# Colorimetric detection of fentanyl using a supramolecular displacement assay

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**Abstract**

Fentanyl is a potent synthetic opioid with an alarmingly low lethal dosage of 2 mg. The equipment necessary to detect fentanyl in field settings (e.g., handheld spectrometers) is restricted to highly trained, well-funded, and specialized personnel. Established point-of-need technologies, such as lateral flow immunochromatographic strips, are available; however, they often involve multiple contact-based steps (e.g., collection, mixing) that pose a higher risk to users handling unknown substances. Herein, we developed a colorimetric displacement assay capable of contactless detection of fentanyl as liquid or solid samples. The basis of our assay relies on the presence of fentanyl to displace a redox mediator, ferrocene carboxylic acid, inclusively bound in the cavity of a supramolecular host, CB[7]. The displacement is only possible in the presence of high affinity binding guests, like fentanyl ($K_A$ ~ $10^6$ $M^{-1}$). The liberated redox guest can then react with indicator reagents that are free in solution, producing either: (i) a distinct blue color to indicate the presence of fentanyl or (ii) remain a pale blue tint in the absence of fentanyl. We demonstrate rapid and specific detection of fentanyl free base and fentanyl derivatives (e.g., acetyl fentanyl, furanyl fentanyl) against a panel of 9 other common drugs of abuse (e.g., morphine, cocaine, heroin). Furthermore, we highlight the intended use of this assay by testing grains of fentanyl derivatives on a surface with a drop (i.e., 25 µL) of assay reagent. We anticipate this approach can be applied broadly to identify the presence of fentanyl at the point of need.

## Introduction

The Centers for Disease Control and Prevention (CDC) has consistently reported synthetic opioids (e.g., fentanyl) as the majority cause of national drug-involved overdose deaths since 2016 with the sharpest monthly increases occurring in 2020.[1] Fentanyl—an opioid 50 times more potent than heroin—has a strikingly low lethal dosage of 2 mg and can absorb into the body via inhalation, ingestion, and even skin contact.[2] The high risk associated with exposure and increased presence of unidentified white powders, such as fentanyl, in drug-related emergency response scenes pose a significant danger to first responders.[3] Currently, methods to identify unknown substances in these settings are severely limited by manual collection of a sample and either (i) off-site characterization in a laboratory[4] or (ii) field analysis using mobile instrumentation (e.g., a handheld Raman or mass spectrometers).[5–8] Reliance on off-site characterization does not provide real-time analysis for first responders, where pertinent information can be critical to guide a proper response for decisions that impact patient treatment and personal safety. Additionally, the cost of mobile instrumentation restricts its access to well-funded police departments and is not amenable for socioeconomically disadvantaged communities where fentanyl use is endemic.[9] With no evidence of the opioid crisis abating,[10] it is crucial to equip first responders with a strategy for the real-time, contactless detection of suspected unknown substances.

To address this need, two colorimetric test kits are available for on-site opioid detection: the Sirchie NARK II fentanyl reagent and the BTNX Rapid Response.[11,12] These kits provide numerous advantages for real-time use (e.g., portability, colorimetric readout, and rapid response time); however, they also require extensive sample manipulation by the responder. For instance, the kit requires the responder to collect the suspected substance into a test pouch followed by vigorous manual shaking. Similarly, the BTNX Rapid Response fentanyl test kit requires the user to: (i) collect a swab of the unknown substance, (ii) insert it into a buffer-containing test tube, (iii) mix, (iv) insert a lateral flow test into the tube, (v) and remove the lateral flow test before placing

it on a flat surface to interpret the readout. These associated user steps present numerous opportunities for handling errors and, more importantly, put the responder at a greater risk of encountering a potentially hazardous substance. Drawing inspiration from the luminol spray, which is used to detect the presence of blood on surfaces, similarly employing a liquid dropper format to detect the presence of fentanyl on surfaces could provide responders with a user-friendly and contactless method of analysis. Ideally, the dropper should facilitate an assay that is specific, uses non-toxic reagents, and requires minimal user steps. However, current commercialized tests utilize chemistries unsuitable for a dropper application, such as indicators for a specific class or functional group and affinity detection using antibodies.[13] For example, antibody capture is highly selective, but it requires immobilization of reagents within devices (usually lateral flow immunochromatographic strips) and is thus not amenable to a dropper format. While these tests permit real-time analysis at the point of need, alternative wet chemistries are necessary for safer methods of analysis of unknown powders to better protect first responders.

