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Best Practices in Novel Psychoactive Substances Testing for Laboratory Practitioners

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Table of Contents

The NIJ Forensic Technology Center of Excellence in Collaboration with the Center for Forensic Science Research and Education	ii
Technical Contacts	ii
Acknowledgments.....	ii
Introduction.....	1
1. Surveillance of the illicit drug market and indicators of drug emergence	1
1.1 Naming conventions	1
1.2 Identification of substances in seized drug casework	2
1.3 Identification of substances in non-targeted toxicological screening	3
1.4 Substances added to federal and local drug schedules	3
1.5 Monitoring scientific and professional literature	4
1.5.1 Peer-reviewed scientific literature	4
1.5.2 Trade magazines, conference proceedings, association discussion boards.....	5
1.5.3 Patents	6
1.6 Online research	6
1.6.1 Government drug policy and drug trend websites.....	6
1.6.2 Drug user websites	8
1.6.3 Drug vendor websites	8
2. Prioritization of targets for incorporation into scope of testing	9
2.1 Analyte popularity	9
2.2 Availability of standard reference materials and labeled internal standards	9
2.3 Metabolite studies.....	9
2.4 Considerations for incorporating targets into testing	10
2.4.1 Estimates of the sensitivity necessary for method development	10
2.4.2 Analytical platform selection considerations	10
3. Targeted chromatography-based and fit for purpose method development strategy	12
3.1 Method specification	13
3.1.1 Sampling Strategy	13
3.1.2 Included analytes	13
3.1.3 Biological Matrices.....	13
3.1.4 Quantitative versus qualitative.....	13
3.1.5 Other considerations	14
3.2 Method selection	14
3.2.1 Adding a new analyte to an existing method	14
3.2.2 Method modifications	14
4. Method validation	15
4.1 Scientific Working Group for Forensic Toxicology (SWGTOX)/Organization of Scientific Area Committees (OSAC) direction	15
4.2 Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG)	15

4.3	Fit-for-purpose validation	16
4.4	Validation experiments.....	16
4.4.1	LOD	17
4.4.2	Calibration model (Linearity)	17
4.4.3	LOQ	17
4.4.4	Precision (Repeatability/Reproducibility)	17
4.4.5	Bias and Uncertainty.....	17
4.4.6	Interference studies (Selectivity)	17
4.4.7	Carryover	18
4.4.8	Matrix effects.....	18
4.4.9	Dilution integrity	18
4.4.10	Stability	18
4.4.11	Matrix matching.....	18
4.5	Validation documentation	19
	Conclusion	19
	Addendum A: Fit-for-Purpose Quantitative Method Validation.....	21
	Addendum B: Quantification by Standard Addition for Toxicology Testing	28

Introduction

New psychoactive substances (NPS) have always presented a challenge to analytical laboratories tasked with identifying drugs in biological and nonbiological material. The pace at which new compounds appeared on the illicit drug market increased exponentially in the late 2000s, thus magnifying this challenge. Laboratories possess the technical expertise to develop and validate appropriate analytical methods for detecting new compounds; however, many laboratories lack the time and resources needed to keep up with the quickly changing landscape. This guidance document provides laboratories and practitioners with the resources to effectively respond to a dynamic drug market; this information proposes to help identify potential new drug targets, prioritize analytical targets, evaluate the best instrumental techniques for monitoring casework for new drugs, and develop and validate appropriate analytical methods.

1. Surveillance of the illicit drug market and indicators of drug emergence

Monitoring multiple resources and information channels to identify new compounds in the illicit drug market is critical for laboratories to stay current and relevant; laboratories garner this information from various areas—including science, law enforcement, public health, media, government, and drug users. Limited time and resources may require a laboratory to prioritize which methods will be most beneficial for their processes. This section highlights several resources that laboratories can monitor to stay up to date about the types of drugs used in specific countries or regions.

1.1 Naming conventions

Naming conventions do not exist for the naming of newly identified psychoactive substances; therefore, identifying these substances can be complicated. It is important to ensure a substance is truly novel and not a previously identified compound with a new or different name. Whenever an individual encounters a new compound using one of the resources described in this section, the first step should be to find the chemical structure of the substance and compare the substance carefully to all known analytes.

Online searches should include all known synonyms, spellings, and variants of the suspected new compound names. For example, a recent online search for fenethylamine, a prodrug emerging as a stimulant compound of interest in the Middle East, resulted in spellings “phenethylamine” and “fenethylamine,” and the drug’s other names, “amphetaminoethyltheophylline” and “amfetamine.” Additionally, this drug has been marketed under the trade names “Captagon,” “Biocapton,” and “Fitton.” A comprehensive search must include all of these names and spellings. Similarly, synthetic cannabinoids are also subject to inconsistent naming conventions, and many drugs have multiple names; for example, FUB-AMB is also known as “AMB-FUBINACA” and “MMB-FUBINACA.”

Naming conventions is a step-wise process:

1. Find the chemical name of the substance (e.g., International Union of Pure and Applied Chemistry [IUPAC] naming conventions).
2. Compare the chemical name to known analytes within laboratory and online keyword searches to include all names and spellings.
3. Search online resources, such as chemical search engines; original developer/manufacturers (e.g., academic institutions, pharmaceutical companies); academic publications; standard reference material sources; and drug user-oriented sites.
4. Review interagency communication, drug laws, drug scheduling.

Visit <https://iupac.org/what-we-do/nomenclature/> for more information.

When attempting to identify all spellings and variants of suspected new compound names, both the original source(s) of information about the new compound and scientific sources—such as academic publications and manufacturers' standard reference materials—constitute good places to start. However, websites oriented toward drug users should not be neglected because these sites can provide valuable insight into slang terms and the branding or stamping of specific products or mixtures.

1.2 Identification of substances in seized drug casework

Identifying the contents of seized material is a primary mechanism by which NPS are recognized. U.S. federal agencies, such as the Drug Enforcement Administration (DEA), Customs and Border Protection (CBP), and the United States Postal Service (USPS) monitor shipments that arrive in the country via land, sea, or air—including those transported by domestic and international mail and private express mail shippers. Packages, containers, vehicles, and persons entering the United States may be detained and searched for drugs or drug-related contraband, precursor chemicals, and pill presses. Given the ongoing opioid crisis in the United States, bulk materials—including finished drug products and precursors used in the illicit manufacturing of fentanyl and fentanyl-related substances—are of particular interest. Additionally, law enforcement agencies at the local, state, and federal levels enforce applicable drug laws and send exhibits seized during case investigation to crime laboratories for identification and analysis.

Inter- and intra-agency communication affect the usefulness of seized drug casework for forensic practitioners. This communication should include information about newly identified substances, derivatives, analogs of controlled substances; novel dosage forms (e.g., counterfeit pharmaceuticals, inhalers, gel tabs, droppers), new and challenging mixtures/matrices, and new suspected—but not yet identified—substances encountered in casework. Even unidentified compounds can be monitored in forensic casework by determining their accurate mass, fragmentation, molecular ion, and key fragment masses; such monitoring can help prompt further investigation if the compounds are detected on a more frequent basis. As unidentified substances become identified, appropriate analytical methods may need to be developed and validated to effectively analyze these substances in both seized drug samples and biological samples. To facilitate this process, seized drug laboratories must quickly and accurately communicate all information about new substances to toxicology laboratories to initiate method development.

Processes for analyzing seized drug samples involve building an analytical scheme, from selected techniques, that achieves a sufficient level of selectivity to enable the analyst to detect and identify the substances of interest. While most seized drug laboratories utilize electron impact gas chromatography-mass spectrometry (EI-GC-MS) for routine casework, NPS analysis often require the use of non-conventional techniques. More frequently, analysts are turning to nuclear magnetic resonance Spectroscopy (NMR) and gas chromatography-infrared spectroscopy (GC-IRD) to help differentiate between structurally similar variations of a substance.

The composition of seized drug evidence has been increasingly changing from mono-drug to poly-drug samples. Samples are not just containing more than one or two psychoactive substances, but in many instances, there may be a combination of five to eight different drugs in one sample. Many of these psychoactive drugs are NPS substances and often variations of one substance, creating analytical challenges for their detection and identification. For instance, positional isomers of fentanyl related substances may generate similar mass spectra due to their structural similarity. Laboratories are having to expand their instrumental capabilities with non-traditional techniques and the development of new analytical methods.

1.3 Identification of substances in non-targeted toxicological screening

Targeted analysis involves examining a sample for a pre-defined list of compounds but does not allow for NPS identification. Most toxicology laboratories currently use this technique for routine casework—typically using gas chromatography-mass spectrometry (GC-MS) and, less commonly, liquid chromatography-tandem mass spectrometry (LC-MS/MS). However, there is increasing interest in using GC-MS, time-of-flight (TOF), and quadrupole time-of-flight (QTOF) mass spectrometry for non-targeted screening; using these methods would allow for previously unknown compounds potentially to be identified.

