Metanephrines in (EDTA) plasma

1060 M MET

Instruction manual for LC-MS/MS assay
for in vitro diagnostic use

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CE IVD Medical Devices

Contents

1. General Information

1.1 Information for the Device

1060 M MET - Metanephrines reagent set
UDI-DI: 0872051431MMETDT
ENDM: W01019099 (see section 2.4 and 2.5 for ENDM codes individual products)

For information on the individual components of this set, refer to chapter 2 of these instructions for use.

1.1.1 IVDR classifications

Class C (not self-test/near patient test/companion diagnostic), based on rule 3(h); (EU) 2017/746, Annex VIII, 2.6.

IVR 0608
IVP 3002 / 3003
IVD 4002
IVS 1004 / 1006
IVT 2006

1.2 Manufacturer

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1.3 Intended Purpose

1.3.1 Measurand

Metanephrine

Normetanephrine

3-methoxytyramine
3-MT

1.3.2 Function

This In Vitro Diagnostics medical device enables the quantitative measurement of metanephrine, normetanephrine and 3-methoxytyrimaine (V3-MT) in human serum. The device

is intended to support the assessment of catecholamine metabolism in individuals undergoing evaluation for conditions that may involve abnormal catecholamine production, including neuroendocrine tumours. The results provide specific information related to the physiological or pathological levels of the measured analytes. The test is intended for use by trained laboratory professionals in a clinical laboratory environment

The device does not provide interpretative statements and is not intended to diagnose or monitor any specific disease or clinical condition.
The components in this kit must be used as stated in the user manual.

1.3.3 Required Specimen

Human plasma or serum.

1.3.3.1 Conditions for collection, handling and preparation of specimen

This method is validated for use with plasma collected in EDTA tubes. Internal comparison studies involving matched samples from multiple donors have shown that lithium heparin plasma and serum may also be suitable matrices, as no clinically relevant differences were observed. However, since these matrices have not been fully validated, users must verify their suitability under their specific pre-analytical conditions before implementation.

Sample collection, handling, transport, and storage procedures must be defined and validated by the end user, taking into account their specific laboratory workflows and materials (e.g., collection tube types, anticoagulants). Minor differences between matrices may occur and should be assessed locally where applicable. It is the user's responsibility to ensure that pre-analytical conditions are suitable for use with this test.

1.3.4 Testing Population

This device is intended for use in patients for whom the quantitative measurement of metanephrine, normetanephrine and 3-MT is requested by a healthcare professional.

1.3.5 Intended User

This kit is designed for use by healthcare laboratory professionals with a basic understanding of LC-MS/MS. Diagnotix recommends that users adhere to ISO 15189 Medical Laboratories.

1.4 Test Principle

1.4.1 Description of the analytical procedure

Metanephrine, normetanephrine and 3-MT are determined from human (EDTA) plasma by UHPLC with positive ion electrospray LC-MS/MS.

Prior to the LC-MS/MS analysis a purification of the sample spiked with internal standard is performed with Weak Cation Exchange (WCX) SPE-columns. Solid-phase extraction (SPE) is a method of sample preparation that concentrates and purifies analytes from solution by adsorption onto a disposable solid-phase cartridge, followed by elution of the analyte with a solvent appropriate for instrumental analysis.

WCX SPE is used to remove all non-basic compounds from the matrix. As the compounds of interests have positive charges at neutral and low pH, and the charge of the WCX SPE is negative at neutral as well as low pH, this method can be used to remove all neutral polar, acidic and hydrophobic compounds as well as proteins, lipids and salts.

The eluate consists of basic compounds with a similar hydrophobicity as the compounds of interest.

After sample preparation, the sample is injected into a LC-MS/MS system. Prior to MS detection, the eluate is injected onto a HILIC analytical column. HILIC is used as the separation method; this technique employs a polar stationary phase and, in contrast to conventional reversed-phase separation, water functions as the strong solvent, while acetonitrile is used as the weak solvent in the mobile phase.

After elution from the analytical column metanephrines, normetanephrines and 3-MT are ionized by electrospray ionization (ESI) and detected by MS/MS.

Measurement of the analytes is carried out in MRM (Multiple Reaction Monitoring) mode. In this mode only selected ions, known as precursor ions, with a defined mass/charge (m/z) ratio are isolated in the first quadrupole and subsequently transferred into the collision cell, where they are fragmented by impact with an inert gas (argon or nitrogen) at defined voltage settings. Among the fragments generated (known as product ions) only those with a defined m/z ratio can pass the third quadrupole for final detection. In this way the MRM mode ensures a selective identification and quantification of the target analytes.

The analytical method enables a quantitation in biological matrices by use of isotope-labelled internal standards. Two MRM-transitions (quantifier, qualifier) are used for each analyte.

1.4.2 Function of the Internal Standard Without a Specific Value

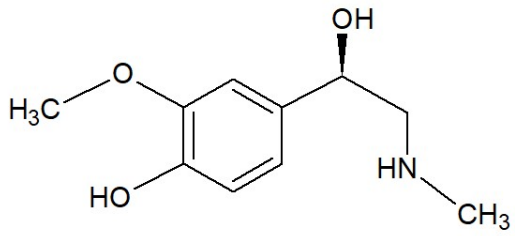
The internal standard solution must be added in the same volume to each calibrator, control, and patient sample. This ensures that every sample has the same internal standard concentration. The specific concentration of the internal standard is not relevant.
The internal standard compensates for interference (suppression) or enhancement from the matrix background. Since the patient matrix, calibrator matrix, and control matrix can all differ, the internal standard response will vary between samples. Because the internal standard has a very similar chemical nature to the analyte of interest, its signal will be suppressed or enhanced in a comparable manner.
When setting up the LC-MS software for quantitative analysis, it is important to assign a specific internal standard to each analyte and to set its concentration to 1.
The internal standard should ideally have a retention time as close as possible to that of its corresponding analyte.
It is also crucial to ensure that the internal standard is added to each sample in a consistent manner. Any variation in solubility, concentration changes over time (e.g., due to solvent evaporation), or differences in the time between sample preparation and measurement could lead to changes in concentration, potentially causing falsely elevated or decreased results. Strict adherence to the method is therefore essential.

