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Toxicology

Context matters: selecting LC-MS sample preparation methods for LC-MS/MS in clinical research and toxicology applications

Keywords

Biological matrices, Tox Explorer collection, TraceFinder software, Orbitrap Exploris 120 mass spectrometer, TSQ Quantis Plus mass spectrometer, triple quadrupole, high-resolution mass spectrometry, HRAM, LC-MS, sample preparation, liquid-liquid extraction, protein precipitation, solid phase extraction

Introduction

Professor Alain Verstraete, MD, PhD, head of the toxicology laboratory at Ghent University Hospital, Belgium, used the Thermo Scientific™ Tox Explorer™ Collection to compare different sample preparation methods for detecting therapeutic and narcotic drugs in small volumes of serum and urine.

Liquid chromatography-mass spectrometry (LC-MS) is a highly versatile analytical technology that accommodates broad and untargeted screening for analytes in biological matrices not attainable with immunoassays. As instruments become increasingly easy to use and the quality and robustness of assays improve, LC-MS is gaining traction in clinical labs to cover a growing spectrum of testing applications.

Beyond toxicology screens, LC-MS is used to detect and measure hormones,[1] therapeutic drugs,[3,4] vitamins,[5,6] and amino acids[7,8]. Its use is also being explored to quantify therapeutic antibodies and coagulation factors.[9,10]

While LC-MS may not replace traditional high-throughput clinical chemistry panels anytime soon, its broad spectrum of applications will drive adoption for new testing needs. Currently, LC-MS is most frequently used in large regional reference labs, private labs, and university clinical labs. Nevertheless, the flexibility of LC-MS and the continued development of end-to-end solutions will go a long way to overcome adoption reluctance in smaller clinical labs.

Today's highly standardized MS workflows for evaluating biological samples enable clinical labs to deliver high-quality, efficient, and accurate testing. However, the analytical performance of LC-MS protocols depends greatly on how a sample is prepared for analysis.

So, what are the key considerations in selecting the right sample preparation method?

Sample preparation approaches: what are your options?

Biological samples used for clinical and toxicological analysis—urine, blood, plasma, serum, hair—are complex. Preanalytical sample processing serves both practical and performance purposes:

- **Extending the working life of your MS instrument**
  Processing removes insoluble sample components that are likely to damage analytical equipment, such as proteins and lipids. These components can precipitate and clog the chromatography column, requiring repeated replacement. Complex matrices, such as those found in biological samples, can also cause pressure build-up in the system. Minimizing the matrix components injected into the chromatography system and mass spectrometer extends the maintenance-free uptime of the instrument.
- **Reducing matrix interference**
  Residual matrix components can interfere with ionization in a sample-dependent manner, causing quantification errors, or even the complete disappearance of a peak. In pre-analysis processing, you can remove components of the sample that may influence analyte separation and ionization. This will improve the sensitivity, precision, accuracy, and robustness of your analyses by ensuring quality chromatographic peaks and decreasing interfering background.

Several sample preparation methods are available for LC-MS and they each differ in their complexity, ability to deplete matrix components and concentrate analytes, and cost (Table 1).

Table 1. Common sample preparation methods for LC-MS

Method	Advantages	Disadvantages
Protein precipitation	• Simple, low-cost method requiring no dedicated equipment	• Minimal matrix depletion • Does not concentrate analytes
Dilution	• Simple, low-cost method requiring no dedicated equipment	• Low matrix depletion • Decreases analyte concentration
Phospholipid removal	• Relatively simple protocol • Commercial solutions available • Removes interfering matrix components	• Specialized equipment increases costs • Does not concentrate analytes
Liquid-liquid extraction	• Removes interfering matrix components • Concentrates analytes • Low cost	• Complex procedure • Relatively time-consuming
Supported liquid extraction	• Speeds up sample preparation compared with liquid-liquid extraction • Simplifies an otherwise time-consuming and highly manual task	• Complex procedure • Relatively time-consuming • High cost per sample
Solid-phase extraction	• Removes interfering matrix components • Concentrates analytes • Amenable to automation	• Complex procedure • Relatively time-consuming • High cost per sample
Online solid-phase extraction	• Removes interfering matrix components • Concentrates analytes • Reduced hands-on time • Amenable to automation	• Complex procedure • High cost per sample • Requires dedicated equipment • May block access to LC-MS for other protocols

Dilution, protein precipitation and phospholipid removal are similar sample preparation methods that all aim to reduce background matrix components:

Dilution and protein precipitation are fast, simple, and cheap procedures. Dilution tends to be used for low-protein matrices like urine, whereas protein precipitation is preferred for high-protein matrices such as blood, serum, and plasma. Phospholipid removal is a simple but more costly procedure because it uses specialized filtration plates with moieties designed to selectively retain phospholipids.