For instance, unidentified peaks are sometimes detected in toxicological screens performed by GC-MS operated in full-scan mode. These peaks should be further investigated as potential new substances, paying careful attention to other peaks in the chromatogram with common ions as these may represent metabolites. In addition, other analytical pitfalls must be considered. For instance, substances may be thermally labile, and thus susceptible to conversion at the inlet to other compounds including a metabolite for that substance. TOF typically provides only the accurate mass of the parent substance, although some substances may undergo in-source fragmentation, which can increase the specificity of an identification. Similarly, the accurate mass of an unidentified peak in a QTOF spectrum is sufficient to determine its molecular formula, and the accurate masses of related fragments provide structural detail that may be pieced together to obtain a novel compound's tentative structure. However, structural determination using this technique does have limits—for example, some isobaric compounds (i.e., compounds that have the same exact mass) and ring positional isomers cannot be differentiated.

These limitations can have important ramifications in seized drug material analyses. For example, some ring positional isomers may be specifically scheduled, whereas others are not. Arguably, legislation that prohibits “any analog” of a controlled substance could be applied to these variants, but absolute identification is the preferred approach when new drugs become a source of litigation. For seized material, this can be achieved by (1) analyzing the seized material using Fourier transform infrared (FTIR) or nuclear magnetic resonance (NMR) spectroscopy or (2) comparing the mass spectrometric results with those obtained for standard reference materials of all candidate positional isomers. However, FTIR and NMR are not generally applicable to biological matrices, complicating the identification of isomeric compounds; therefore, care should be taken in reporting potential isomers in toxicological analysis. Another benefit of commercially available TOF and QTOF instruments is that they can be configured to collect “all the data, all the time,” which allows for retrospective data mining. This allows previously acquired data files to be reprocessed to look for these new additions without having to extract and analyze the samples again once reference standards become available and are added to the library.

1.4 Substances added to federal and local drug schedules

Laboratories should strive to test for all controlled substances. Drugs are typically scheduled on both the local and federal levels. The federal drug schedules are based on the abuse potential and accepted medical use, if any, of the compounds. The DEA publishes a “Notice of Intent” to temporarily schedule new compounds in the Federal Register; these notices can be found in the Rules section on the DEA Diversion Control Division website (https://www.deadiversion.usdoj.gov/fed_regs/index.html). Temporary scheduling typically goes into effect 1 month after the “Notice of Intent” is published, and temporary orders expire after 2 years unless the DEA takes further action to make them permanent.

Drug possession laws and the mechanisms used to communicate additions of newly scheduled substances vary from state to state. A single, generally reliable source to easily locate a specific state's laws does not exist, but the website “Findlaw” provides a page that links to individual state statutes (<https://statelaws.findlaw.com/criminal-laws/drug-possession.html>). The National Organization for the Reform of Marijuana Laws (NORML) provides an accurate and

up-to-date compendium on marijuana-related legislation and, using state-specific information, can help identify where drugs are addressed in state statutes. For example, a review of the NORML page on Pennsylvania Laws & Penalties for marijuana (<http://norml.org/laws/item/pennsylvania-penalties-2>) indicates that laws regarding drug possession are covered in Title 35 of the Pennsylvania statutes; NPS are also listed there.

1.5 Monitoring scientific and professional literature

Regularly reviewing relevant scientific and professional literature is essential in keeping up to date on changes in NPS drug markets; this literature includes peer-reviewed journal articles, trade magazines, conference proceedings, relevant association discussion boards, patents, and instrument vendor application notes. However, when using these materials as NPS indicators, it is important to note that NPS may lag significantly behind what is actually going on in NPS drug markets because of publication delays and the time that passes between abstract submission and when conferences occur.

1.5.1 Peer-reviewed scientific literature

Many databases are available for searching peer-reviewed scientific literature; however, some require a subscription, which often includes an annual fee. These paid resources may be available to individuals with academic appointments and, thus, access to institutional online library systems. Public universities often allow in-person visitors to use library resources, including online resources; however, they may not permit online access outside the facility. Even without access to paid databases, other pathways exist for forensic laboratory staff to search scientific literature. Currently two open databases are available to any interested party: PubMed and Google Scholar. These databases are described in more detail below.

The National Center for Biotechnology Information (NCBI) offers access to multiple scientific databases, the most well-known of which is PubMed. The U.S. National Library of Medicine and the National Institutes of Health administer PubMed; anyone with an internet connection can access the database. Although academic credentials are required to access many database articles at no charge, many other articles are available for free without such credentials—especially articles funded by public research grants. Clicking the “free full text” filter on the search page allows these articles to be easily identified. PubMed Central is a subdatabase that indexes free full-text articles. Registering with NCBI for free allows users to create saved searches to automatically search the database at a specified interval and receive emails with the results.

Authors frequently provide access to their articles on their personal webpages or self-archive platforms. Authors can also be contacted through these sites or by using their email address, which is usually listed on PubMed, to request reprints, which they are often happy to provide. Most articles that are not available through one of the previously mentioned options can be “rented” (i.e., limited access time, cannot be downloaded) or purchased through the journal in which the article was published.

Google Scholar searches published scientific literature; the tool uses the same search technology as Google’s website, and Google Scholar searches can be further narrowed by date range. Searching Google Scholar for an article’s exact title may indicate other archived online repositories where the article can be accessed. Governmental reports, media publications, patents and other informational formats beyond peer-reviewed publications are also accessible through Google Scholar.

Links to PubMed, Google Scholar, and some additional resources are listed below:

- PubMed: <https://www.ncbi.nlm.nih.gov/pubmed/>
- PubMed Central: <https://www.ncbi.nlm.nih.gov/pmc/>

- Google Scholar: <https://scholar.google.com/>
- ResearchGate: <https://www.researchgate.net/>
- Academia: <https://www.academia.edu/>
- New York Public Library: <https://www.nypl.org/>

1.5.2 Trade magazines, conference proceedings, association discussion boards

Conference proceedings include abstracts published for meetings that are held by professional organizations. Some organizations publish their conference proceedings in a specific journal, which may be found through a PubMed or Google Scholar search.

Trade magazines are publications dedicated to specific topics; professional societies and other organizations produce these publications. Access may require membership in the organization, although some trade magazines.

Discussion boards (Listserv) are user groups that interact through electronic notifications that are automatically broadcast to everyone on the list. A user can reply which feed into a discussion on a particular topic.

Table 1 presents a list of professional organizations that may publish NPS-related information (1) as online conference proceedings or (2) in trade magazine or newsletter articles.

Table 1. Professional organization conference proceedings, trade newsletters, and association discussion boards

Organization	Conference Proceedings	Trade Newsletter/Discussion Boards
American Academy of Clinical Toxicology (North American Congress of Clinical Toxicology [NACCT]/European Association of Poisons Centers and Clinical Toxicologists [EAPCCT])	http://www.clintox.org/resources/abstracts (free)	N/A
American Academy of Forensic Sciences (AAFS)	https://webdata.aafs.org/RefLibrary/RefSearch/Search.aspx (free and searchable)	Academy News https://www.aafs.org/resources/academy-news-pdf-library (free, archived back to 2003)
American Association for Clinical Chemistry (AACC)	https://www.aacc.org/science-and-practice/annual-meeting-abstracts-archive (free)	https://www.aacc.org/publications/clinical-and-forensic-toxicology-news (membership required)
American College of Medical Toxicology (ACMT)	Abstracts are published annually in Issue 1 of the <i>Journal of Medical Toxicology</i> : https://link.springer.com/journal/volumesAndIssues/13181	N/A
Clandestine Laboratory Investigators Association's (CLIA)	https://clialabs.com (meeting proceedings not available, meeting registration required)	CSAlert https://www.nesglobal.net/csalert-newsletter/ (access for law enforcement officers)
Clandestine Laboratory Investigating Chemists' (CLIC)	Meeting Proceedings not available, meeting registration required	membership@clic.org to request access to Listserv (membership required)

Table 1 (continued).

Organization	Conference Proceedings	Trade Newsletter/Discussion Boards
Pittsburgh Conference and Expo (PITTCON)	https://pittcon.org/pittcon-archives (free and searchable, last 2 years only)	N/A
Society of Forensic Toxicologists (SOFT)	http://www.soft-tox.org/past_meetings (free)	http://www.soft-tox.org/toxtalk (free online and searchable)
The International Association of Forensic Toxicologists (TIAFT)	http://www.tiaft.org/tiaft-meetings.html (free)	Bulletin http://www.tiaft.org/tiaft-bulletin.html (free hard copy to members, available for a fee to non-members)

1.5.3 Patents

Many NPS are compounds that pharmaceutical companies originally synthesized during their search for new therapeutic remedies. Patents may contain the documented results of these syntheses, detailed instructions for synthesizing the compounds, and information from the initial studies about drug activity (e.g., receptor binding studies, in vitro functional assays, animal studies). Therefore, these patents can become ‘virtual menus’ for illicit chemists looking for new compounds to introduce to drug markets. In addition, patents can be extremely useful for identifying substances related to previously identified drugs. Patents can be accessed through Google Scholar, as previously mentioned, or Google Patents (<https://patents.google.com/>).