1.5 Clinical background

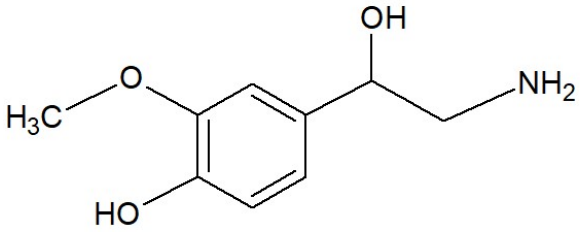
The Free Metanephrines test in (EDTA) plasma measures three metabolites of the catecholamines epinephrine, norepinephrine and dopamine. These catecholamines are made in the adrenal gland to regulate the heartbeat, blood pressure and glucose

concentrations. They can also be released in excess by rare tumors, such as pheochromocytomas and paragangliomas, on adrenal and extra-adrenal chromaffin tissue. The majority of these tumors release epinephrine and norepinephrine. Some rare tumors however excrete mostly, or sometimes solely, dopamine.

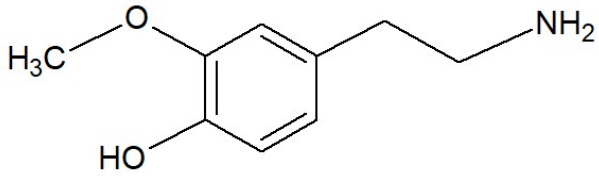
This is why all three the catecholamines are important to be determined when one of these tumors is suspected. There are however some difficulties with determining catecholamines, some tumors, for example, are biochemically silent or only periodically secrete these catecholamines making false negatives a possibility. False positives may also arise because of panic disorders or congestive heart failure. Their metabolites, metanephrine, normetanephrine and 3-methoxytyramine (3-MT) have superior diagnostic sensitivity and specificity compared to urinary and plasma catecholamines and so are recommended for the diagnosis of pheochromocytomas and paragangliomas.[1]



Metanephrine



Normetanephrine



3-methoxytyramine

[1] Weismann, D. et al (2015)

1.6 Notice Regarding Serious Incidents

Following (EU) 2017/746 Annex I, Chapter III, 20.4.1 af), any serious incident that has occurred in relation to this device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

1.7 IVD symbols

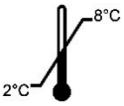
Order Number

Lot Number

For in vitro diagnostic use

See instructions for use

Manufacturer



Temperature limits

Contains sufficient for < n > tests

Expiry date

2. Components and Accessories

2.1 Safety information

1 Before starting the assay, ensure that you have thoroughly read and fully understood the instructions.

2 Some components are chemical preparations and may contain hazardous substances. For safety information, please refer to the Material Safety Data Sheet (MSDS) for each component.

3 The donor blood used as raw material was tested for HBsAg, anti-HIV 1/2, and anti-HCV. However, since no test method can guarantee that products derived from human sources are completely free from infectious agents, it is recommended to handle this product with the same precautions as patient samples.

4 Dispose of all reagents as hazardous waste in accordance with your national biohazard and safety guidelines or regulations. It is important to ensure that everyone who may come into contact with the products is informed about the proper handling and disposal procedures.

5 Any serious incidents related to the device must be reported to the manufacturer and the relevant competent authority.

2.2 Storage conditions and lifetime of kit components

1 Upon receipt, immediately unpack the kit components from the transport packaging and follow the storage instructions indicated on the product labels.

2 If any component is damaged, collect physical evidence (e.g., by taking a photo) and contact Diagnotix within one week. Do not use any damaged product and store it in a safe location.

3 Handle broken glass with care to prevent injury.

4 Do not use expired products. Do not mix reagents of the same type.

2.3 Storage conditions and lifetime of kit components

All components are intended for LC-MS/MS use only. They may also contain ingredients other than the active ingredients listed below, which could influence the measurement. All stated stability conditions are only valid in the absence of bacterial contamination.

Please unpack the kit components from the transport packaging *immediately upon receipt* and follow the instructions for storage conditions indicated on the product labels.

2.3.1 Calibrators and controls

1061 CAL M MET | Metanephrines Calibrator Set
UDI: 8720514310670

A six-point lyophilized blood calibrator at clinically relevant levels, refer to the value data sheet provided with each set for specific values per production batch.

1062 CON M MET | Metanephrines Control Set
UDI: 8720514310687
1075 M MET | Metanephrines Control I
UDI: 8720514310816
1075.10 M MET | Metanephrines Control I (10-pack)
UDI: 8720514313633
1076 M MET | Metanephrines Control II
UDI: 8720514310823
1076.10 M MET | Metanephrines Control II (10-pack)
UDI: 8720514313640
1077 M MET | Metanephrines Control III
UDI: 8720514310830
1077.10 M MET | Metanephrines Control III (10-pack)
UDI: 8720514313657

Three levels of lyophilized blood controls at clinically relevant levels for quality control purposes, refer to the value data sheet provided with each set for specific values per production batch.

2.3.1.1 Handling

Reconstitute the calibrators and controls as follows:

1. Carefully remove the cap and rubber plug avoiding any loss of contents.
2. Reconstitute Metanephrines Calibrator Set and Controls with exactly 2,0 ml distilled or deionised water using a volumetric pipette.
3. Replace the plug and let stand during 15 minutes.
4. Swirl the vial carefully and mix thoroughly making sure that all traces of dry material have been dissolved, do not shake. Avoid foaming.
5. Let stand for another 15 minutes at room temperature.
6. Swirl the vial carefully, do not shake. Avoid foaming.
7. Use the preparation as a patient sample.

2.3.1.2 Stability and storage

The stability of the calibrators and controls are:

Before reconstitution:	2 - 8 °C	Until expiry date printed on the product label.
After reconstitution:	2 - 8 °C	2 weeks.
After reconstitution:	- 20 °C	1 month.

The declared stated stabilities are only valid in case of no bacterial contamination.

2.3.2 Internal standard

1069 M MET | Metanephrines Internal Standard D3 D4
UDI: 8720514310755

1069.10 M MET | Metanephrines Internal Standard D3 D4 (10-pack)
UDI: 8720514313626

A lyophilized deuterated version of the measurand, dissolved in an inert substance. Used to identify and correct potential deviating values, due to errors or varying circumstances in sample preparation or within the LC-MS.