In contrast to class-specific indicators and antibodies, the molecular recognition features of supramolecular host macrocycles (e.g., cucurbiturils, calabadions, and cyclodextrins) allow for specific detection of drugs of abuse.[14,15] These supramolecular hosts have demonstrated high binding affinities ($K_A$ ~ $10^6$–$10^8$) as sequestration agents for opioids (e.g., methamphetamine, fentanyl). [14] Furthermore, supramolecular hosts have previously been employed as sensors for direct [16] and indirect (i.e., displacement)[17,18] detection of analytes with fluorescent output, thereby demonstrating the potential for adaptation to colorimetric outputs. Supramolecular hosts, such as β-cyclodextrins, have found success in commercial applications (e.g., air fresheners)[19] where they sequester hydrophobic guest molecules (e.g., aromatics) while dispersed in droplets to effectively minimize odors. The real-time formation of these inclusion complexes aligns with our intended application to detect fentanyl.[20,21]

Herein, we report a method for the real-time colorimetric detection of fentanyl using a contactless dropper format. Our method employs a sequestrant macrocycle, cucurbit[7]uril

(CB[7]), that is pre-loaded with a reaction catalyst, ferrocene carboxylic acid (FCA), which has a moderate-binding affinity to CB[7], but is bound as in inclusion complex rendering it inactive. When present in a sample, fentanyl displaces FCA and is ultimately sequestered by CB[7] (i.e., $K_{A,host\text{-}catalyst} < K_{A,host\text{-}fentanyl}$). The liberated FCA is free to react with a chromogenic substrate in solution to produce a colorimetric signal (**Figure 1**). This chemistry can be employed simply by adding a drop of the solution onto the unknown substance from a distance, waiting 60 seconds, and directly visualizing the colorimetric readout. Furthermore, we show high specificity to fentanyl free base and fentanyl derivatives, as expected, with only one cross-reaction (i.e., methamphetamine) out of a larger panel of drugs of abuse. Our indirect detection method using a ferrocene displacement strategy can allow for rapid detection of fentanyl on surfaces at the point-of-need without requiring first responders to come into contact with unknown substances that are potentially fatal.

## Results and Discussion

### *Development of assay conditions*

We designed our displacement assay with the motivation of achieving (i) specific binding to fentanyl, (ii) rapid displacement, and (iii) colorimetric readout. We selected CB[7] as the host macrocycle for its strong binding to fentanyl ($K_A \sim 10^6$–$10^7$ $M^{-1}$).[14] CB[7] forms strong hydrogen bonds with the phenethyl ammonium group of fentanyl, making it an excellent host candidate for sequestration and detection of fentanyl. The ideal host-guest system for our displacement assay would also mediate a redox reaction upon displacement of a pre-bound reagent and produce a colorimetric response upon liberation. Our assay requires a two-state system where sequestration of the reaction catalyst, by inclusion into the host cavity, renders it inactive. While some oxidizing agents (e.g., methylene blue, acridine orange, riboflavin) are known to bind moderately to CB[7],[22–24] they are not entirely encapsulated by CB[7], which can lead to interactions with solution-phase indicator reagents and produce false positives. With this in mind, we identified

ferrocene[25] as our redox mediator because it is an inclusive binder to CB[7] and possesses peroxidase-like activity. Using this catalytic activity, we selected the 3,3′,5,5′-tetramethylbenzidine (TMB) and hydrogen peroxide ($H_2O_2$) reaction for our colorimetric output as TMB oxidation is known to be highly sensitive and rapid.[26] Ferrocene, however, is insoluble in water and thereby incompatible with our assay. Therefore, we tested several water-soluble ferrocene derivatives, including ferrocene methanol and 1,1′ ferrocene dimethanol, and found that ferrocene carboxylic acid (FCA) exhibited the highest and fastest signal output with the TMB (**Figure S1A**). Furthermore, the peroxidase-like activity of ferrocene was initially studied with TMB,[27] so we expected to observe a similar reactivity trend for FCA.