1.6 Online research

1.6.1 Government drug policy and drug trend websites

Many government agencies monitor drug use in the United States and abroad. These agencies publish regular updates, which can be useful for identifying NPS. The following list highlights some of these agencies:

- The National Institute of Justice furthers efforts to combat the opioid epidemic through supporting a [research portfolio](#) focused on reducing the incidence of drug abuse.
 - The Forensic Technology Center of Excellence is an NIJ program that has developed more than 20 deliverables related to opioids and emerging drug threats, including novel psychoactive substances. These deliverables include the [2019 National Opioids and Emerging Drug Threats Policy and Practice Forum](#), the three-part online workshop series with CFSRE, “[Best Practices Guidance for Advancing Research Initiatives and Combatting the Synthetic Drug Epidemic](#),” and “[Just Throwing Darts at the Opioid Crisis](#),” part of the center’s Just Science podcast series.
- The National Institute of Standards and Technology (NIST) has collaborated with the DEA and Federal Criminal Police Office of Germany-Bundeskriminalamt (BKA) to create the [Novel Psychoactive Substance \(NPS\) Data Hub](#), which serves as a real-time dissemination measure to help forensic laboratories identify newly appearing NPSs.
- National Forensic Laboratory Information System (NFLIS): NFLIS is a DEA-funded resource that catalogs seized drug testing results submitted by federal, state, and local laboratories. Participating laboratories can access somewhat real-time reporting information or request such information from the DEA. NFLIS is enhancing its data collection through the participation of toxicology laboratories and medical examiner and coroner offices. Reports are published twice annually.
 - The NFLIS website: <https://www.nflis.deadiversion.usdoj.gov/NFLISHome.aspx>

- Laboratories and offices interested in participating in NFLIS should contact DEANFLIS@rti.org for more information.
- National Drug Early Warning System (NDEWS): NDEWS is funded at the Center for Substance Abuse Research by the National Institute on Drug Abuse (NIDA). This system provides multiple NPS-related resources to the community. One such resource—the DEA Emerging Threat Reports, which are published six times per year—can be found at the following link: <https://ndews.umd.edu/resources/dea-emerging-threat-reports>
- NIDA: Individual states report trends and alerts related to drug use, which NIDA then publishes.
- The Emerging Trends and Alerts page on NIDA's website: <https://www.drugabuse.gov/drugs-abuse/emerging-trends-alerts>
- European Monitoring Centre for Drugs and Drug Abuse (EMCDDA): Many European Union member countries have national early warning systems to monitor the appearance of new drugs. These member countries then report this information to the EMCDDA, which evaluates the information to determine if further action such as risk assessments is required and can provide valuable information to the forensic community, and regular publications about the state of NPS in Europe.
 - The EMCDDA's Early Warning System on NPS page: <http://www.emcdda.europa.eu/activities/action-on-new-drugs>
 - In addition, several countries maintain websites that are regularly updated with current news about NPS. In 2012, EMCDDA issued a report summarizing European Union member countries' early warning systems; links to individual countries can be found in that report.¹
- United Nations Office on Drugs and Crime (UNODC): UNODC provides basic information on NPS to the general public. More in-depth information about specific chemical structures, laboratory analysis, and legal response is available to registered users.
 - The UNODC Early Warning Advisory (EWA) on New Psychoactive Substances (NPS): <https://www.unodc.org/LSS/Home/NPS>
- Designer Drugs Online: An international comprehensive mass spectral database, but this service requires registering and paying a fee.
 - Designer Drugs Online website: <https://db12.designer-drugs.de/login.pl>
- Center for Forensic Science Research and Education at the Fredric Rieders Family Foundation (CFSRE): CFSRE is a nonprofit organization that engages in research to monitor NPS. NIJ-supported research projects have included toxicological testing of attendees at electronic music dance festivals, testing of drug materials entering the United States, and data mining of data collected during routine toxicology testing at a large reference laboratory. The NPS Discovery page is a repository of valuable, up to date information on drug trends, including monographs, reports, and an NPS Discovery dashboard.

The Center for Forensic Science Research and Education (CFSRE), in association with the FTCOE, delivered a [three-part online workshop series](#) to promote improved capabilities for NPS detection and measurement:

Session I: The Synthetic Drug Crisis- Identifying NPS in Forensic Casework

Session II: Analysis of NPS- Practical Considerations and Analytical Approaches

Session III: Interpretative Toxicology for NPS in Forensic Casework

¹ Gallegos, A., & Sedefov, R. (2012). *Early warning system—national profiles*. Retrieved April 22, 2019, from <http://www.emcdda.europa.eu/thematic-papers/ews>

- NPS Discovery page on the CFSRE website: <https://www.forensicscienceeducation.org/resources/nps-discovery/>
- DEA Real-Time Communication Network: A communication network to assist in the rapid identification of unknown substances, highlight analytical challenges associated with low level and structurally similar substances, and facilitate a collaborative effort in detecting and identifying NPS.
 - For more information, contact Synth-Opoids@usdoj.gov

Table 2 lists some additional databases that contain NPS-specific data and information.

Table 2. Additional databases that contain NPS-specific data and information

Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG)	http://swgdrug.org/
Southern Association of Forensic Scientists	http://forendex.safs1966.org/index.php/home/index
European Project Response to Challenges in Forensic Drug Analyses	http://www.policija.si/apps/nfl_response_web/seznam.php
UNODC Fentanyl/Fentanyl Analogs Monograph (2017)	https://www.unodc.org/unodc/en/scientists/recommended-methods-for-the-identification-and-analysis-of-fentanyl-and-its-analogues-in-biological-specimens.html
Cayman Chemical	https://www.caymanchem.com/Home
Spectral Database for Organic Compounds (SDBS)	http://sdb.sdb.aist.go.jp/sdb/cgi-bin/direct_frame_top.cgi
NPS Data Hub	https://www.nps-datahub.com/
Data Search System for New Psychoactive Substances	http://npsdb.nihs.go.jp/Search/Default_e.aspx
MassBank of North America	http://mona.fiehnlab.ucdavis.edu/

1.6.2 Drug user websites

Multiple internet forums are dedicated to discussions about all aspects of illicit drug use. Drug users often own and operate these forums. Additionally, these forums can offer a plethora of information about which drugs are gaining popularity. These websites may be subject to your agency's online search policies as user information can be retrieved by internet cookies and shared with third parties. The following list presents some examples of websites with pages dedicated to NPS:

- Reddit: <https://www.reddit.com/r/researchchemicals/>
- Drugs Forum: <https://drugs-forum.com/forums/research-chemicals.21/>
- Erowid: https://www.erowid.org/experiences/exp_front.shtml
- Blue Light: <http://www.bluelight.org/vb/forums/58-Other-Drugs>

1.6.3 Drug vendor websites

Many drug users obtain their NPS via the internet. Online vendors often sell products packaged with street names that provide no information about which drugs the packages may contain. However, many vendors do identify the drugs in their inventory by chemical name or abbreviation. These websites change regularly, making it difficult to provide a current list; a simple internet search, such as "buy Furanyl fentanyl online," will result (subject to your agency's online search policies) in a list of vendors that sell a wide range of NPS. Searching for a product by its CAS number by way of image results may also lead to uncovering NPS vendors.

2. Prioritization of targets for incorporation into scope of testing

2.1 Analyte popularity

Because of time and resource constraints, it is impossible to develop and validate testing for every compound rumored to be on the market. Thus, laboratories must develop a plan for filtering and screening the data from all of the previously mentioned intelligence channels and prioritizing the incorporation of analytes into their scope of testing. For example, a few years ago, the internet was filled with stories about desomorphine (krokodil), a heroin-like codeine derivative that was killing many users in other countries. In response, some U.S. laboratories developed methods for identifying this drug, but there has yet to be a single verified case in the United States in either seized drug or toxicological analysis². Therefore, a plan should (1) emphasize the importance of cross-referencing multiple data points and (2) consider evidence of the rise in a drug's popularity from multiple channels and regional prevalence before deciding to prioritize that compound for method development and validation. Resources, such as the DEA [National Drug Threat Assessment \(NDTA\)](#), which provides information about the abuse of illicit drugs, can be used to provide insight. This assessment combines federal, state, local and tribal law enforcement reporting; public health data; open source reporting; and intelligence from other government agencies.