Active ingredients: 3-MT-D4, Metanephrine-D3, Normetanephrine-D3

2.3.2.1 Handling

Reconstitute the internal standard as follows:

1. Carefully remove the cap and rubber plug avoiding any loss of contents.
2. Reconstitute Metanephrines Internal Standard with exactly 6 ml buffer (1079 M MET) using a volumetric pipette.
3. Swirl the vial carefully and mix thoroughly making sure that all traces of dry material have dissolved, do not shake. Avoid foaming.
4. Let stand for 15 minutes at room temperature.
5. Swirl the vial carefully, do not shake. Avoid foaming.
6. After reconstitution pour this vial into the buffer bottle and mix thoroughly.

2.3.2.2 Stability and storage

Store at 2 - 8 °C	After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C.

2.3.3 Mobile Phases

1070 M MET | Metanephrines Mobile Phase I
UDI: 8720514310762
1071 M MET | Metanephrines Mobile Phase II
UDI: 8720514310779

Two mobile phases are added to tune and carry the sample through the LC-MS/MS. Different ratios of the mobile phases will allow different components to eluate from the analytical column at differing speeds.

Active ingredient(s):

Mobile Phase I:	75 - < 100% Water
Mobile Phase II:	75 - < 100% Acetonitrile

2.3.3.1 Handling

The Reagents are liquid and ready for use.

2.3.3.2 Stability and storage

Store at 2 - 8 °C	After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C or 4 weeks on the UHPLC.
Store at RT	Before first opening the Reagent can be stored for 12 weeks at Room Temperature

2.3.4 Washing Solutions

1072 M MET | Metanephrines Washing Solution 1
UDI: 8720514310786
1073 M MET | Metanephrines Washing Solution 2
UDI: 8720514310793
1074 M MET | Metanephrines Washing Solution 3
UDI: 8720514310809

Three solvent solutions are used during SPE sample preparation to remove specific molecules that could otherwise interfere with the later LC-MS/MS analysis.

Active ingredient(s):
Washing solution 1: 75 - < 100% Methanol
Washing solution 2: 0 - < 2.5% Ammonium phosphate (w/v)
Washing solution 3: 75 - < 100% Acetonitrile

2.3.4.1 Handling

The Reagents are liquid and ready for use.

2.3.4.2 Stability and storage

Store at 2 - 8 °C — After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C.

2.3.5 Elution solution

1078 M MET | Metanephrines Elution Solution
UDI: 8720514310847

The elution solvent is used to elute the desired analytes from the SPE sorbent.

Active ingredients: 75 - < 100% Acetonitrile; 0 - < 2.5% Formic Acid

2.3.5.1 Handling

The Reagent is liquid and ready for use.

2.3.5.2 Stability and storage

Store at 2 - 8 °C — After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C.

2.3.6 Buffer

1079 M MET | Metanephrines Buffer
UDI: 8720514310854

The buffer solvent is used to dissolve the internal standard and to adjust the sample to the appropriate pH for SPE sample preparation.

Active ingredient(s): 0 - < 2.5% Ammonium phosphate (w/v)

2.3.6.1 Handling

The Reagent is liquid and ready for use.

2.3.6.2 Stability and storage

Store at 2 - 8 °C — After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C.

2.3.7 Autosampler washing solution

10601 M MET | Methanephrines Autosampler Washing Solution
UDI: 8720514310861

A solution used to clean the LC-MS/MS system after use, specifically designed to remove residue from testing the measurand.

Active ingredient: 75 - < 100% Acetonitrile

2.3.7.1 Handling

The Reagent is liquid and ready for use.

2.3.7.2 Stability and storage

Store at 2 - 8 °C — After first opening the Reagent can be used for 6 weeks if closed and stored at 2 - 8 °C or 4 weeks on the UHPLC.

2.4 List of components provided

1060 KIT M MET - Complete Kit for Metanephrines in (EDTA) plasma
UDI: 8720514311721

Contents (for 200 assays):

Description	**Part number**	**Content**	**EMDN code**
Metanephrines Calibrator Set (Calibrator 1 – 6)	1061 CAL M MET	6 x 2 x 2 ml	W0101050301
Metanephrines Internal Standard D3 D4	1069 M MET	4 x 6 ml	W0101050303
Metanephrines Mobile Phase I	1070 M MET	1 x 250 ml	W01019099
Metanephrines Mobile Phase II	1071 M MET	2 x 500 ml	W01019099
Metanephrines Washing Solution 1	1072 M MET	2 x 220 ml	W01019099
Metanephrines Washing Solution 2	1073 M MET	2 x 220 ml	W01019099
Metanephrines Washing Solution 3	1074 M MET	2 x 100ml	W01019099
Metanephrines	1078 M MET	2 x 30 ml	W01019099

Elution Solution			
Metanephrines Buffer	1079 M MET	4 x 30 ml	W01019099
Metanephrines Autosampler Washing Solution	10601 M MET	1 x 1000 ml	W01019099
Metanephrines Manual			

2.5 Separately available materials and components

Metanephrines Calibrator Set (Calibrator 1 – 6)	1061 CAL M MET	6 x 2 x 2 ml	W0101050301
Metanephrines Controls Set (control I – III)	1062 CON M MET	3 x 3 x 2 ml	W0101050101
Metanephrines Internal Standard D3 D4	1069.10 M MET	10 x 6 ml	W0101050303
Metanephrines Mobile Phase I	1070 M MET	250 ml	W01019099
Metanephrines Mobile Phase II	1071 M MET	500 ml	W01019099
Metanephrines Washing Solution 1	1072 M MET	220 ml	W01019099
Metanephrines Washing Solution 2	1073 M MET	220 ml	W01019099
Metanephrines Washing Solution 3	1074 M MET	100 ml	W01019099
Metanephrines Elution Solution	1078 M MET	30 ml	W01019099
Metanephrines Buffer	1079 M MET	30 ml	W01019099

