viewed as a detriment, it could also be viewed as an advantage in the case where the First Responder is more concerned with sensitive, rapid detection of any member of the OPR class.

ACRONYMS AND ABBREVIATIONS

AVMA	American Veterinary Medical Association
BD	Becton, Dickson, and Company
CDC	Centers for Disease Control and Prevention
diH_2O	deionized water
ECBC	U.S. Army Edgewood Chemical Biological Center
ECT	effective concentration–time
ETOH	ethanol
HHA	hand-held assay
IACUC	Institutional Animal Care and Use Committee
LC	liquid chromatography
LOD	limit of detection
MS-MS	tandem mass spectrometry
OPR	opiate pain reliever
PBS	phosphate-buffered saline

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APPENDIX: Market Survey

Principal Investigator (PI): Jennifer Sekowski, Ph.D. and Co-PI: Jennifer Gibbons, Ph.D.

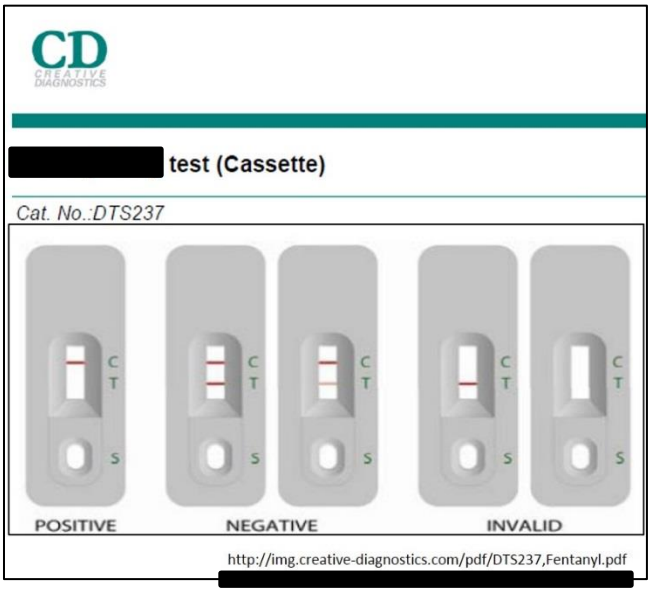
There are currently multiple hand-held assays (HHAs) that test for one member of the opiate pain reliever (OPR) class of compounds in the United States. However, most tests are only certified for urine samples, and have not been tested for OPR analogs or metabolites. In addition, the available HHAs have not been tested and evaluated for use with law enforcement, first responders, or military applications. The goal of our market survey was to identify the U.S.-based companies that sell immunoassays specific to the proposed class of compounds. Based on the survey results, we chose the top three available HHA products for bench evaluation.

Our first effort was to define what was meant by a "U.S.-based" company because many companies sell their products globally and operate at multiple locations. We first examined only tests that were produced by companies that have their primary address in the United States, although it was acceptable if they had subsidiary offices in other countries. Second, some companies were distributors; therefore, we also looked at the location where each product was manufactured to choose only products that were physically manufactured in the United States. The key component in an HHA is the antibody, so as a third step, we attempted to determine where the antibody was produced for each product. All companies either produced their own antibody or purchased the antibody through a U.S.-based vendor.

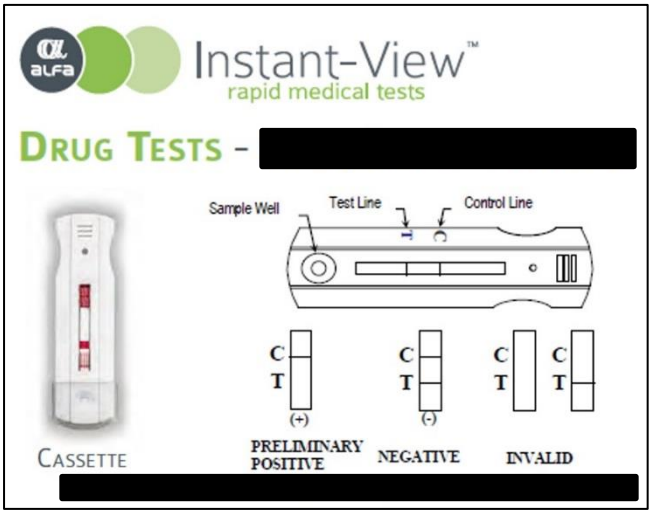
The next effort was to determine the relevant characteristics of each HHA product. First, all products were intended for use with urine and had not been tested with other biological fluids. Second, there are two major types of HHAs sold: dipsticks, which are dipped into a container of liquid sample for analysis, and cassettes, where the liquid sample is dripped into the appropriate well. Because the most-sensitive European assay (from Diagnostik Nord GmbH; Schwerin, Germany) is a dipstick type, we prioritized the same format for the U.S.-based HHAs. Third, we looked for assay sensitivity and specificity and determined the cutoff as well as any cross-reactivity, if these characteristics were noted in the product literature. In humans, Compound 1 is rapidly metabolized and excreted; therefore, cross-reactivity between Compound 1 and the metabolite of Compound 1 (meta-Compound 1) was appropriate.[*] Other cross-reactivity notes could enable us to determine if the test could identify Compound 1 derivatives as well. Finally, we focused on single assays. Many companies produce multi-analyte kits to test for multiple compounds simultaneously and can even include tests to check for sample adulteration. However, due to the focused nature of this project, we desired to reduce cost and only purchase assays for the analyte of interest. We also determined whether we could procure the necessary products, as described on the next several pages.