2.2 Availability of standard reference materials and labeled internal standards

The availability of standard reference materials is a key factor in whether a laboratory will be able to develop and validate a method for the identification and/or quantification of an NPS. Developing a relationship with a vendor that produces certified reference materials can aid in this task, but the lead time in the manufacturing of new compounds can be a limiting factor for method development. In 2019, the Center for Disease Control and Prevention (CDC) developed [Traceable Opioid Material Kits](#) to support qualified U.S. laboratories in the detection of emerging opioids. This kit contains 44 solutions, 32 of which are US DEA controlled standards.

In the absence of certified reference materials, it is possible to begin development using evidence from a seized drug case. It is critical that the laboratory first verify the identity and estimate the purity of the substance using QTOF, FTIR, and/or NMR analyses. Using a seized drug exhibit can, for example, be useful in expediting the first steps of developing a toxicological assay when there is a public health emergency. However, using seized drug exhibits as standards for validating or reporting biological specimen results is not best practice. Therefore, if this strategy is used, the laboratory should always change to using a certified reference material if and when one becomes available.

For seized drug analysis, if a reference material cannot be obtained, comparisons to external reference data are and can often be used. The veracity of the data, however, should be assessed and documented prior to use in case work. In situations when reference materials nor external reference data can be obtained, structural elucidation techniques can be performed, and the data interpreted.

2.3 Metabolite studies

For many classes of NPS—such as opioids, cathinones, and benzodiazepines—the parent compound is expected to be detected in both blood and urine following ingestion. However, for synthetic cannabinoids, the parent compound is unlikely to be excreted in urine. For these compounds, metabolite studies using in-vitro pooled human

² Agarwal V, Levounis P. Krokodil: 'Zombie Drug' Scare Hits U.S.

Psychiatric News. American Psychiatric Association. <https://psychnews.psychiatryonline.org/doi/full/10.1176/appi.pn.2014.3a23>.

liver microsome incubations or hepatocyte studies can identify targets for urine testing.³ These analyses may give direction and provide evidence as to the most common metabolites likely to be encountered in biological samples. These experiments are relatively easy to perform; however, not every metabolite identified in in vitro studies is found in measurable quantities in human specimens. NIJ supports research to elucidate metabolic pathways and has listed it as a needs requirement based on its technology working groups. It is worth noting that vendors may synthesize a metabolite based on the suspected metabolism of a parent compound without having strong evidence of the metabolite's prevalence in human blood and urine. Therefore, it is important to verify the metabolite will actually be formed before investing time and resources in developing an analytical method to detect it.

2.4 Considerations for incorporating targets into testing

2.4.1 Estimates of the sensitivity necessary for method development

Typically, little or no pharmacological data are available for NPS when they first appear on the market. Careful review of the available literature and reported concentrations for similar compounds can offer some guidance about the sensitivity that may be required to detect the new compound in casework. It is also advisable to do a thorough patent search (1) to determine if any initial activity studies have been performed on the compound and (2) to be aware of any structure-activity relationship studies performed on the class of compounds in question, which may provide clues about their expected potency. In cases in which no information is available, using a limit of detection (LOD) approach to create a curve across the assay's linear range can work until enough authentic samples have been tested to provide better guidance about the appropriate range for routine use.

2.4.2 Analytical platform selection considerations

There are many considerations when selecting an analytical platform—including sensitivity, specificity, availability, and cost. Commonly available instrumentation, such as GC-MS and LC-MS/MS, is sufficient for the identification and quantification of many NPS classes. In contrast, high resolution mass spectrometry techniques (e.g., LC-TOF, LC-QTOF) should be considered as screening techniques, with LC-QTOF also offering quantitative testing capabilities. It should be noted that identifying compounds in seized materials is analytically easier than identifying them in biological matrices. Analytical techniques—such as Raman spectroscopy—allow for presumptive identification through packaging material, but the specificity of this technique degrades in complex mixtures. Since samples being submitted to seized drug laboratories are frequently poly-drug in nature, it is often best practice to utilize separation techniques for initial screening. Full-scan GC-MS is commonly employed by forensic drug chemistry laboratories for analysis of seized drug casework. Additionally, carefully evaluating unidentified GC-MS peaks using available external libraries may reveal that new compounds have been detected. It is also important to be familiar with the analytical limitations associated with NPS classes. For instance, some compounds may decompose to other substances when exposed to thermal techniques. Thus, using LC-MS may be a better suited technique, instead of a GC-MS method without derivatization.

Table 3 highlights some of the 'pros and cons' of different analytical platforms commonly employed for screening and confirmation testing in forensic laboratories.

³ See, for example, Identification and Prevalence Determination of Novel Recreational Drugs and Discovery of Their Metabolites in Blood, Urine and Oral Fluid, Amanda L.A. Mohr, Melissa Friscia, Barry K. Logan, NCJ 250338, 2016, <https://www.ncjrs.gov/pdffiles1/nij/grants/250338.pdf>.

Table 3. Benefits and limitations of commonly employed analytical platforms

Platform	Benefits	Limitation(s)	Type of Testing
Color Tests	• Sensitivity • Cost	• Mixtures • NPS studies	• Seized Drugs
Immunoassay	• Commercial kits • Fast validation • Cross-reactivity	• Non-specific	• Toxicology
IR	• Simple sample prep • Virtually any sample state • Non-destructive • Qualitative and Quantification • Portable	• No molecular weight • Mixtures	• Seized Drugs
GC-IR	• High specificity • Quantification	• Sensitivity • Sample preparation • Polar, volatile, thermally labile compounds	• Seized Drugs
GC-MS	• Readily available • High specificity • Commercial libraries • Full scan allows unknown identification • Quantification	• Sensitivity • Sample preparation • Polar, volatile, thermally labile compounds	• Seized Drugs • Toxicology
LC-MS/MS	• Higher sensitivity • Easier sample prep • Co-eluting compounds • Quantification	• Matrix effect • Unknown identification	• Toxicology
LC-QTOF	• Structural information • More sensitive than TOF • Quantification • Exact mass information • Data mining	• Expensive • Isomers resolution • Large data files, requiring additional training	• Toxicology
LC-TOF	• Simple sample prep • Exact mass information • Data mining	• Low resolution • Isomers resolution • Large data files, requiring additional training	• Toxicology
Microcrystalline	• High specificity • Sensitivity • Cost	• Expertise • Mixtures	• Seized Drugs

Table 4 (continued)

Platform	Benefits	Limitation(s)	Type of Testing
NMR	• High specificity • Structural information	• Expensive • Expertise • Sensitivity • Mixtures	• Seized Drugs
Pharmaceutical Identifiers	• High specificity • Commercial databases	• Illicit manufacture	• Seized Drugs
Raman	• Sample prep • Non-destructive • Portable	• Substances with strong fluorescence • Mixtures	• Seized Drugs
TLC	• Sensitivity • Non-destructive • Cost	• Mixtures	• Seized Drugs

3. Targeted chromatography-based and fit for purpose method development strategy

3.1 Method specification

Before method development begins, the project goals must be clearly identified. A method specification should be written that provides guidance to the developer and establishes these goals. The method specification should include information about the analytes to be included in the method, the type of test needed (qualitative or quantitative), and the matrices that will be used.

3.1.1 Sampling Strategy

For seized drug analysis, the appropriate sampling strategy is important and highly dependent on the purpose of the investigation and anticipated use of the results. Decisions should be made to answer questions about a population to address legal and scientific requirements. The sampling strategy utilized may be statistical or non-statistical in nature. ASTM International published a standard guide for sampling seized drugs for both qualitative and quantitative purposes.⁴

3.1.2 Included analytes

When the decision has been made to develop and validate a test for a specific NPS, it is worthwhile to evaluate the available resources and determine whether there are any similar compounds that should be included in the method and if the metabolite activity is known or can be surmised. Similar compounds with documented activity may be worth including in the method at the time of development, even if there is no evidence that they have yet to appear in the illicit marketplace. Active metabolites should be included in analytical methods. Drugs with short half-lives that have some inactive metabolites that are markers of recent use should also be included (e.g., norfentanyl or N-phenethyl-4-piperidinone [4-ANPP] for fentanyl and its analogs).

For seized drug analysis, it is important to be familiar with the current mixtures being used in the illicit market and include those substances in the validation. Often, mixtures of structurally similar substances create the most difficult analytical challenges, that if not addressed during validation can lead to misidentifications. Method limitations should be noted and reported.

3.1.3 Biological Matrices

The primary matrix used for forensic testing is usually whole blood. The inclusion of serum and/or plasma is advised, when possible, to ensure the ability to test hospital admission samples, which may be the best specimens available in cases of prolonged death and driving under the influence of drugs. The ability to test urine is also beneficial in cases in which an individual survives for a period of time after hospital admission, but no admission blood, serum, or plasma samples are available. When evaluating matrices, it is important to consider whether parent compounds will likely be detected in the sample. If metabolite testing is necessary, the samples may need to be hydrolyzed.