Metanephrines Autosampler Washing Solution	10601 M MET	1000 ml	W01019099
Metanephrines Control I	1075.10 M MET	10 x 2 ml	W0101050101
Metanephrines Control II	1076.10 M MET	10 x 2 ml	W0101050101
Metanephrines Control III	1077.10 M MET	10 x 2 ml	W0101050101

2.6 List of required additional products not supplied

Category		**Examples**
General laboratory equipment	UHPLC-MS/MS	Sciex 4500 or higher Shimadzu LC8050 or higher Waters TQS-µ or higher
	Shaker for sample tubes	Ohaus Orbital Shaker
	Vortex shaker	VWR VV 3
	Calibrated pipette 20-200 µl	
	Calibrated pipette 100-1000 µl	

	2-8°C controlled refrigerator	
	-20°C controlled freezer	
	Centrifuge	Eppendorf Centrifuge 5425
	Tube roller	Phoenix instrument RS-TR05
	SPE-Vacuum system, SPE-positive pressure system, or centrifuge system capable for SPE	Phenomenex Presston 1000 positive pressure system
General laboratory material	Sample tubes	
	Autosampler vials, inserts, caps or well plates	
	Pipette tips 20-200 µl	
	Pipette tips 100-1000 µl	
	Analytical column & SPE material	See section 4.1.6

3. Warnings, precautions, measures and limitations of use

3.1 General

The device and its components must only be used in line with the intended purpose by the intended user as stated in chapter 1. Due to their nature, most reagents of this device contain or are largely composed of hazardous substances. Please refer to the Safety Data Sheets (SDS) for each of the components for specific hazards and measures to be taken.

Used components should be discarded and are not suitable for re-use.

3.1.1 Potentially infectious material

The human material used for manufacturing calibrators and controls was tested for various markers for infectious diseases and/or pathogens and found negative. Nevertheless, the Whole blood calibrators & controls should be considered as potentially infectious and treated with appropriate care.

3.2 Interferences & Limitations

Visual evidence of lipemia, homolysis, or icterus (hyperbilirubinemia) and/or older age of the specimen may affect the performance of the device.

3.3 CMR substances

Methanol is present in the deproteinization solution. Methanol has been classified as a Category 2 reproductive toxicant according to Annex VI of Regulation (EC) No. 1272/2008. Although not classified as carcinogenic or mutagenic, methanol may pose a risk to human reproduction and is suspected of causing harm to the unborn child.

3.4 Disposal

For the safe disposal of the components of this kit, please refer to the safety data sheet of the component in question.

4. Assay procedure

4.1 System suitability & Method installation

4.1.1 Minimum requirements instrument and LC modules

Using this test kit requires a UHPLC system with tandem mass spectrometer (LC-MS/MS) with the following modules:

- Autosampler
- UHPLC gradient pump
- Column heater
- Degasser

- Proper gas supply is required for the specific MS/MS system in use

4.1.2 Minimum requirements tandem mass spectrometer

The tandem mass spectrometer should be sensitive enough to achieve a signal-to-noise ratio of at least 10 for calibrator 2.

To the best of our knowledge, no two LC-MS/MS systems react exactly the same due to minor technical differences between manufacturers, sensitivity, age, time of usage, location settings, and individual machinery conditions. Therefore, the MS settings, including the m/z transitions mentioned in section 5.4.3, should be considered as a guideline. It is strongly recommended that users tune and optimize their instrumentation as part of the method installation.

4.1.3 Minimum requirements UHPLC

The UHPLC should have a backpressure limit of at least 600 bar. It is strongly recommended to use a binary pump.

The use of a loop with a minimum volume of 10 µL is recommended.

4.1.4 Minimum requirements chromatography

Due to minor differences between UHPLC and MS devices, the chromatographic conditions, such as temperature, gradient, and injection volume, may need to be adjusted to ensure the following conditions are met:

- A minimum retention time of 3 column volumes
- A resolution of at least 1 between all analytes, but advised is at least 1.5 (baseline separation)
- Repeatable retention times for all analytes between runs. If retention times fluctuate by more than 0.1 minutes, this may indicate that the gradient is not properly set or the column is not adequately conditioned.
- For quaternary pumps, it is advisable to extend the reconditioning step of the gradient to improve the repeatability of retention times.
- Separation of possible isobars/interferences, see section 6.9. In case separation of isobars are not been demonstrated, one should take notice of possible false elevated concentrations and therefore further patient specific research can be necessary.

4.1.5 Column conditioning and maintenance

In most cased the analytical column is ready to use.

he column should be stored in the storage solution recommended by the manufacturer. Before reuse after storage, it may be necessary to flush the column with the initial gradient conditions until a stable backpressure is achieved, ensuring complete removal of the storage solvent. Store the column in its original box, in a dark, temperature-controlled environment, protected from continuous movement and vibrations.

4.1.6 Examples of suitable columns and SPE materials

The list below contains columns that Diagnotix has used or is using for this method. However, other columns may also suffice, as long as the minimum chromatography requirements as stated in section 4.1.4 are met.

Columns:

Supplier	**Product name**	**Part number**
Phenomenex	Kinetex Hilic 1.7 µm 150 x 2.1 mm	00F-4474-AN
Waters	Acquity UPLC BEH Amide 1.7µm 2.1 x 100mm	186004801

The list below contains SPE 96-wells plates that Diagnotix has used or is using for this method. However other SPE 96-wells plates may also suffice, as long as they can handle small volumes, like Waters µElution plates.

SPE 96-wells Plates:

Supplier	**Product name**	**Part number**
Waters	Oasis WCX 10mg	186006342

4.1.7 Verification, Validation & Quality management

We recommend that users perform at least a verification of the method using independent controls and correlation studies. A full validation is advised due to potential LC-MS/MS system-specific variations.

A comparison study with an existing method is highly recommended. Diagnotix offers the possibility to conduct comparison studies. Please contact us for more information.

Additionally, we recommend incorporating multiple-level controls in each batch of runs and participating in proficiency testing programs. Diagnotix provides three levels of lyophilised whole blood controls (low, medium, and high), which are available separately from the reagent kit. These controls are not an integral part of the reagent set, to allow laboratories the flexibility to use fully independent, third-party control materials where required. This supports unbiased internal validation and is aligned with best practices for quality assurance and regulatory expectations.