The trade-off between the simplicity and affordability of these methods is the higher limits of detection obtained, because none of the simpler, cheaper approaches concentrate the analytes within the sample.

By contrast, liquid-liquid extraction (LLE), supported liquid extraction (SLE), and solid-phase extraction (SPE) increase the concentration of analytes to enhance the sensitivity of detection while still depleting matrix components to increase selectivity. However, these multi-step procedures are more cost-, labor-, and time-intensive than dilution or precipitation.

The throughput of these methods can be improved by automation, allowing the sample preparation process to proceed overnight or while analysts are working on other tasks. For example, when fully automated plasma separation tasks—including serial dilutions, pipetting of plasma samples, and addition of standard solutions—were used for SPE sample preparation in a high-throughput assay of very-late antigen-4 (VLA-4) antagonists, the sample preparation time was less than two hours for 51 samples with 24 standards and 9 quality controls.[11] In another example, a robotic system based on automated liquid handling with additional components capable of handling the entire sample preparation process was used in the quantification of 31 illicit and medicinal drugs from whole blood. In this case, the SPE process for 96 samples was performed in less than three hours while demonstrating robustness and precision of results.[12]

To cleave or not to cleave?

For some matrices, mainly urine, cleavage of phase II metabolism conjugates, such as glucuronide and sulfate groups from the parent drug or phase I metabolites, may also be necessary, if they are not being measured directly. For example, urine testing is often used to assess adherence to opiates and benzodiazepines, and these drug classes are known to undergo extensive glucuronidation or sulfation. Cleavage of conjugated forms of the drug by hydrolysis (Figure 1) increases analytical sensitivity as the combined sum of the free and cleaved metabolites yields higher signals.

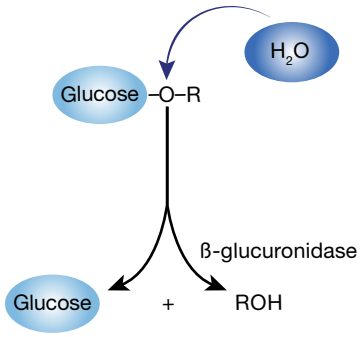


Figure 1. Hydrolysis mechanism

Cleavage by hydrolysis was particularly important when GC-MS was the prevalent analysis method, because intact phase II metabolites were not as readily analyzed by capillary GC as phase I metabolites. This was primarily because of their higher polarity, and, in the case of glucuronic acid, the need for chemical derivatization of the multiple OH groups prior to analysis. By contrast, cleaved drug residues are far more amenable to derivatization often required for gas chromatography.

With the advent of LC-MS and the use of full-scan techniques such as high-resolution accurate mass spectrometry (HRAM), it is now feasible to concurrently detect the parent drug, phase I metabolites, and phase II metabolites, obtaining a more complete picture of the metabolic clearance of any given drug. However, in forensic/clinical toxicology, most reference materials used to confirm analytical findings by MS techniques are either parent drug or phase I metabolites. This means that the conversion of phase II metabolites to parent drug or phase I metabolites by hydrolysis is still required to unequivocally identify a substance.

Hydrolysis can be performed by chemical (acid or base) or enzymatic (e.g., glucuronidase) approaches, either pre- or mid-extraction, depending on the compounds being tested and whether or not an unconjugated fraction is required as well as a conjugated fraction. However, preanalytical hydrolysis can be time-consuming and extend the turnaround time for obtaining results.[13] Moreover, chemical hydrolysis or exposure to temperatures exceeding 50 °C during hydrolysis can lead to sample degradation and loss of the target analyte. Finally, the efficiency of hydrolysis processes varies, and, depending on the drug, it is possible that not all of the conjugated drug will be cleaved. Therefore, when designing sample preparation methodologies, it is important to carefully consider whether a cleavage step is required and which hydrolysis method will be optimal.