We determined binding affinities for CB[7] and each guest (i.e., fentanyl and FCA) by isothermal titration calorimetry (ITC) to be $4.24 \times 10^6$ $M^{-1}$ and $2.67 \times 10^5$ $M^{-1}$, respectively (**Table 1**). The results confirmed the desired relationship, where $K_{A,host\text{-}catalyst} < K_{A,host\text{-}fentanyl}$, under our assay conditions (0.2 M acetate, pH 4). For the identification of assay conditions, it is desirable to identify the ratio of CB[7] to FCA where the creation of that complex results in: (i) minimal free FCA present in solution, which would otherwise contribute to background signal or false positives; (ii) sufficient FCA still present to detect fentanyl at field-appropriate concentrations with rapid signal development upon liberation (ca. minutes) (**Figure S1B**). We experimentally determined that an approximate 10-fold excess of CB[7] to FCA—12 mM CB[7] and 1.25 mM FCA—ablated the background activity of FCA (**Figure 2**), which is consistent with the magnitude of the association constant. Reagent systems with lower concentrations of CB[7] retained solution FCA activity and could lead to false positives. Analogously, systems with higher concentrations of CB[7] could lead to false negatives.

We mimicked displacement of FCA from fentanyl using a model analyte, adamantyl amine (AM), which showed a higher binding affinity ($K_A = 1.49 \times 10^8$ $M^{-1}$; **Table 1**) for CB[7] than FCA and is safer to use in a laboratory setting than fentanyl (**Figure S1B**). Using our established assay conditions and samples containing a range of concentrations of AM (2–8 mM), we monitored the

development of colorimetric signal over time using a spectrophotometer to determine the duration that would lead to the greatest visual signal contrast between positive and negative samples (**Figure 3A**). A 15-minute duration provided sufficient visual signal contrast for interpreting the results of the displacement assay. A faint blue color, caused by a low amount of free FCA liberated due to the change in system equilibrium upon dilution by the added sample, indicated a negative result. In contrast, an intense blue color indicated displacement of ferrocene and a positive result (**Figure 3B**). This evidence supported the efficacy of the displacement assay as designed—rapid displacement of the reaction catalyst and strong signal formation for interpretation by the naked eye—and allowed us to proceed with further testing.

To select a final form factor for our assay, we investigated assay performance by dispensing reagents via dropper bottle or by spray. For these experiments, we chose grains of adamantyl amine as our positive sample and grains of glucose as our negative sample. Glucose is a known interferent (i.e., results in false positives) for the Marquis reagent test because its hydroxyl groups can form detection complexes in a similar way as alkaloids.[28] We placed a grain (approximately 1 mg) of each reagent on a white surface and used a camera to record signal development over time. For solid samples, we noticed the read time should be adjusted to 2 minutes, presumably due to the creation of high localized concentrations of analyte as the grain dissolves and reacts with reagents. Additionally, TMB is sensitive to light and, thus, partially influences the speed of color formation for samples treated on the benchtop (i.e., grains). At this newly established read time, there is distinguishable signal contrast between the positive and negative samples (**Figure S2**). Furthermore, signal contrast between the positive and negative samples was greater when using the dropper format than the spray format. By dropper, assay reagents were localized around the grain and generated a uniform signal. By spray, the assay regents were dispersed over a wider area and resulted in signal disparities among sprayed droplets. Therefore, we selected the dropper as our final form factor to achieve user-friendly signal interpretation and maintain user safety (i.e., avoiding the movement or aerosolization of fentanyl).