Using multiple control levels is important to verify calibration accuracy across the entire clinically relevant measuring range. Controls should be selected to represent low-end, mid-range, and high-end concentrations, ensuring the analytical system performs reliably at all points on the calibration curve.

In addition, it is recommended to repeat control measurements at regular intervals during large sample series. This allows the early detection of drift, matrix interferences, or technical variation over time. Control tracking supports stability monitoring and ensures timely corrective actions can be taken before patient results are affected.

The routine use of matrix-matched control materials is a cornerstone of laboratory quality assurance. It improves result traceability, supports conformity with regulatory requirements, and enables performance verification over time.

Diagnotix strongly recommends implementing such control strategies within a quality management system in line with ISO 15189. This includes maintaining documented procedures

for control evaluation, trend analysis, and root cause investigation. Participation in proficiency testing schemes (external quality assessment, EQA) is also advised to ensure inter-laboratory comparability and long-term analytical robustness.

4.1.8 Installation requirements

Diagnotix or its partners can be consulted to assist with or perform the installation of this kit on your LC-MS/MS device. Please contact Diagnotix for more information.

We strongly recommend that the method installation be performed by a skilled staff member who is familiar with your LC-MS/MS device, software, data analysis, and the general principles of LC-MS/MS analysis for clinical applications. The staff member should, at a minimum, be able to tune the system, evaluate and optimize the gradient, and generate and interpret data.

4.1.9 General Advice for Gradient Management

This section provides a general explanation. There may be method-specific requirements which can be found in the section *System Suitability & Method Installation*. In case of doubt, please contact Diagnotix at techsupport@diagnotix.com or consult your officially supplying partner.

Although Diagnotix kits specify fixed compositions for Mobile Phase I and II, the gradient profile may be adapted to suit the specifications of the LC system in use, provided that critical functional requirements are maintained.

Due to the complexity of human matrices, it is crucial to adhere strictly to general gradient principles. LC-MS/MS is an excellent tool for detecting and isolating molecules of interest; however, one should not underestimate the presence of other molecules within the sample. These can affect reproducibility, sensitivity, and overall system suitability.

It is important to note that gradients are typically designed by increasing or decreasing the percentage of Mobile Phase II, which is the organic component. Correspondingly, Mobile Phase I is the aqueous (water-based) component.

Generally speaking, all proper gradients consist of the following parts: a beginning phase starting at a low percentage of organic solvent; an elution phase as a linear increase towards high organic; a washing phase to wash off strongly retained compounds; and a conditioning phase, which returns to the initial low percentage organic either linearly or immediately.

Beginning phase
This phase is intended to wash off highly polar molecules (such as sugars, salts, etc.). When analysing relatively hydrophobic compounds, one can choose to increase the starting organic percentage, enabling the removal of more unwanted molecules.

Elution phase
In this phase, the percentage of organic solvent is increased to allow the analytes of interest to elute. The elution phase must be designed to ensure the method remains reproducible and validated. Complete chromatographic separation of compounds is not a necessity for LC-MS/MS, although it can help improve sensitivity. Isobaric compounds, however, must be baseline separated, as MS/MS detection cannot distinguish between them due to their identical mass-to-charge ratios.

Washing phase
The washing phase is extremely important for removing highly hydrophobic molecules that may adhere to the column or system and require sufficient time to dissolve in the mobile phase. These molecules can influence reproducibility and sensitivity over time and may

interfere with other methods if they remain in the system. It is advisable to wash for at least five column volumes—more if possible. The flow rate may be increased to reduce washing time, if the system and column can tolerate the increased backpressure. Besides the washing step after each injection, it is strongly recommended to routinely wash the system with high organic content during runs to prevent contaminant build-up.

Conditioning phase
It is important that all injections start under identical conditions. Therefore, it is crucial to ensure that the entire system returns to the starting conditions by washing with the initial gradient at the end of each run. Ideally, this step should last for at least 6–10 column volumes, depending on the LC pump's performance and the system's dwell volume. These values will differ between LC-MS systems, laboratories, and with system ageing.

Calculating column volume
To determine your column's volume, consult the column manufacturer. As a rough estimate, consider the column as a cylinder and calculate its total volume. For columns packed with fully porous material, 60% of the total volume is a reasonable estimate of the actual column volume. For core-shell material, this would be 50% of the total cylinder volume.

4.1.10 Other method specific parameters to consider

This section provides a general explanation. There may be method-specific requirements which can be found in the section *System Suitability & Method Installation*. In case of doubt, please contact Diagnotix at techsupport@diagnotix.com or consult your officially supplying partner.

Injection mode
In pre-injection gradient start, the gradient begins before injection; dwell volume must be accounted for to ensure correct elution timing. In post-injection gradient start, the gradient begins after injection. This can improve reproducibility for early eluting compounds but requires careful timing to avoid band broadening or peak distortion.

Injection volume
Decreasing the injection volume can reduce matrix contamination and improve peak shape for abundant compounds. Increasing the injection volume may enhance sensitivity and reduce the risk of background noise being misinterpreted as part of the analyte peak. When analysing compound panels, it is advised to reduce the injection volume to ensure that the most sensitive peak remains clearly measurable. However, if this leads to overloading of high-abundance peaks, one may choose to down-tune the MS settings to reduce signal intensity.

Sample loop
The sample loop size must match the injection volume to prevent dispersion or loss. Typically, the injected volume should be 50–80% of the loop capacity. For small-volume injections, direct injection or bypassing the sample loop may prevent dilution in the mobile phase. Although dilution can negatively affect peak shape, this effect is usually negligible at moderate to high flow rates. Additionally, dilution may lower the organic content of the sample in the loop, potentially improving the peak shape of early eluting compounds.

Column temperature
It is recommended to maintain the specified column temperature as standard. Higher temperatures generally reduce backpressure and allow faster flow rates, though they may shorten column lifespan. It is essential not to exceed the manufacturer's temperature limits. Columns should always be temperature-controlled, as fluctuations between runs can affect chromatographic behaviour and reduce reproducibility. Some systems allow for pre-heating of

the mobile phase prior to entering the column, which may help maintain consistent column temperature but is not strictly necessary with Diagnotix products.