Sample preparation in practice: what are others doing?

All the above sample preparation methods, or variations of them, are used in clinical laboratories focused on clinical drug analysis and forensic toxicology. The choice of method is usually aligned to the type of biological sample being analyzed, but those extraction methods that enable both matrix depletion and analyte concentration are preferred.

In a review of the applications of LC-MS in clinical research and forensic toxicology,[14] a range of untargeted screening and targeted multi-analyte screening studies were highlighted as applications of LC-MS. Table 2 summarizes the studies by sample type and preparation method used.

This analysis shows that dilution and precipitation were more likely to be used in targeted screening, where the impact of preparation methods on the analytes of interest can be clearly assessed. For untargeted screening, the authors note that the sample work-up should be as unselective as possible, and suggest dilution and precipitation are suitable, resource-efficient options.

Nonetheless, under the right extraction conditions, LLE and SPE can be suitable for a wide variety of analytes and may have performance advantages over dilution and precipitation, especially for certain analytes and small biological sample volumes. For clinical labs that run both types of screening, generalized LLE and SPE may be good preanalytical protocols to establish as additional options.

Sample preparation compared: how do methods perform?

To examine the impact of different sample preparation methods on LC-MS detection sensitivity, we carried out a comparison drug bioanalysis on serum samples at our clinical lab at Ghent University Hospital using a HRAM mass spectrometer.

The three sample preparation methods—precipitation, LLE and SPE—were tested in targeted screening using the compound database and high-resolution spectral library of the Tox Explorer Collection (a complete workflow solution for toxicology). The acquisition parameters were as follows and remained constant for all three sample preparations:

- Resolution of 35,000 for the full scan and 17,500 for MS-MS scan
- Isolation window of 2.0 *m/z*
- Polarity switching to capture positively and negatively ionizing compounds

The same inclusion list of targeted compounds was used across all sample preparation methods, with exact mass, polarity, and retention time.

As Table 3 shows, detection sensitivity was highest for SPE and lowest for precipitation. The higher detection limit with precipitation may result from the lower sample volume used compared to LLE and SPE (50 µL versus 250 µL serum). However, increasing the sample volume in precipitation does not improve analyte concentration, and the low volume requirement may be advantageous when specimens are used for a battery of tests. Evaporating the supernatant after precipitation may improve sensitivity but also extends sample preparation time.

It is worth noting the generally weaker results for certain drug classes. Regardless of the sample preparation process, typically only one in three barbiturates and one in four cannabinoids were detected. This might be due to incomplete ionization of the analytes with the mobile phase being used.

The extensive panel of drugs tested includes some that ionize in positive mode and others, such as barbiturates and tetrahydrocannabinol metabolites, that typically ionize in negative mode. This required analysis of MS spectra in both modes, achievable in a single analytical run on the Thermo Scientific™ Orbitrap Exploris™ mass spectrometer, with its fast polarity switching.

Table 2. Frequency of four preparation methods in untargeted (52 screenings) and targeted (90 screenings) LC-MS

	Untargeted screening (<7,500 analytes)				Targeted multi-analyte screening (<132 analytes)			
Method	• Blood • Plasma • Serum	• Urine	• Hair	• Oral fluid • Tissue • Other	• Blood • Plasma • Serum	• Urine	• Hair	• Oral fluid • Tissue • Other
Dilution	1	6	–	–	3	12	–	1
Precipitation	2	6	–	–	6	3	–	1
Liquid-liquid extraction	10	7	3	1	20	6	11	4
Solid-phase extraction	18	16	–	1	11	11	4	7

Table 3. Number of drugs detected at low and medium concentrations after precipitation, LLE, and SPE