*Assay performance with fentanyl*

Once we determined the necessary conditions for our assay with the model analyte, we moved forward with evaluating assay performance with our target analyte, fentanyl. To approximate our limit of detection with grains of opioid, we first compared the color development of a negative sample against solutions of fentanyl free base (0, 0.05, 0.1, 0.25, 0.5, 1, 5, and 10 mM). For safety and experimental considerations when using solutions containing fentanyl, we separated the assay reagents into two components: (i) the sequestration complex (CB[7]–FCA) and (ii) the indicator reagent (TMB/$H_2O_2$). A detailed experimental procedure can be found in the Supporting Information. Solutions of fentanyl above 5 mM developed a distinct blue signal immediately upon indicator addition (<10 seconds; **Figure 4**). Conversely, solutions of fentanyl below 5 mM were indiscernible from negative samples for a duration up to 5 minutes (**Figure S3**). Because of the order of addition of reagents, absorbance measurements for samples of 0.05, 0.10, and 0.25 mM fentanyl are slightly lower than others. Signal development around 5 mM fentanyl was expected as the sample is expected to bind both occupied (i.e., CB[7]–FCA) and unoccupied CB[7]. Thus, higher concentrations of fentanyl are required to displace sufficient FCA to generate a colorimetric signal. Though the approximate limit of detection (LOD) for solutions of fentanyl (ca. 5 mM or 1.7 mg/mL) is greater than other platforms (e.g., lateral flow immunochromatographic strips at 150 ng/mL),[11] our goal and intended application was not to achieve a low LOD but rather to validate performance with grains of unknown composition. Situationally, in our anticipated use case, a 25-µL volume of assay reagents dispersed onto a 1 mg grain of fentanyl will result in an effective concentration of 118 mM—vastly exceeding our assay LOD.

We then evaluated the assay against a panel of 8 drugs of abuse (morphine, phencyclidine, ketamine, heroin, codeine, methamphetamine, oxycodone, and cocaine) and 2 additional fentanyl derivatives (acetyl fentanyl and furanyl fentanyl). When challenged with solutions of these drugs at 10 mM, a concentration above the expected LOD, our assay demonstrated high specificity to

all the fentanyl compounds and only produced signal for one other drug—methamphetamine (**Figure 5**). This false positive was an expected result, as methamphetamine is known to bind strongly to CB[7], $1.2x10^8$ $M^{-1}$.[13] For all positive samples, signal development was rapid and observed immediately upon addition of indicator (<60 seconds). In addition to drugs of abuse, we tested other substances that exist as white powders (e.g., baking soda, starch, laundry detergent) to demonstrate broader applicability in identifying unknowns in response scenarios. All of these substances produced no detectable color, except the powder cleanser which produced a blue signal, indicative of a false positive (**Figure S4**). We hypothesize this color was formed because the powder cleanser contains bleach, a strong oxidant, which reacted with TMB to generate the false positive.

*Demonstration of detection dropper on surfaces*

As a final proof-of-concept demonstration of our intended use case, we placed grains of all the drugs on a clean surface and observed signal development when we added drops of our reagent. For this experiment, we created a grid to spatially separate the grains and included a paired area for each that was left empty (i.e., clean surface). We added a 25-µL drop of assay reagent to each side (control and experimental) and observed any development of color over time. After a few minutes (ca. 4 min), droplets added to grains of acetyl fentanyl and furanyl fentanyl generated a distinct blue signal while their corresponding blank sample remained a pale blue tint (**Figure 6**), indicating that our assay can accurately detect the presence of fentanyl grains on a surface. Similar to the results of the specificity screen using solutions of drugs, the grain of methamphetamine also generated a signal that was visibly differentiable from the negative control. For the fentanyl free base, to evaluate a different presentation of the drug on a surface, we prepared solution at 10 mM and dried a 25-µL drop onto the surface for 30 minutes. When the drop of reagent was added, this sample of fentanyl sample did not produce any obvious change in color (**Figure S5**). This result supports the anticipated mechanism of our assay that relies on

the production of relatively high concentrations of drug (ca. hundreds of mM) caused by grains dissolving in, and reacting with, the reagent; these conditions are not replicated if the drug was solubilized and then dried as a film, which leads to concentrations below the LOD of the assay (ca. <10 mM).