Flow rate
Flow rate should align with column width and efficiency. Adjusting flow rate affects chromatographic performance but is not inherently problematic. Increasing the flow rate may shorten run time but raises backpressure, while decreasing it lowers backpressure. However, without adjusting gradient timing proportionally, changes in flow rate can affect chromatographic behaviour, so gradient adjustments are recommended. The relation between column material and optimal flow rate impacts peak shape and resolution. Though knowing the optimal flow rate is useful, operating under suboptimal conditions may be beneficial—for example, to reduce backpressure or shorten run times. Consistency between runs and within patient sample batches is most important, as is preventing carry-over by using appropriate washing and reconditioning steps.

Column length
Column length is proportional to efficiency. Longer columns allow for better separation and improved peak shape but also increase run time and backpressure. This limits the possibility of using higher flow rates or may reduce column lifespan. Conversely, shortening the column may improve throughput and reduce analysis time, provided that the loss of chromatographic resolution is acceptable.

Guard columns or column protection products
Diagnotix does not advise against the use of column protection products, such as guard columns. However, these can influence the dwell volume and chromatographic behaviour of the method. It is important to replace such products on time to avoid excessive backpressure or chromatographic disturbances during analytical runs.

Protein precipitation or phospholipid removal plates
Although Diagnotix has not validated the use of protein precipitation or phospholipid removal plates within the workflow of its methods (unless specifically stated), such tools may offer practical benefits during sample preparation. These plates allow the deproteinisation step to be performed without requiring a centrifugation step to collect the eluate. The user remains solely responsible for validation. As part of this validation process, a comparison study must be carried out using real patient samples across the entire clinically relevant concentration range.

4.2 The analytical system

4.2.1 Preparing the LC-MS system

- Use the solvents supplied with the kit.
- Flush the LC system to remove any residues or contaminants from previous analyses. You may use the mobile phases or autosampler Washing Solution provided in the kit for this purpose.
- Install and connect the analytical column, ensuring correct orientation and secure fittings.
- Set system parameters (flow rate, temperature, gradient) and equilibrate according to the intended method.
- Verify stable baseline and system pressure.

Note: Always follow the safety guidelines and operation manual for the LC-MS system. Method-specific conditions should be applied as validated in your laboratory.

4.2.2 Starting the analytical system

- Equilibrate the system.
- Check the temperature of the column.
- Initialize the injector.
- Start the programme on the LC-MS/MS system.
- Perform several test runs with an easy-to-measure standard (e.g. a standard with a sufficiently high concentration) until consistent and reproducible results are obtained (System Suitability Test).

4.3 LC-MS/MS Parameters and Conditions

4.3.1 LC Parameters

UHPLC pump Flow rate 0.4 ml/min.

Mobile Phases I and II Close the bottles to avoid alteration of RT's through evaporation of the mobile phases.

Column The column is installed in the column heater 30 °C for the complete UHPLC system the back pressure should not exceed 800 bar.
1 bar = 14.5 PSI

4.3.2 Autosampler Conditions

Needle volume:	30 µL
Injection volume:	5-20 µL
Sample syringe volume:	250 µL
Sample temperature:	10 °C
Runtime:	4.0 min
Column temperature:	30 °C ± 2 °C alarm
Needle wash:	15 seconds
Seal Wash:	90:10 H2O:ACN
Wash Solvent:	Metanephrines Autosampler washing solution

Or: the injection needle has to be flushed after sampling (minimising sample carryover). For this purpose, please use the settings recommended by the manufacturer of the auto sampler in use.

4.3.3 Gradient

Time (min)	**Flow Rate (mL/min)**	**%A**	**%B**	**Curve**
0.00	0.40	0	100	Initial
0.75	0.40	0	100	6
1.50	0.40	0	100	6
3.00	0.40	20	80	1
4.00	0.40	0	100	1

Please note that the gradient is dependent on the analyser used. End users will need to define the optimal gradient for the analyser in use.

4.3.4 MS Conditions (e.g. Waters Xevo TQS)

The mass transitions are an indication, the optima can differ vary slightly between different LC-MS/MS systems.

Analyte/IS	Quantifier MRM		Qualifier MRM	
	precursor	product	precursor	product
3-MT	151.2	91	151.2	119
Metanephrine	180	148	180	165
Normetanephrine	165.9	134.0	165.9	106.0
3-MT-D4	154.9	95	154.9	123
Metanephrine-D3	183	151	183	168
Normetanephrine-D3	168.9	137	168.9	109

Analyte	Rt(min)	Internal standard (IS)	Rt(min)
3-MT	1.69	3-MT-D4	1.69
Metanephrine	1.86	Metanephrine-D3	1.86
Normetanephrine	2.48	Normetanephrine-D3	2.48

5. Sample

5.1 Sample material

Use blood plasma (EDTA-tubes) after sample collection directly centrifuge and separate the plasma and blood cells.

Plasma samples can be stored: 3 months (- 20 °C)
Avoid freeze-thaw cycles.