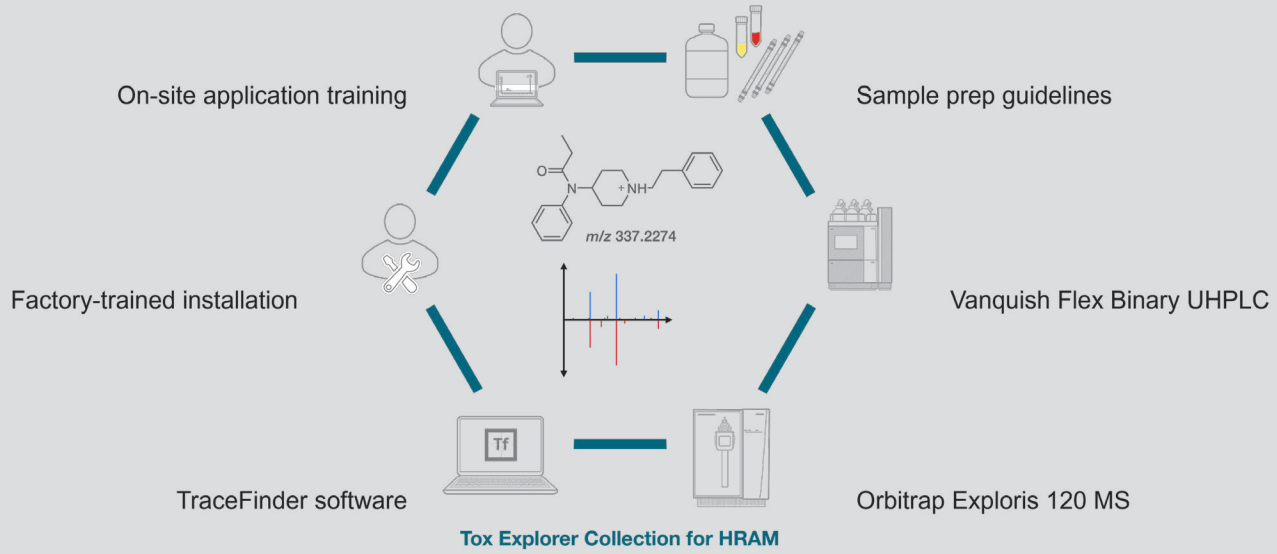
		Precipitation		LLE		SPE	
	n	Low	Medium	Low	Medium	Low	Medium
Amphetamines	17	3	13	16	16	16	17
Cocaine	4	0	4	4	4	3	4
Opiates	23	2	17	18	18	22	22
Benzodiazepines	26	11	22	23	23	23	24
Barbiturates	3	0	1	1	1	1	1
Cannabinoids	4	0	1	1	1	1	2
LSD	1	0	1	1	1	1	1
Replacement drugs	4	1	1	2	3	3	3
Others	20	9	14	14	14	12	12
Total	**102**	**29**	**77**	**82**	**84**	**85**	**89**

Thermo Scientific Tox Explorer Collection

The Tox Explorer Collection is a standardized workflow for chromatographic separation and MS detection, enabling fast identification and targeted screening of large panels of therapeutic drugs, drugs of abuse, and pesticides.

An all-in-one LC-MS solution, it comes equipped with sample prep guidelines, a standardized liquid chromatography method with (U)HPLC column and Thermo Scientific™ TraceFinder™ software with Tox Explorer methods, and an experimentally obtained compound database with retention times. On-site LC-MS instrument set-up and Tox Explorer application training enables smooth start-up and implementation.

It is available in either Thermo Scientific™ High-Resolution, Accurate-Mass (HRAM) Orbitrap™ or Thermo Scientific™ TSQ Quantis™ Plus triple quadrupole MS platform options to boost confidence in data collection and enhance productivity in the laboratory.



Tox Explorer Collection for HRAM

From resolution to robustness

We also included several analytical standards to ensure robustness of the method and were able to demonstrate high mass accuracy and mass stability across multiple injections (Figure 2).[15] The availability of commercial multi-analyte calibrators and controls, sometimes containing 100+ compounds, greatly facilitates the implementation of an LC-MS method for the clinical lab.