## Conclusions

In this work, we developed a colorimetric displacement assay that can detect the presence of fentanyl on surfaces using a contactless dropper format. Our method employs CB[7], a supramolecular macrocycle, as an indirect sensor where the displacement of a pre-loaded reaction catalyst (ferrocene carboxylic acid) is only possible by guests with higher affinity to CB[7], such as fentanyl. The displacement of FCA allows for the development of a colorimetric signal that is fentanyl-specific, rapid, and user-friendly. We demonstrate the successful application of our displacement assay with liquid and solid samples of fentanyl and other drugs of abuse. Additionally, we demonstrate a dropper platform as our final form factor for an approach that we designed to be more user-friendly than current commercial techniques (i.e., test strips and instrumentation). The assay dropper can (i) open availability of use to more than just skilled technicians and (ii) directly address often-neglected considerations in assay development, usability and user safety. [29]

While our displacement assay works as intended for samples of fentanyl, further development is required to improve the dropper format for best performance at the intended point of use. Ideally, the assay should have no background signal and be specific to fentanyl only (e.g., no false positives from methamphetamine). Future work could explore functionalized CB[7] alternatives that could improve the specificity of CB[7]–fentanyl binding. However, even in these cases, the rapid onset of signal can be a differentiator of negatives and positives, and a positive signal, while equivocal for fentanyls or methamphetamine, would still indicate the presence of a hazardous substance for a responder. Furthermore, while we showcased the assay utility for on-

site detection of fentanyl, signal generation is non-destructive and can enable sample collection for subsequent confirmation or further analysis. Essentially, our assay presents a new approach towards rapid, point-of-need fentanyl detection that requires minimal user steps.

## Notes

ACM and CRM are co-inventors on a patent application related to the described displacement assay.

## Supporting Information

Materials list. Descriptions of methods to conduct ITC measurements, quantitative displacement assays, and qualitative displacement assays.

## Acknowledgements

This material is based upon work supported by the Army Contracting Command – Aberdeen Proving Ground (ACC-APG) under Contract No. W911SR-21-C-0019. This work was further supported by a generous gift from James Kanagy.

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**Figure 1.** Schematic of a ferrocene-displacement strategy to detect fentanyl. (**A**) Cucurbit[7]uril (CB[7]) is preloaded with ferrocene carboxylic acid (FCA) to produce a CB[7]–FCA inclusion complex where FCA loses its catalytic activity. (**B**) In the presence of fentanyl, FCA is displaced and thereby free to (**C**) react with indicator reagents, TMB and $H_2O_2$, in the assay solution. The resulting oxidized TMB product generates a blue color that is quantifiable by spectrophotometry or discernable by eye.

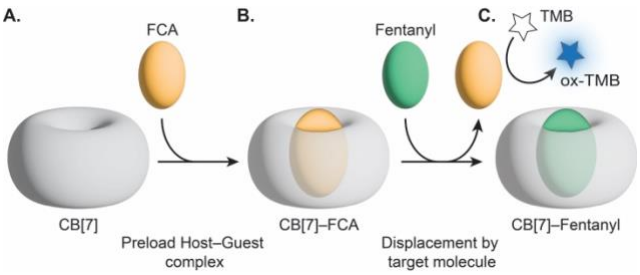

**Table 1.** Association constant ($K_A$) for CB[7]–ligand complexes as determined by isothermal titration calorimetry

| Host | Ligand | $K_A$ ($M^{-1}$) |
|---|---|---|
| CB[7] | Fentanyl free base | $4.24 \times 10^6$ |
| CB[7] | Ferrocene carboxylic acid | $2.67 \times 10^5$ |
| CB[7] | Adamantyl amine | $1.49 \times 10^8$ |