3.1.4 Quantitative versus qualitative

Deciding whether to develop a quantitative or qualitative test is difficult. It is often easier and faster to develop a qualitative-only test, especially if no labeled internal standards are available. When a compound first appears on the market, quantitative results do not add value with respect to interpretation because no results are available for comparison. However, unless laboratories perform quantitative analyses, concentrations will never become available for comparative purposes. An alternative is becoming familiar with the purity levels reported from seized

drug samples, which can give an indication to what users may be ingesting. Collecting quantitative results from even a short case series can begin to develop context for interpreting future identifications of the substance, which is especially useful when many of the cases are polysubstance cases. One example that shows the value of collecting quantitative results, was the 2017 reported discovery in impaired driving casework of certain carfentanil concentrations.⁴ Although these concentrations would have been considered lethal by clinical standards, this case demonstrated that users may develop a tolerance to this drug's toxicity.

3.1.5 Other considerations

Although the goals of any method development project should be outlined at the outset, flexibility is also important. For example, consider a case in which it was determined that three analytes should be included in a test, but one of those analytes was causing difficulty during development due to an inability to resolve that analyte from another one or due to poor chromatography. In this case, eliminating this analyte from the scope may be necessary to avoid delaying the ability to test for the other two analytes. In this case, the specificity of the test would be updated, and documentation of the decision-making process would be documented as part of the method development. As another example, if the initial decision was to develop quantitative testing, proceeding with qualitative test development may be the best option if the quantitative method does not meet the method validation criteria. Similarly, one may start out with a goal of achieving a specific analytical cutoff or LOD, but the validation may not support reaching that goal. In this situation, obtaining a qualitative result may be sufficient—or subsequent analyses of authentic patient samples may reveal that the higher, attainable cutoff is fit for purpose.

3.2 Method selection

3.2.1 Adding a new analyte to an existing method

The most efficient way of testing a new analyte usually involves inserting it into an existing method for similar compounds. In this case, development requires only verification that the extraction and instrumental methods provide sufficient sensitivity and specificity for the new analyte and that the addition of the new analyte does not affect the performance of the analytes currently included in the scope. In addition, during validation, only the new analyte needs to be evaluated; revalidating all existing analytes is not necessary.

3.2.2 Method modifications

If it is not possible to directly insert a new analyte into an existing method, it may be necessary to slightly modify the method to add the new analyte while maintaining the method's integrity for existing compounds. Modifications to consider may include the following:

- Mobile phase modification (e.g., solvent type and ratio, pH, modifiers)
- Stationary phase modification (e.g., column type, column length, column diameter, film thickness)
- Temperature profile

Modifying these parameters can affect analytes' retention times and will require validation experiments to ensure that the new and existing analytes perform as expected. Specifically, evaluating potential changes in interfering substances and matrix effects is important. Reanalysis of previously tested positive samples can confirm the validity of the method for existing analytes; new analytes should undergo more extensive validation.

⁴ Papsun, D., Isenschmid, D., & Logan, B.K. (2017). Observed Carfentanil Concentrations in 355 Blood Specimens from Forensic Investigations. *Journal of Analytical Toxicology*, 41(9), 777–78. <https://doi.org/10.1093/jat/bkx068>

4. Method validation

4.1 Scientific Working Group for Forensic Toxicology (SWGTOX)/Organization of Scientific Area Committees (OSAC) direction

SWGTOX published the following recommendations regarding the minimum validation parameters that should be evaluated based on the intended purpose of the method (**Table 5**).⁵ The American Academy of Forensic Sciences (AAFS) Standards Board (ASB) adopted these recommendations, and they are currently under review for final publication.⁶

Table 5. SWGTOX-recommended minimum validation parameters for different types of analysis

Parameter	Immunoassay Screen	Other Screen*	Qualitative Identification	Quantitative Analysis
LOD	✓	✓	✓	✓
Limit of Quantification (LOQ)				✓
Calibration Model				✓
Precision	✓			✓
Bias				✓
Interference Studies		✓	✓	✓
Carryover			✓	✓
Ion Suppression/Enhancement (Matrix Effect)			✓	✓
Dilution Integrity (if applicable)	✓	✓	✓	✓
Stability (if applicable)	✓	✓	✓	✓

* Examples of “Other Screen” methods are full-scan GC-MS and TOF.

4.2 Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG)

For seized drug analysis, SWGDRUG published recommendations to assist laboratories create a validation plan when validating seized drug analytical methods.⁷ The performance characteristics recommended (**Table 5**) were adopted by ASTM International to publish a standard practice for the validation of seized drug analytical methods.²

⁵ Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. (2013). *Journal of Analytical Toxicology*, 37(7), 452–474. <https://doi.org/10.1093/jat/bkt054>

⁶ ASB. (2018). DRAFT standard practices for method validation in forensic toxicology. ASB Standard 036. Washington, DC: ASB. <https://www.asbstandardsboard.org/>

⁷ Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) Recommendations. <https://www.swgdrug.org/Recommendations> Part IVB Quality Assurance/Validation of Analytical Methods.

Table 5. SWGDRUG-recommended minimum validation parameters for seized drug analysis

Parameter			Qualitative Identification	Quantitative Analysis
Selectivity			✓	✓
Matrix Effects			✓	✓
Recovery				✓
Accuracy	Precision	Repeatability	✓	✓
		Reproducibility	✓	✓
	Trueness			✓
Range	LOD		✓	
	LOQ			✓
	Linearity			✓
Robustness			✓	✓
Ruggedness			✓	✓
Uncertainty				✓

4.3 Fit-for-purpose validation

In some cases, performing a full validation before analyzing patient specimens may not be necessary. This is especially true when the laboratory must test a single case. If no reference laboratory offers a validated test, the next best option is for the laboratory to develop its own limited fit-for-purpose validation. Such a validation provides a forensically defensible result but does not require the expense or time necessary for a comprehensive validation. For example, a fit-for-purpose validation might include determination of limit of detection, limit of quantification (LOQ), carryover, limited interference, precision, and accuracy; however, this validation does not include stability, matrix effect, or dilution integrity. Using fit-for-purpose validation is an acceptable practice provided that the result is reported with the understanding of its limitations. If a substance appears to be gaining traction in the market, the laboratory should move forward with a complete validation. Another option for laboratories is to use the method of standard addition, in which each patient sample acts as its own control and provides additional assurance about the performance of an assay that has not been fully validated. When relying on a validation that does not include all the recommended experiments, careful consideration should be given to what is required to defend the analytical results.

4.4 Validation experiments

There are many approaches to method validation. The following is a brief overview of the most common validation parameters. Addendum A provides an example of a 3-day fit-for-purpose method validation protocol for quantitative methods—including setup, data analysis, and acceptance criteria. Documentation and a protocol for quantification by standard addition can be found in Addendum B. It is best practice to attempt to validate a quantitative method; however, if the method does not achieve acceptable results for parameters such as precision and bias, employing the method for qualitative identification only may be acceptable.

4.4.1 LOD

The LOD, or lowest concentration at which the method can positively identify the analyte of interest, can be determined analytically; however, for the purposes of NPS testing, defining the lowest calibrator or cutoff calibrator as the method's LOD may be sufficient. It is important to note that if this approach is used, it may preclude the reporting of positive findings below the lowest calibrator or cutoff concentration.

4.4.2 Calibration model (Linearity)

The calibration model defines the range over which the method can be used to quantify the analyte of interest. Evaluation criteria for correlation coefficient and equal distribution of standard residuals is important to establish. Although "equally distributed" is subjective, there are statistical approaches that can be used to provide a more objective final selection.⁸

Matrix-matched calibrators are recommended, if possible. As this is often not practical, matrix-matching experiments can be performed as an alternative.

The best calibration model should be identified during the method development process and then validated as part of the method validation plan.

4.4.3 LOQ

The LOQ, or lowest concentration that can be reproducibly quantified, must be established for all quantitative methods. This can be accomplished by evaluating the accuracy and precision at a variety of concentrations.

4.4.4 Precision (Repeatability/Reproducibility)

Precision measures an assay's reproducibility. Both within- and between-run precision should be evaluated for all quantitative methods. Although it is called "precision," it is typically documented as the percent coefficient of variation of replicate analyses of control samples, which actually represents the method's imprecision.

4.4.5 Bias and Uncertainty

Bias is the relationship between (1) the measured concentration of an analyte and (2) the known or nominal concentration. Bias studies must be performed for all quantitative methods. In addition, uncertainty studies must evaluate the contribution of systematic and random errors to cause a measured value to deviate from the true value.

4.4.6 Interference studies (Selectivity)

An interfering substance is anything present in a sample that impedes the identification or correct quantification of the analyte of interest. Such substances can include matrix components, internal standards, and commonly encountered drugs. Matrix interference can be evaluated by analyzing multiple sources of blank matrix. The contribution of an internal standard can be evaluated by comparing the response of analytes of interest with and without internal standard present.