The within-run coefficient of variation (CV) for the internal standards was 10%.[15] Moreover, across all sample preparation methods, over 75% of analytes could be quantified with a CV of 30% or less. This CV is sufficiently precise to classify analyte concentrations as "therapeutic", "above therapeutic", and "toxic", in accordance with guidelines for emergency toxicology in some countries.[16] For forensic analysis, the U.S. Academy Standards Board (ASB) states that the % CV shall not exceed 20% at each analyte concentration, and some analytical methods (e.g., blood alcohol analysis) require a much lower CV (≤10%).[17] Meanwhile in Germany, for emergency toxicology, the precision requirements for emergency toxicology analyses are higher: <30% of two quality controls at the upper (80%) and lower (20%) ends of the measurement range, determined on five different days in duplicate.[18]

Clearly, selecting sample preparation methods for LC-MS in clinical applications is not a simple choice. It will depend on what analytes are being sought, the quantification precision required by relevant guidelines, and the impact of different sample preparation protocols on overall lab routines.

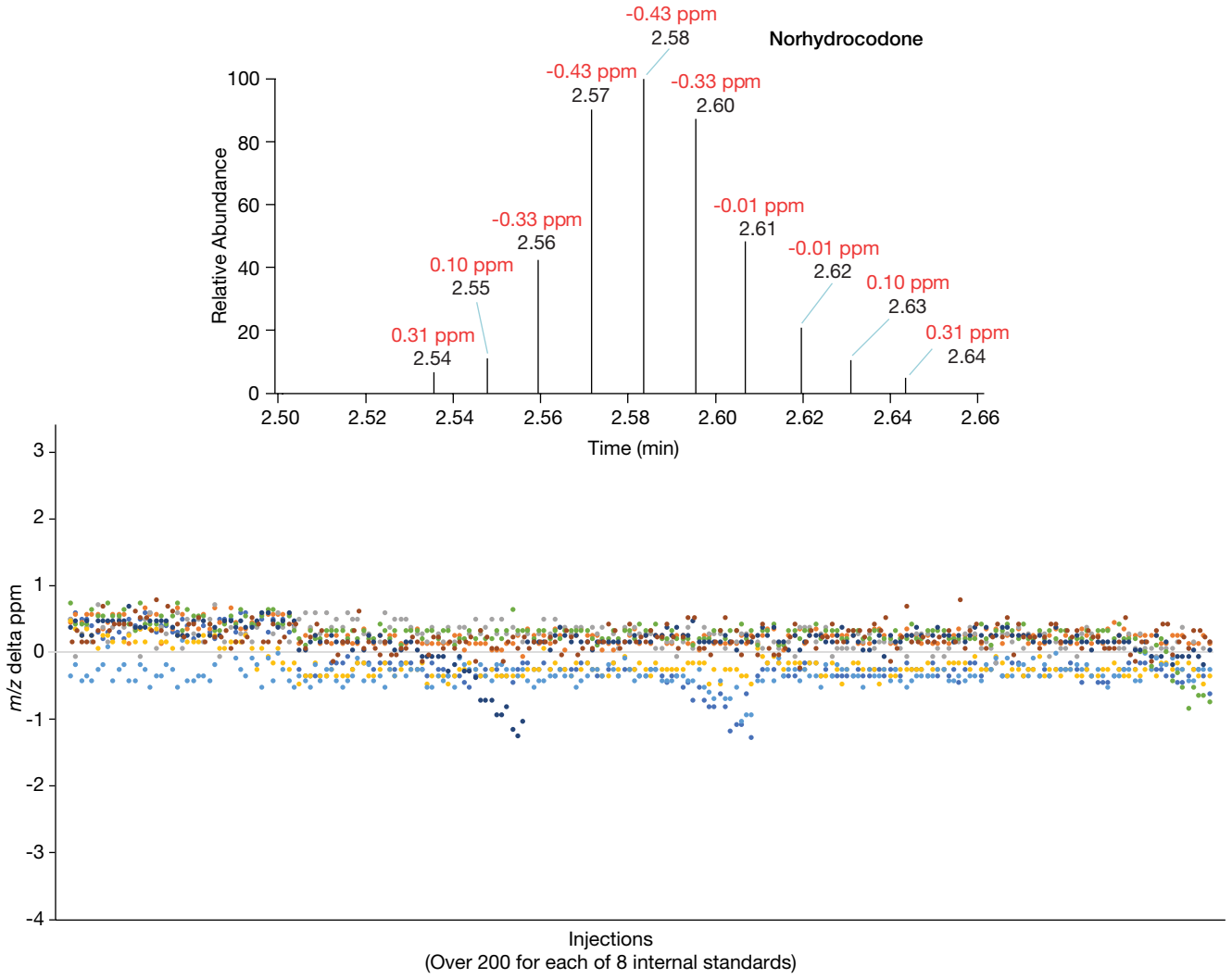


Figure 2. Extracted ion chromatogram for one compound at 0.1 ng/mL and mass deviation for several compounds across an analytical run