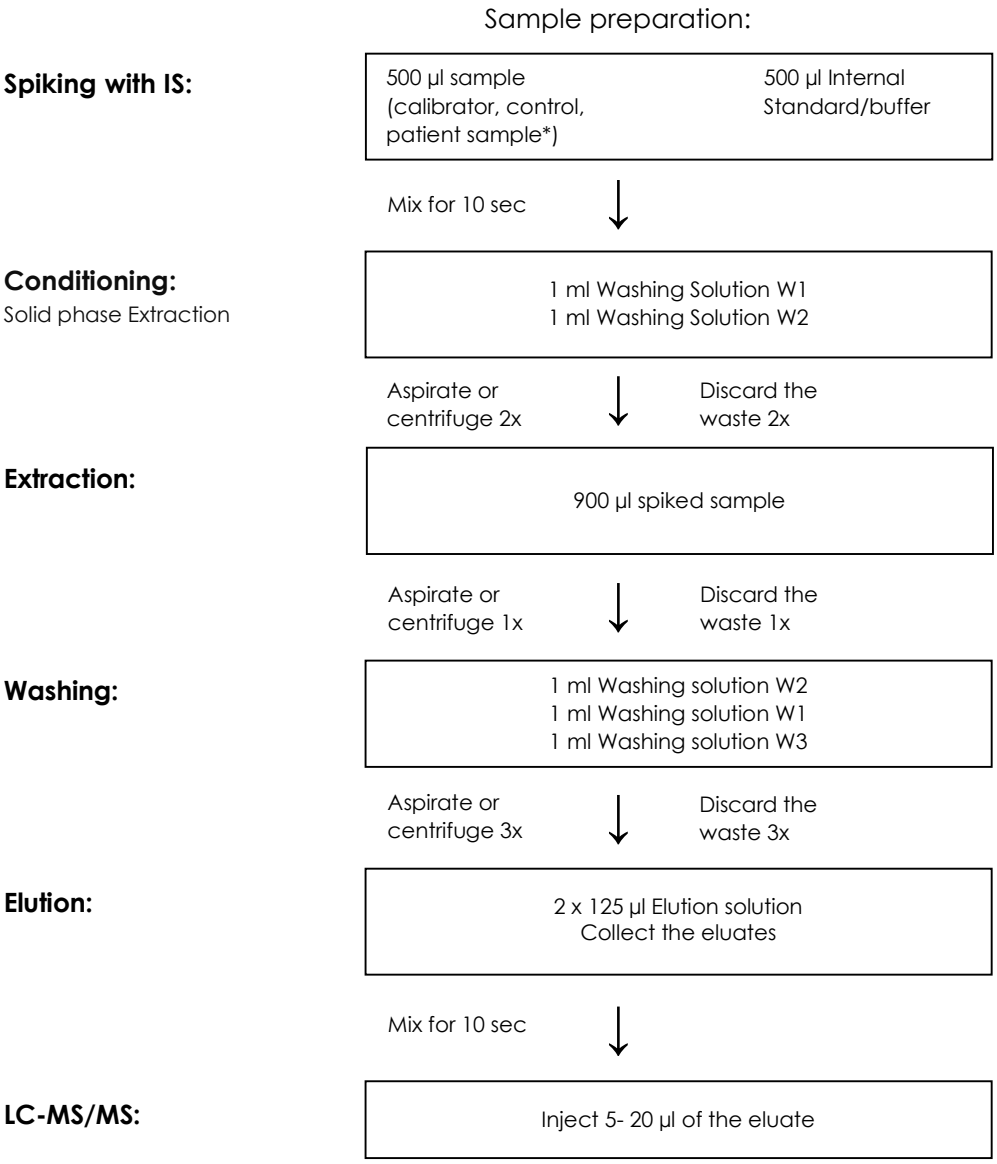
5.2 Sample preparation

5.2.1 Reconstitution of the lyophilised Calibrators / Controls.

See 2.3.1.1 and the product data sheets

5.2.2 Sample Preparation

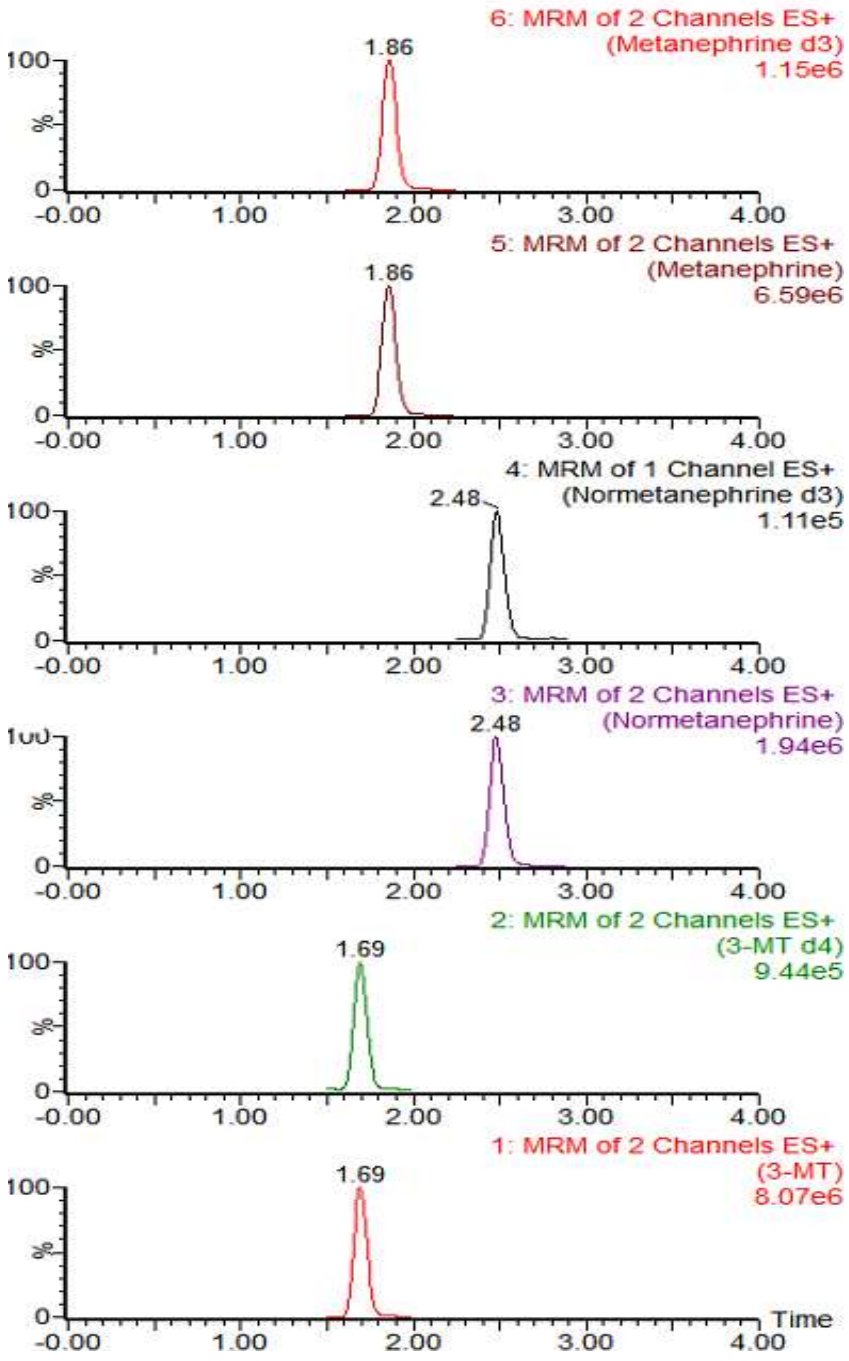
Note: After each step, apply a gentle vacuum, if needed, to ensure all solvent passes through the SPE sorbent, using centrifugating or an SPE vacuum or SPE positive pressure system. Experience with, or prior training in, the use of SPE is strongly recommended. For appropriate training opportunities, please contact Diagnotix at techsupport@diagnotix.com or consult your official supplier.