**Figure 2.** Loading capacity for CB[7] to result in complete inhibition of FCA catalytic activity. We fit a non-linear response curve modeling FCA inhibition (via monitoring oxidation of TMB through the production of a colorimetric product) by increasing concentrations of CB[7] at a single concentration of FCA (1.25 mM). The signal generated by FCA is entirely inhibited by 12 mM CB[7]. Concentrations of CB[7] are represented on a log scale. Experiments were performed in triplicate and error bars indicate standard error of the mean).

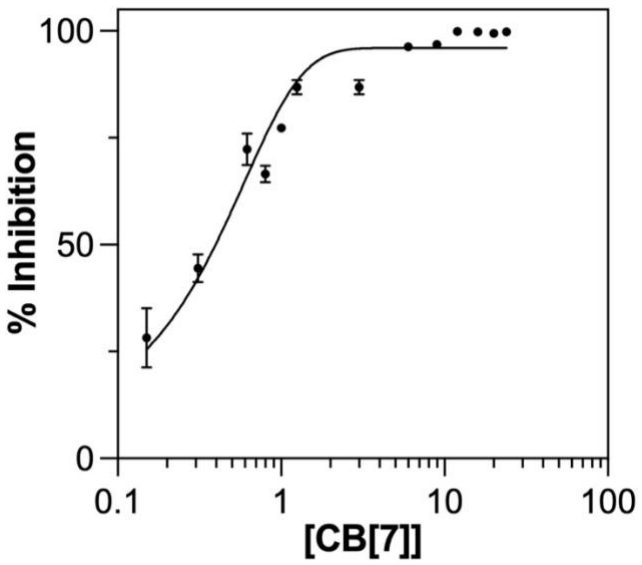

**Figure 3.** Dose response curves and representative images of signal development from the CB[7]–FCA inclusion complex with increasing concentrations (0–8 mM) of adamantyl amine (AM) over time. (**A**) Curves for the signal generated by the introduction of AM (N=3, error bars indicate standard error of the mean). Absorbance measurements were taken at 653 nm at intervals of 2 minutes. (**B**) Representative images of signal development in each well, corresponding to concentrations from 0–8 mM, at t = 5, 10, and 15 min.

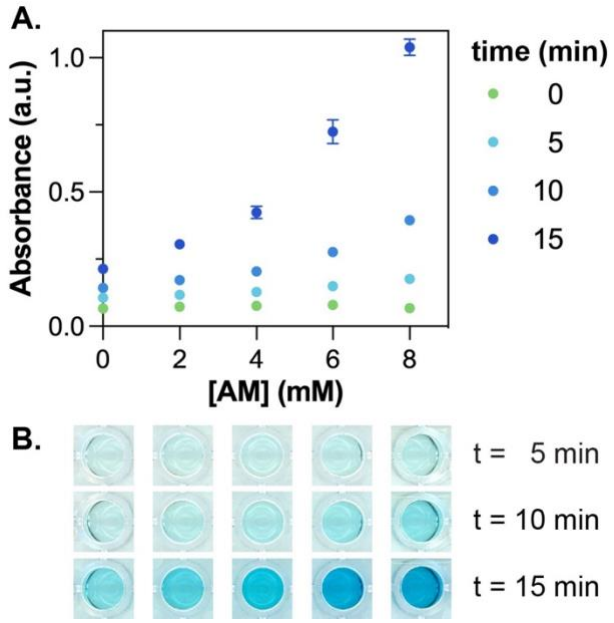

**Figure 4.** Signal development from assay reagents using solutions of fentanyl free base. (**A**) Graph of absorbance measurements of the signal generated by increasing concentrations of fentanyl free base. Measurements were performed in triplicate with each result displayed as an individual point, and error bars indicate the standard error of the mean of those measurements. Absorbance measurements were acquired at 653 nm after 1 minute. (**B**) Representative images of wells containing 0 mM (negative) and 5 mM (positive) fentanyl immediately after addition of assay reagents.