⁸ Almeida, A. M., Castel-Branco, M. M., & Falcão, A. C. (2002). Linear regression for calibration lines revisited: Weighting schemes for bioanalytical methods. *Journal of Chromatography B*, 774(2), 215–222. doi:10.1016/s1570-0232(02)00244-1

4.4.7 Carryover

Carryover may occur at very high analyte concentrations. This could result in an analyte from a sample being detected in the next injected sample. The potential for carryover can be evaluated during method validation by injecting a blank sample after a control fortified at high concentrations of the analyte of interest. Alternatively, blank samples can be injected between each sample to ensure that no carryover occurs.

4.4.8 Matrix effects

LC-MS methods are susceptible to matrix effects. This enhancement or suppression of analyte ionization resulting from the presence of co-eluting compounds is most likely to impact samples at or near the method LOQ/LOD. Typically, deuterated internal standards compensate for this effect, but these are often not available for NPS. Therefore, it is important to not only determine the potential matrix effect of both the analyte of interest and internal standards but also to confirm that any observed matrix effect does not affect the quantification.

4.4.9 Dilution integrity

If sample dilution is required due to low sample volume or a concentration that is higher than the established calibration range, its effect must be determined. This can be done during validation or contemporaneously with the analysis of a diluted sample by analyzing the high control diluted at the same factor as the patient specimen.

4.4.10 Stability

Understanding the effects of storage conditions and sample processing on the analyte of interest is important because instability can result in quantitative results that do not accurately reflect the concentration at the time of sample collection. Ideally analyte stability is determined under a variety of storage conditions; however, analyte stability may be beyond the scope of the validation protocol for NPS testing. If stability studies are not conducted, then it should be clearly communicated that no information is available about how storage or delays in analysis after sample preparation may affect the ability to detect the analyte or determine its concentration.

4.4.11 Matrix matching

If analysis will be performed in multiple matrices, matrix matching can be evaluated during method validation to ensure that the calibrators in one matrix can accurately quantify the analyte in different body fluids. This analysis can be performed by repeating the bias and precision experiments in each matrix.

4.4.12 Robustness/Ruggedness

Evaluating variations of individual method parameters and their subsequent effects to accuracy is important to understand and identify potential method limitations. Factors external to the method need may also affect the reliability of the method results and should be assessed.

4.5 Validation documentation

Commercially available and open-source software packages can be used to document validations, but they are typically designed for clinical laboratory applications and may be cumbersome to modify for a fit-for-purpose validation for the analysis of NPS in biological specimens. Laboratories may find it easier to develop an internal template using a spreadsheet program that can be set up to automate calculations and summarize the validation results. It should be noted that these programs may not have the built-in ability to calculate the parameters of weighted calibration curves, meaning the user must manually type in the formulas for these calculations. Freely available templates containing the appropriate formulas are available for download (<http://terpconnect.umd.edu/~toh/models/CalibrationCurve.html>).

Conclusion

By dedicating resources to monitoring online activities and published literature, a laboratory or practitioner can stay informed about NPS-related trends. Best practices guidelines provide a framework for identifying drugs on the illicit market, determining the best analytical approach for detecting and quantifying relevant compounds in casework, and developing/validating analytical methods that will provide reliable results.

The Forensic Technology Center of Excellence

RTI International (RTI) and its academic and community based-consortium of partnerships, including its Forensic Science Education Programs Accreditation Commission partners, work to meet all tasks and objectives put forward under the National Institute of Justice (NIJ) Forensic Technology Center of Excellence (FTCoE) Cooperative Agreement (award number 2016-MU-BX-K110). These efforts include determining technology needs; developing technology program plans to address those needs; developing solutions; demonstrating, testing, evaluating, and adopting potential solutions into practice; developing and updating technology guidelines; and building capacity and conducting outreach. The FTCoE is led by RTI, a global research institute dedicated to improving the human condition by turning knowledge into practice. The FTCoE builds on RTI's expertise in forensic science, innovation, technology application, economics, data analytics, statistics, program evaluation, public health and information science.



Addendum A: Fit-for-Purpose Quantitative Method Validation

A. Purpose

This procedure defines the minimum parameters and sets of experiments to validate a fit-for-purpose quantitative method in 3 days. Due to the limited availability of deuterated internal standards and the potential limited interpretive value of quantitative results for NPS, the suggested acceptance criteria are wider than those allowed by the Scientific Working Group for Forensic Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology⁹. Acceptance criteria for seized drug analysis should be established by the laboratory based on standards and guidelines developed by the Scientific Working Group for Analysis of Seized Drug (SWGDRUG) and the OSAC Seized Drug Subcommittee.

B. Validation Parameters

The validation parameters are consistent with those required by SWGTOX and SWGDRUG.

1. Calibration Model
2. Limit of Detection (LOD)
3. Limit of Quantification (LOQ)
4. Precision and Accuracy (Bias)
5. Interference Studies
6. Carryover
7. Matrix Effect (for Instrumental Techniques Susceptible to Ionization Suppression and Enhancement)
8. Matrix Matching (If Required)

C. Validation Preparation

Before validation begins, assemble the materials required to complete the entire validation. If analyte stability in control material has been verified during method development, it is advisable to prepare bulk controls before beginning the validation, aliquoted into containers (one for each replicate) and stored frozen. Enough material should be prepared to perform the entire validation. The following samples will be required:

⁹ Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology. (2013). *Journal of Analytical Toxicology*, 37(7), 452–474. <https://doi.org/10.1093/jat/bkt054>

1. LOD and LOQ Control

Prepare a minimum of nine samples at the target LOD/LOQ (lowest calibrator) using three different matrix sources. Three replicates in each of the three matrix lots on each of the 3 validation days (nine samples per day) will be analyzed.

2. Imprecision and Accuracy (Bias) Controls Are Performed at Low, Medium, and High Concentrations

Prepare a minimum of 15 samples at each concentration to be used over the validation days. There will be five replicates of each concentration to be run on each of the 3 validation days. The low control should be no more than three times the lowest calibrator (LOQ) and the high control should be no less than 80% of the upper limit of linearity.

3. Interference Study

a. Matrix Interference

A minimum of 10 sources of blank matrix should be collected and tested on the first validation day.

b. Interference from Stable-Isotope Internal Standards (IS)

One blank matrix sample fortified with IS and analyzed each validation day. This sample will also be analyzed after the carryover sample on day 1 to evaluate carryover.

c. Interference from Commonly Encountered Analytes

A low control sample is fortified with high concentrations of analyte or a group of analytes being evaluated as interferents. More than one low control may need to be prepared to cover all potential interferents.

4. Carryover

Prepare a sample at 10 times the highest calibrator concentration. This will be analyzed on the first validation day.

5. Ion Suppression and Enhancement (Matrix Effect) (If Required)

A minimum of 10 sources of blank matrix will be required. Matrix effect should be evaluated on analytes of interest and internal standard in each matrix that will be quantified using the validation method. Matrix effect is quantified as percent suppression or enhancement. If the average suppression or enhancement is more than 30%, then the method should be evaluated for potential impact on quantification of low concentrations of the analyte of interest in patient specimens. See Section F.7. for more details about how to prepare these specimens.

6. Matrix Matching (If Required)

If the method will be used to analyze matrices other than those used to prepare the calibration curve, prepare controls to perform the bias and imprecision experiments in each matrix.

D. Calculations

The following calculations will be used in the validation:

1. Residual

$$\text{Residual} = \text{Observed } Y - \text{Predicted } Y$$

2. %Relative Error

$$\%RE = \frac{\text{Calculated Concentrations} - \text{Nominal Concentration}}{\text{Nominal Concentration}} \times 100$$

3. Bias

$$\text{Bias} = \frac{\text{Average of Calculated Concentrations} - \text{Nominal Concentration}}{\text{Nominal Concentration}} \times 100$$

4. Within-Run Imprecision

$$\text{Within - Run Imprecision (\%CV)} = \frac{\text{Standard Deviation of Single Run}}{\text{Average of Single Run}} \times 100$$

5. Between-Run Imprecision

$$\text{Between - Run Imprecision (\%CV)} = \frac{\text{Standard Deviation of All Runs}}{\text{Average of All Runs}} \times 100$$

6. Matrix Effect

$$\text{Matrix Suppression or Enhancement (\%)} = \frac{\text{Average Area of Extracted Samples}}{\text{Average Area of Neat Samples}} \times 100$$

E. Validation Plan

This validation can be completed in a minimum of three validation runs. These runs should be performed over at least 3 days. Additional validation runs may be needed if matrix matching is required. The following table provides an example of distributing the samples across the 3-day validation.