[*] Feierman, D.E., Lasker, J.M. Metabolism of Fentanyl, a Synthetic Opiod Analgesic, by Human Liver Microsomes - Role of CYP3A4. *Drug Metab. Dispos*. **1996,** 24 (9), 932–939.

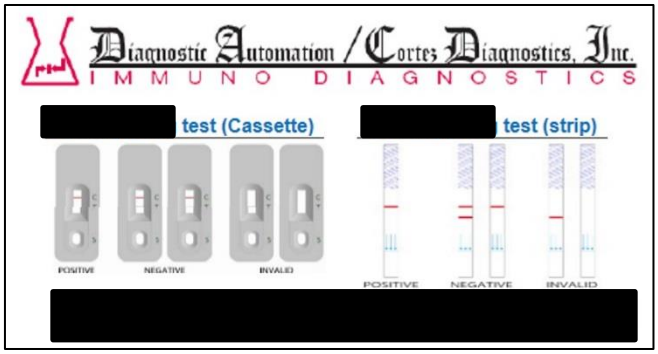
Creative Diagnostics is a biotechnology company, based in Shirley, NY, that is focused on the antibody market. They produce test cassettes and antibodies in-house and include the antibody used for their Compound 1 product. Their Compound 1 test was a cassette type that was used for urine samples only and was sold as a point-of-care (POC) product. The cassette's cutoff was roughly equivalent to some of the other products (200 ng/mL). No cross-reactivity was listed on the product insert. They also had contract assay and custom antibody segments of their company that could assist clients in developing and manufacturing immunoassays.



Alfa Scientific Designs, Inc., is a biotechnology company based in Poway, CA. This company follows the Current Good Manufacturing Practices (CGMP) and is International Organization for Standardization (ISO) 13485 and ISO9001 registered. They produce in vitro diagnostic immunoassay products for the point-of-care market. The antibody used in their Compound 1 product was provided by a U.S. distributor; however, they did not wish to provide the name of the distributor, nor did they know where the distributor received the antibody. Their Compound 1 test was also a cassette type, with the same cutoff as the Creative Diagnostics HHA (200 ng/mL). It was an in vitro diagnostics (IVD) product but was not cleared by the U.S. Food and Drug Administration (FDA), and therefore, was not available in the United States. However, they were willing to sell the product for use with the label "For Forensic/Research Use Only". They also had cross-reactivity data for meta-Compound 1, with a higher cutoff of 500 ng/mL. The cassettes were made-to-order and required a lead time of 2–3 weeks. A dipstick version of the Compound 1 test was listed in their product brochure; however, that version was discontinued.

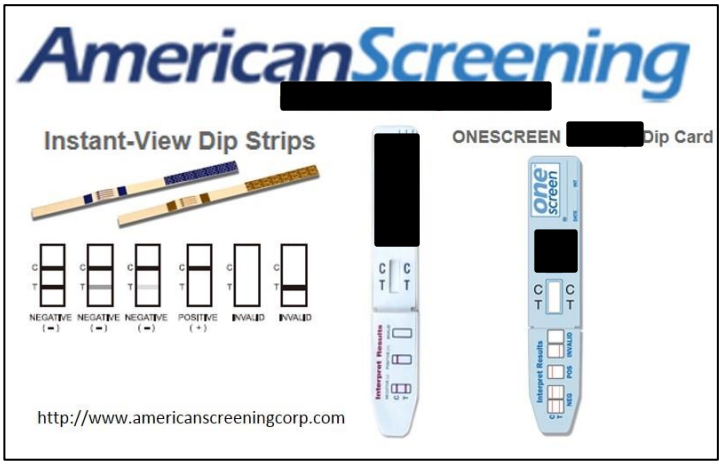


Cortez Diagnostics, Inc., (also called Diagnostic Automation) is a privately owned biotechnology company based in Calabasas, CA. The company produces both cassette and dipstick type Compound 1 tests. Both products are IVD, but not FDA-approved. However, this company was unwilling to sell their products to us, even under a "Forensic Use Only (FUO)/Research Use Only" label. In addition, the company was unwilling to give us additional information about the products, such as where the relevant antibody was produced. Based on the product sheets, the cutoff for both assays was the same as those for the Creative Diagnostics and Alfa Scientific HHAs at 200 ng/mL. No cross-reactivity was listed. Their Compound 1 test was also listed on the website as being available in a cup format, although no catalog number was listed.