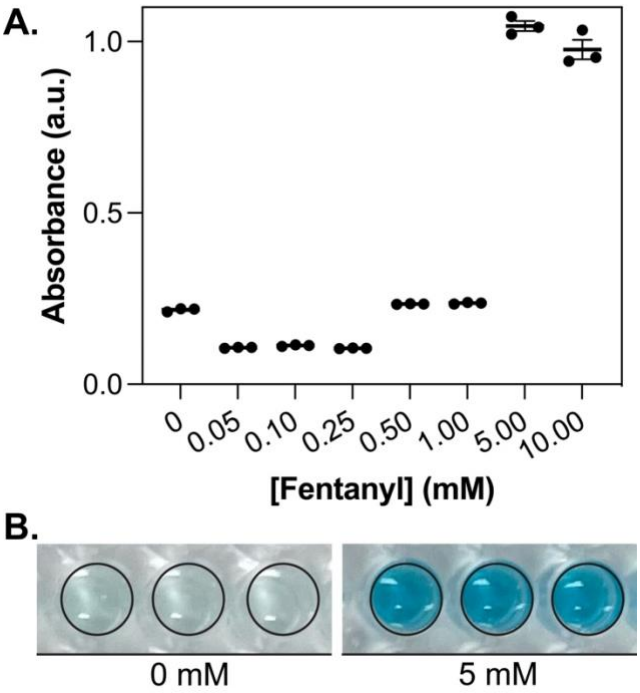

**Figure 5.** Assay specificity screen. Signal development due to addition of solutions containing various drugs of abuse at a concentration of 10 mM: fentanyl free base and fentanyl derivatives (indicated in dark blue), other drugs (indicated in light blue), and methamphetamine (indicated in green). The negative sample (indicated in black) is assay buffer. Experiments were performed in triplicate, where the black line represents the mean absorbance measured (at 653 nm) after the addition of each drug.

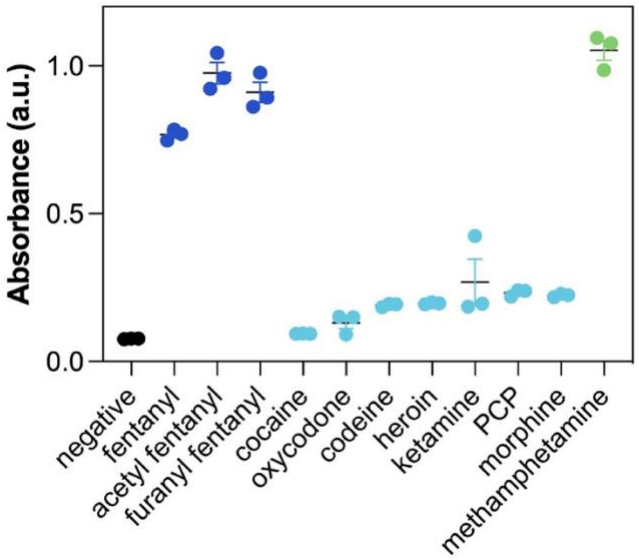

**Figure 6.** Assay performance with grains on surfaces. Representative images of signals developed when a drop of assay reagents is added to a clean surface (paired negative) compared to a surface containing a grain of a drug of abuse (positive): morphine, acetyl fentanyl, furanyl fentanyl, ketamine, and methamphetamine. The image was taken at 4 minutes and 30 seconds after 25 µL droplets of assay reagents were added.

| Drug of abuse | Grain on surface | | Result |
|---|---|---|---|
| | - | + | |
| Morphine |  |  | – |
| Acetyl fentanyl |  |  | + |
| Furanyl fentanyl |  |  | + |
| Ketamine |  |  | – |
| Methamphetamine |  |  | + |

## Table of Contents Graphic