	Day 1	Day 2	Day 3
Calibration Model	6	6	6
LOD/LOQ	3	3	3
Bias and Precision	15	15	15
Matrix Interference	10	-	-
IS Interference	1	1	1
Common Analyte Interferences	n ¹	n	n
Carryover	1	-	-
Matrix Effect (If Required)	20	20	10 ²
Matrix Matching (If Required) ³	15	15	15

¹ The number of samples required to evaluate interferences from commonly encountered analytes will depend on the number of analytes being studied. These can be distributed throughout the validation as space permits on each analytical run.

² If required (more than 30% suppression or enhancement)

³ Repeat Bias and Precision studies for each matrix being evaluated

F. Validation Experiments

1. Calibration model

a. Procedure

- 1) Prepare a series of at least 6 (nonzero) calibrators covering the concentration range of interest with no more than a 5-fold increase in concentration between sequential calibrators.
- 2) Run a calibration curve on each of the three validation days

b. Data Analysis

- 1) Visually evaluate the individual run calibration curves on each of the 3 days
- 2) Prepare a single calibration curve using the data from all three runs
- 3) Prepare a standard residual plot of the combined calibration curve data
- 4) Calculate the $\sum\%RE$

c. Acceptance Criteria

- 1) For each curve $r^2 > 0.990$
- 2) Standard residual plot must have random distribution
- 3) Selected weighting should be simplest model that minimizes $\sum\%RE$

2. LOD and LOQ

a. Procedure

- 1) The LOD will be defined as the concentration of the lowest calibrator.
- 2) Prepare controls at the same concentration as the lowest calibrator.
- 3) Analyze three replicates each validation run.

b. Data Analysis

- 1) Evaluate retention time and ion ratios.
- 2) Conduct a visual inspection of the chromatograms to evaluate the peak shape and any other criteria used to identify the analyte of interest.
- 3) Use the instrument software to evaluate the signal-to-noise ratio in the lowest calibrator samples.
- 4) Use the established calibration curve to quantify the analyte of interest.

- 5) Calculate the bias.
- 6) Calculate the within-run and between-run precision.

c. Acceptance Criteria

- 1) Lowest calibrator must yield a reproducible signal-to-noise ratio greater than or equal to 3.3.
- 2) Controls must achieve acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios)
- 3) Bias: % Bias less than or equal to 30
- 4) Within-Run Precision: Coefficient of variation (% CV) less than or equal to 30
- 5) Between-Run Precision: % CV less than or equal to 30

3. Precision and Accuracy (Bias)

a. Procedure

- 1) Analyze a minimum of five replicates at each control concentration (i.e., low, medium, and high) for each validation run.

b. Data Analysis

- 1) Use the established calibration curve to quantify the analyte of interest on each run day.
- 2) Calculate the bias, within-run, and between-run precision.

c. Acceptance Criteria

- 1) Bias: % Bias less than or equal to 25
- 2) Within-Run Precision: % CV less than or equal to 25
- 3) Between-Run Precision: % CV less than or equal to 25

4. Interferences

a. Procedure

- 1) Matrix interference—Analyze a minimum of 10 different sources of blank matrix (without IS).
- 2) Interference from stable-isotope internal standards—Analyze a blank matrix fortified with IS but without an analyte of interest in each validation run.
- 3) Interference from commonly encountered exogenous analytes—Analyze low-control specimens that have been fortified with potentially interfering compounds at high concentrations.

b. Data Analysis

- 1) Matrix interference—Evaluate response of any peak at the analyte of interest's retention time.

- 2) Interference from stable-isotope internal standards—Evaluate response of any peak at the analyte of interest's retention time.
- 3) Interference from commonly encountered exogenous analytes—Use the established calibration curve to calculate the analyte of interest's concentration.

c. Acceptance Criteria

- 1) Matrix interference—Response of blank matrix must be less than or equal to 10% of the average response of LOQ.
- 2) Interference from stable-isotope internal standards—Response of IS fortified blank must be less than or equal to 10% of the average response of LOQ.
- 3) Interference from commonly encountered exogenous analytes—The concentration of analytes of interest in the interferant-fortified low control must quantitate within acceptable bias of the target concentration.

5. Carryover

a. Procedure

- 1) Analyze IS-fortified blank specimen after a control fortified at 10 times the highest calibrator.

b. Data Analysis

- 1) Evaluate response of any peak at the analyte of interest's retention time.

c. Acceptance Criteria

- 1) Response of IS fortified blank must be less than or equal to 10% of the average response of LOQ.

6. Matrix Effect

a. Procedure

- 1) Neat Samples: Prepare 10 replicates each of a neat sample at low- and high-control concentrations with internal standard(s).
- 2) Extracted Samples: Extract each blank matrix and then fortify the extracts at the low- and high-control concentrations with internal standard(s).
- 3) Set 3 (If Required): Fortify each source of blank matrix at the low-control concentration and extract following the method.

b. Data Analysis

- 1) Determination of Matrix Suppression (-) or Enhancement (+): Use calculation (6) to determine percent suppression or enhancement.

- 2) Impact on patient sample quantification (if required):
 - a. Use the established calibration curve to quantify the analyte of interest in set 3.
 - b. Calculate the average bias.

c. Acceptance Criteria

- 1) Quantification of Matrix Suppression/Enhancement: |Average Percent Suppression or Enhancement| less than or equal to 30
- 2) Impact on Quantification of Patient Specimens: If average suppression or enhancement greater than 30% is identified, then perform the impact on patient sample quantification experiment.
 - a. Average Bias: |Percent Bias| less than or equal to 25

7. Matrix Matching

a. Procedure

- 1) Prepare controls in blank matrix.
- 2) Analyze a minimum of five replicates of each concentration for every validation run.

b. Data Analysis

- 1) Use the established calibration curve to quantify the analyte of interest.
- 2) Calculate the bias, within-run, and between-run precision.

c. Acceptance Criteria

- 1) Bias: Percent Bias less than or equal to 25%
- 2) Within-Run Precision: Percent CV less than or equal to 25%
- 3) Between-Run Precision: Percent CV less than or equal to 25%

Addendum B: Quantification by Standard Addition for Toxicology Testing

A. Purpose

1. This document outlines an approach to defining the minimum set of experiments necessary to use standard addition for quantitative purposes. Standard addition involves adding different known amounts of a target analyte to fixed amounts of a sample to compensate for a sample matrix effect that enhances or depresses the analyte signal. This process is referred to as “up-spiking” in this document. No formal standards or guidance documents exist for the use of standard addition in forensic casework; therefore, this document serves as a guide for laboratories intending to use standard addition to quantitate NPS in biological samples. Standard addition, often referred to as a “self-validating” approach to quantitation, is susceptible to the same issues regarding error and/or bias that traditional quantitative methods are susceptible to—so in good practice, these experiments should be considered.
2. The processes described herein can be conducted for a single drug of interest in a specimen or for multiple drugs. Although this protocol describes the process for a single drug, furanylfentanyl, more than one drug can be incorporated into a standard addition protocol with proper standard preparation.

B. Preliminary Method Evaluation/Test Setup

1. Develop a method for sample extraction and analysis prior to quantification by standard addition. Extensive validation is not required; however, the following parameters should be evaluated:
 - a. **Limit of Detection:** Determine the lowest concentration with reproducible signal-to-noise ratio greater than or equal to 3.3 and acceptable predefined detection and identification criteria (e.g., retention time, peak shape, mass spectral ion ratios).
 - b. **Linearity Range:** Use the prepared spiking mixes, extraction protocol, and analytical method to prepare a calibration curve over the desired standard addition points to ensure the selected quantitation range is linear. Ideally, the fitted curve should approach the origin (i.e., the y-intercept should be less than 20% of the response ratio for the LOQ). If it does not, consider actions to remedy. It is imperative that the analytical calibration is truly linear over the whole concentration range that will be used in the quantification.
 - c. **Carryover:** Run a blank sample following the largest concentration of up-spike to determine whether the designed method is free from carryover.
 - d. **Interferences:** Analyze sample mixes of commonly encountered substances (specifically those that are also present with the sample of interest) to determine whether the designed method is free from interferences.

C. Standard Addition Assessment

1. **Quantitation:** Conduct a series of three (n=3) “test” experiments to assess the quantitative validity of your standard addition protocol. Use the best available facsimile to the test matrix. For example, you might use beef liver or pork liver if your test matrix is human liver.
 - a. Divide the drug-free matrix into three pools and fortify each pool with drug at a different target concentration, being careful that the target concentrations do not fall below the lower limit of linearity (as determined in Bii) and/or exceed the upper limit of linearity after the third up-spike amount is added. Plan your target concentrations using up-spikes of 50%, 100%, and 150% of the target concentration. For example, for an assay with linear range of 1–100 ng/mL, your lowest target pool should not be less than 1 ng/mL and your highest target pool should not exceed 40 ng/mL.
 - b. Aliquot each spiked-pool source into four replicates.
 - 1) **Target:** Do not include an additional drug standard.
 - 2) **Up-spike Samples 1–3:** Fortify with a drug that is 50% (up-spike sample 1), 100% (up-spike sample 2), and 150% (up-spike sample 3) of target concentration.
 - c. Analyze sample according to established method.
 - 1) Calculate the concentration of a drug in **target** samples using the method of standard addition described in the following example (E.1.). Quantitative value should be within plus or minus 20% of nominal concentration.