Express Diagnostics International, Inc., is a biotechnology company based in Blue Earth, MN. They are ISO 13485:2003 registered and Conformité Européene (CE) marked. They produce their products in-house, but the antibody used in the Compound 1 test was provided by an outside vendor that was still based in the United States. The company produced both cassette and dipstick type Compound 1 assays, which were both listed as FUO. In addition, the company requires potential customers to sign an FUO letter before ordering the tests. These assays had the lowest cutoff among the surveyed companies at 10 ng/mL. They also provided cross-reactivity data for the meta-Compound 1 (5 ng/mL). The HHAs were made to order and required a lead time of 15 business days with a shelf-life of 18 months.

American Screening Corporation is a biotechnology company based in Shreveport, LA. They are ISO13485 registered and CE marked. Their tests were manufactured in-house, as were the antibodies used for their Compound 1 tests. American Screening Corporation offered two different types of Compound 1 HHA immunoassays: one dipstick and one dip card type. However, their dip card (ONESCREEN), was assembled in China. Both HHA types are FUO, and the dipstick type was 510k. The



dipstick and dip card assays had a 24 month shelf life and cutoffs of 200 and 300 ng/mL, respectively. Both HHA tests cross-react with meta-Compound 1 with a cutoff of 50 µg/mL.

Compound 1 tests are also offered by two U.S.-based distributors, Test Kits at Home, LLC, based in Coldwater, OH, and Source Medical Products, Inc., based in Lake Forest, IL. Test Kits at Home is a medical supply company, and Source Medical manufactures and distributes products that focus on the histology market. The Compound 1 test offered by both companies is the same as the test produced by Express Diagnostics. Neither company required a signed FUO letter, but both companies only allowed the test to be ordered over the phone. As with Express Diagnostics, both companies noted that the product was made-to-order and had a lead time of 2 weeks.

We also surveyed a highly sensitive European Compound 1 test produced by Diagnostik Nord GmbH, which is a CE-marked company based in Schwerin, Germany. The company is also ISO13485 and ISO9001 registered. Their test was an IVD dipstick that was not FDA-approved. They were unwilling to allow anyone based in the U.S. to purchase the product, even for research use. Their Compound 1 cutoff was in the midrange, compared with the others at 100 ng/mL. However, they had the most-comprehensive cross-reactivity data, with results on meta-Compound 1 (20 ng/mL).

Based on the criteria stated in this appendix, we developed a table showing the variables for all U.S.-based products (Table A-1). Using this table, we planned to evaluate Compound 1 tests from Creative Diagnostics (DTS237), Express Diagnostics (30102), and American Screening (03-4877). The Express Diagnostics dipstick type HHA was chosen because it was listed as the most sensitive and could be purchased directly from the manufacturer. The dipstick type HHA from American Screening was chosen because it was the only other available dipstick and had a sensitivity equivalent to that of the other products. The results of this market survey allowed us to determine the best assays for bench evaluation and identify alternative available tests, if desired.

Table A-1. Product Variables

Company	Location	HHA Type	Catalog No.	Time to Result (min)	LOD (ng/mL)	Cross Reactivity (ng/mL)	Manufacturer (Kit)	Manufacturer (Antibody)	Approvals
Creative* Diagnostics	Shirley, NY	cassette	DTS237	5	200	meta-Compound 1 (375)	in-house	in-house	POC
Alfa Scientific*	Poway, CA	cassette	03-4872	4–7	200	meta-Compound 1 (500)	in-house	outside U.S. vendor	IVD
Cortez Diagnostics	Calabasas, CA	dip	121152-1	5	200	none listed	in-house	—	cannot be sold in U.S.
Cortez Diagnostics	Calabasas, CA	cassette	121151-1	5	200	none listed	in-house	—	cannot be sold in U.S.
Express* Diagnostics	Blue Earth, MN	dip	30102	5	10	meta-Compound 1 (5)	in-house	outside U.S. vendor	FUO
Express Diagnostics	Blue Earth, MN	cassette	30102C	5	10	meta-Compound 1 (5)	in-house	outside U.S. vendor	FUO
Test Kits at Home†	Coldwater, OH	dip	DRU105[1]	5	10	meta-Compound 1 (5)	Express Diagnostics	—	FUO
Source Medical Products†	Lake Forest, IL	dip	S.FSD25	5	10	meta-Compound 1 (5)	Express Diagnostics	—	FUO
American Screening*	Shreveport, LA	dip	03-4877	4–7	200	meta-Compound 1 (50,000)	in-house	in-house	FUO; 510k
American Screening	Shreveport, LA	dip card	ASC-DFE-114	5	100	meta-Compound 1 (50,000)	manufactured in United States; assembled in China	in-house	FUO
Diagnostik Nord GmbH‡	Schwerin, Germany	dip	FYL-S20	5	100	meta-Compound 1 (20); Al-Compound 1 (562,500)	—	—	IVD; not FDA-approved, cannot be sold in U.S.

*Selected for analysis.
†Same products as Express Diagnostics 30102.
‡European assay.
—, unknown.

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