Selecting a method: beyond sensitivity, look at context

There is no single ideal clinical sample preparation method for LC-MS. Selection is instead a balancing act between ease of use and sensitivity and between the time and cost of upfront processing versus downstream complications. It must also take into account the between-analysis cost of processing samples, replacing analytical columns, and maintaining the analytical instrument.

Although boosting sensitivity and minimizing matrix effects on detection and process reliability are important objectives in the selection of a sample preparation method, the decision must also consider the context in which LC-MS is being used.

When considering which sample preparation method to use, the following key questions may help:

- What is the sample type?
- How much volume is available?
- Do you need high sensitivity or are drugs present in high concentrations?
- What SPE equipment is available and could you have a dedicated instrument for this analysis?
- How many lab personnel are trained in extraction techniques?
- How many samples will you analyze in a typical run?
- How fast is your required turn-around time?

It is also important to ground your method selection in the reality of your laboratory routines. To do so, there are some important considerations.

Stay flexible

Keeping sample preparation as unselective as possible is important not only for untargeted analyses or for detecting as many analytes as possible (acidic and basic) in a single assay, but it also accommodates the addition of further analytes as testing demands change over time. Today's MS instruments, such as Thermo Scientific™ Orbitrap™ systems, are versatile, and flexible sample preparation allows you to realize their full analytical potential.

Consider complementary assay methods

Leveraging other detection methods in the lab may offer easier ways of detecting compounds that would otherwise require labor- and cost-intensive sample preparation. For example, some of the newer designer benzodiazepines go undetected by LC-MS methods but are easily screened for with immunoassays. Using both methods together to cover a full toxicology profile is more efficient than developing and validating several dedicated LC-MS screens.

Check proficiency

Participate in external proficiency testing to assess and improve performance of developed protocols. One approach is to start with a simple preparation method like precipitation, and then critically assess which concentrations can be reliably measured. If proficiency testing demonstrates insufficiency in detecting toxic concentrations of prominent analytes, a more complex sample preparation method may be necessary. It is worth noting that the ASB has developed guidance for the analytical scope and sensitivity of methods for specific analyses, including drug-facilitated sexual assault DFSA (Standard #121), driving under the influence (Standard #120), and medicolegal death investigations (Standard #119).[19-21]

Keep validation practical

Even if your sample preparation phase is not labor-intensive, your validation phase may well be. For many validation tests (e.g., matrix effect, limit of detection, interference), labs will need to develop analytical standards and spike blank matrices, a time- and cost-intensive endeavor. It can simplify matters considerably if you can use commercial controls and calibrators for validation, and the sensitivity of today's MS instruments makes this more feasible. Some national guidelines now list the validation tests required for certain analyses, such as in ANSI/ASB standard 36 (Annex C).[17]

LC-MS: a staple of the modern clinical lab

The application of LC-MS in clinical labs has several advantages over other detection methods for many substances. LC-MS detection is more specific and reliable than UV or diode-array methods and accommodates broad and untargeted screenings that are unattainable with immunoassays. Plus, given the rapid diversification of analytes for clinical testing, adoption of LC-MS futureproofs the service offering of a clinical lab.

Commercial LC-MS solutions are rapidly evolving towards ease of use and robust performance so that even less-specialized laboratories can confidently adopt these analytical systems. Also, the sensitivity of today's MS instruments makes using commercial controls and calibrators economically feasible, eliminating the tedious process of creating them in-house.

While appropriate and optimal sample preparation will likely always play a role in clinical routines for LC-MS, the range of available methods offers ways to balance ease of use, costs, and assay performance. The optimal choice will be the procedure that meets testing demands and keeps laboratory routines practical and lean.

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