D. Authentic Sample Analysis

1. Estimate the sample’s drug concentration.
 - a. Analyze the sample of interest alongside known standard concentrations (e.g., 1 ng/mL, 10 ng/mL, 100 ng/mL) if sufficient sample volume is available.
 - b. Examine the data previously acquired by the screening methodology (e.g., GC-MS, LC-TOF, LC-QTOF) in comparison to known standard concentrations (or previously quantitated case samples) if sufficient sample volume is not available.
 - c. Use the peak area or a peak area ratio to determine the relative concentration of the drug.
 - 1) For example, if sample area is 10,000 and standard (10 ng/mL) area is 20,000, then the relative drug concentration is 5 ng/mL.
 - d. Determine sufficiency of the sample volume. If insufficient sample volume does not allow for screening, you will need to estimate the sample’s drug concentration based on literature and case history, or you will need to run additional points of standard addition to ensure that sufficient separation exists between the concentration in the sample and the up-spikes (e.g., up-spike concentrations that cover the assay’s linear range).
2. Aliquot biological sample for analysis—typically four replicates.

3. **Target Sample 1:** Ensure this sample remains free of drug standard.
4. **Up-Spike Samples 2–4:** Spike drug standard in increasing amounts. For example, 2 is at 50%, 3 is at 100%, and 4 is at 150% of initial estimated concentration.
5. Analyze sample according to established method.
6. Calculate peak area ratio (PAR) for each sample (area drug/area internal standard).
7. Compare plot added (or spiked) concentration to PAR.
8. Calculate x-intercept (concentration of drug in biological sample).

E. Example

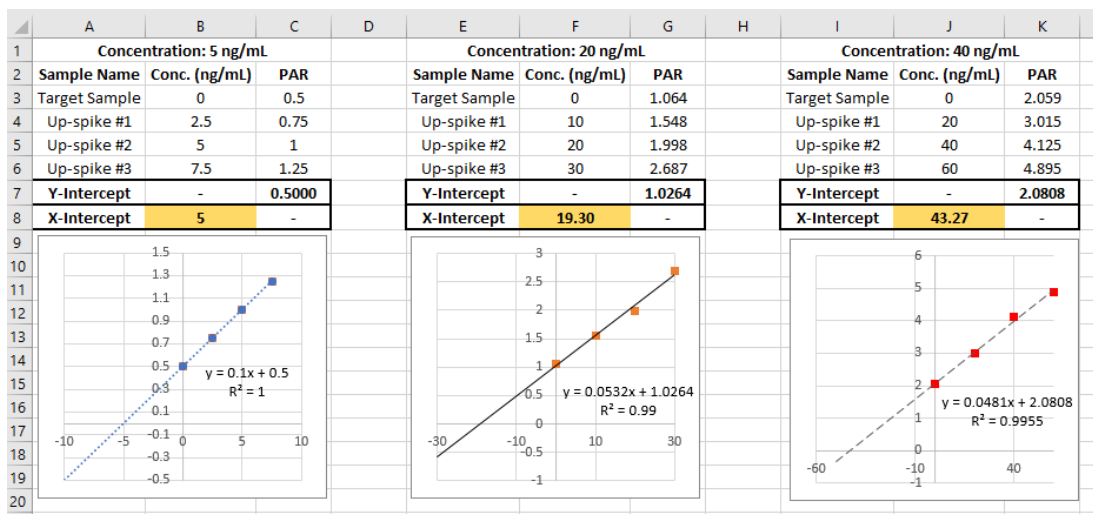
1. Laboratory receives a blood sample that requires the quantitative confirmation of NPS.
2. Initial screening analysis shows the presence of furanylfentanyl (Fu-F) at 35,498 area counts
3. Fu-F standard at 20 ng/mL (63,459 area counts) is analyzed the next day and the relative concentration of Fu-F in the case sample is determined to be 11 ng/mL ($20/63,459 \dots \times 35,498 = 11.2$).
4. Laboratory uses a basic (pH 10) liquid-liquid extraction for screening. The protocol is determined to be optimal for quantitative analysis in this case.
5. Analytical method is developed for Fu-F using fentanyl-D5 as an internal standard.
6. Spiking mixes are prepared at 0.1 ng/μL, 1 ng/μL, and 10 ng/μL.
7. Mock calibration curve is prepared in blood from 1 ng/mL to 100 ng/mL.
 - a. Extracted using the basic liquid-liquid extraction protocol.
 - b. Analyzed using the developed analytical method. The resulting data show linearity between 1 and 100 ng/mL for Fu-F.
8. Standard addition protocol developed for quantitation of Fu-F is determined to be suitable for assessment.
9. Standard addition assessment is performed using three pools of drug-free human blood at 5 ng/mL, 10 ng/mL, and 20 ng/mL.
 - a. Spike pooled blank blood (5 mL) to desired concentration.
 - 1) 5 ng/mL–250 μL of 0.1 ng/μL
 - 2) 10 ng/mL–50 μL of 1 ng/μL
 - 3) 20 ng/mL–10 μL of 10 ng/μL
 - b. Aliquot blood into four 0.5-mL increments.
 - 1) **Target Samples:** No drug added.
 - c. Add drug standard to up-spike samples.

- 1) 5 ng/mL
 - a) **Up-spike 1 (50%, 2.5 ng/mL):** Add 12.5 µL of 0.1 ng/µL.
 - b) **Up-spike 2 (100%, 5 ng/mL):** Add 25 µL of 0.1 ng/µL.
 - c) **Up-spike 3 (150%, 7.5 ng/mL):** Add 37.5 µL of 0.1 ng/µL.
- 1) 20 ng/mL
 - a) **Up-spike 1 (50%, 10 ng/mL):** Add 5 µL of 1 ng/µL.
 - b) **Up-spike 2 (100%, 20 ng/mL):** Add 10 µL of 1 ng/µL.
 - c) **Up-spike 3 (150%, 30 ng/mL):** Add 15 µL of 1 ng/mL.
- 2) 40 ng/mL
 - a) **Up-spike 1 (50%, 20 ng/mL):** Add 10 µL of 1 ng/µL.
 - b) **Up-spike 2 (100%, 40 ng/mL):** Add 20 µL of 1 ng/mL.
 - c) **Up-spike 3 (150%, 60 ng/mL):** Add 30 µL of 1 ng/mL.

d. Prepare and analyze samples.

e. Process resulting data.

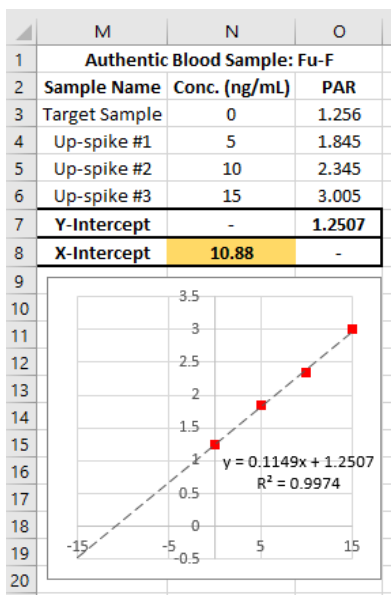
- 1) Calculate and plot peak area ratios against added drug concentration.
 - a) All R^2 values are greater than 0.98.



- 2) Calculate the x-intercept for each set of samples and compare it to the target sample concentration.
 - a) Use the absolute value because the x-intercept will be negative.
 - b) Ensure all values are within 20% of target.

f. Notice that all carryover and interference assessments are negative.

10. Standard addition protocol developed for quantitation of Fu-F is determined to be suitable for application to authentic casework blood samples.
11. Blood sample is analyzed by standard addition.
 - a. Authentic blood sample is aliquoted into four 0.5-mL increments.
 - b. Drug standard is added to three up-spike samples. (Target: estimated to be 11.2 ng/mL, no drug added.)
 - 1) **Up-spike 1 (50%, 5 ng/mL):** Add 25 μ L of 0.1 ng/ μ L.
 - 2) **Up-spike 2 (100%, 10 ng/mL):** Add 50 μ L of 0.1 ng/ μ L.
 - 3) **Up-spike 3 (150%, 15 ng/mL):** Add 15 μ L of 1.0 ng/ μ L.
 - c. Samples are prepared and analyzed.
 - d. Data are processed.



- e. Concentration of Fu-F in the blood samples is 10.9 ng/mL.

Reference

Ellison, S. L., & Thompson, M. (2008). Standard additions: Myth and reality. *The Analyst*, 133(8), 992.
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