*** Note that patient samples need to be centrifuged (10 minutes at 3000 g) prior to spiking with internal standard.**

5.3 Examples of chromatograms

Example chromatogram of a sample, measured with the Waters UHPLC I-Class and the LC-MS/MS system Xevo TQS.



5.4 Results from LC-MS/MS and Reference Values

5.4.1 Data acquisition

It is advised that users are fully trained onto using the supplied LC-MS/MS software and the concepts of performing quantify MS calculations.

Assign the concentration of the internal standard on 1.
Use a weighting factor of 1/x and do not force the calibration curve through zero.

with some systems, and in some cases, the lowest calibrator level may not be measured accurately. Therefore, evaluate your calibration curve both with and without calibrator 1, and choose the calculation that shows the least deviation and the best fit to your controls.

It is recommended to always review the chromatographic results of standards to ensure proper integration. Manually correct the integration if necessary.

Fragmentation Ratio Stability

Always monitor the fragmentation ratio for each compound. This ratio should remain stable under normal conditions. Any observed deviations may indicate contamination or a malfunctioning instrument.
If changes are detected:

1. **Do not use** the obtained results for data acquisition.
2. **Contact your service provider** immediately for further diagnosis and maintenance.

5.4 Clinical performance and reference values

5.4.1 Clinical performance

Measurement of plasma free metanephrines (metanephrine and normetanephrine) and 3-methoxytyramine (3-MT) is clinically validated as a diagnostic approach for the detection and follow-up of pheochromocytoma and paraganglioma (PPGL).
When performed under recommended pre-analytical conditions (supine or seated with rest), method-class literature reports diagnostic sensitivity and specificity of approximately:

Analyte / Approach	Sensitivity	Specificity	Reference
Plasma free metanephrines	~94%	~93%	Därr *et al* 2017[2]
Urinary fractionated metanephrines	~91%	~93%	Därr *et al* 2017[2]
Supine sampling (vs seated)	95% vs 89%	95% vs 90%	Lenders *et al* 2007[3]

[2] Därr R, Kuhn M, Bode C, Bornstein SR, Pacak K, Lenders JWM, et al. Accuracy of recommended sampling and assay methods for the determination of plasma-free and urinary fractionated metanephrines in the diagnosis of pheochromocytoma and paraganglioma: a systematic review. *Endocrine*. 2017;56(3):495–503.
[3] Lenders JWM, Willemsen JJ, Eisenhofer G, Ross HA, Pacak K, Timmers HJLM, et al. Is supine rest necessary before blood sampling for plasma metanephrines? *Clin Chem*. 2007;53(2):352–4.

These values represent method-class evidence and are provided for informational purposes. Device-specific performance data are available in the technical documentation.

5.4.2 Reference values

	Adult reference range (nmol/l)	Peadiatric (5-17 y) (nmol/l)
metanephrine	0.07 - 0.33	0.045 – 0.333
normetanephrine	0.23 - 1.07	0.048 – 0.470
3-MT	< 0.17	n/a

The indicated reference ranges are taken from scientific literature and were determined from healthy individuals. The blood was drawn in sitting position.[4,5]

It is recommended that each laboratory establishes its own reference ranges.

Important:

- For 3-MT measurement, fasting is recommended to avoid dietary interference.
- Certain medications (e.g., tricyclic antidepressants, phenoxybenzamine, some β-blockers) may affect results.
- Interpretation must be performed by qualified medical professionals in the context of the full clinical picture.

Note:
The inclusion of this information is required by Annex I, Section 20.4.1 (v) of the IVDR.
Diagnotix does not employ medically trained professionals and can only suggest possible ways to interpret results.
Always consult a trained medical professional with expertise in the relevant field for the interpretation of results.
The interpretation of test results also significantly depends on the individual characteristics of the patient. Diagnotix recommends considering these factors as well.

[4] De Jong, W.H.A., et al

[5] https://erasmusmc.getincontrol.eu/bepalingen

6. Summary of Analytical Performance Characteristics

Analytical performance characteristics have been defined by validation of the assay according to IVDR parameters, and using EP Evaluator to extract statistical data from the acquired raw data.

6.1 Repeatability (Simple Precision)

The repeatability, or simple precision, was analyzed by measuring a patient sample, Metanephrines control I and Metanephrines control III twenty times from one sample within two hours from each other. From these results the Coefficient of Variation (CV) was calculated and compared to the precision verification goal which in turn is calculated using the biological variation with the statistical program EP Evaluator.

Sample	**Simple precision 3-MT (CV%)**	**Simple precision Metanephrine (CV%)**	**Simple precision Normetanephrine (CV%)**
Low level	0.6	0.9	1.1
High level	1.6	1.9	2.8
Patient	1.6	1.3	1.4

6.2 Reproducibility (Complex Precision)

The Reproducibility, or Complex Precision, was analyzed by measuring a patient sample, Metanephrines control I and Metanephrines control III in duplicate twenty times. Each time the sample preparation had a variance (different analyst, pipet, day, reagent temperature and/or calibration). This to simulate twenty different days in a laboratory. From these results the Coefficient of Variation (CV) is calculated and compared to the precision verification goal which in turn is calculated using the biological variation with the statistical program EP Evaluator.

Sample	**Complex precision 3-MT (CV%)**	**Complex precision Metanephrine (CV%)**	**Complex precision Normetanephrine (CV%)**
Low level	4.6	4.2	5.6
High level	2.6	1.8	2.7
Patient	6.1	5.3	4.6

Linearity

The linearity was analyzed by preparing a series of known incrementally increasing metanephrines concentrations. These samples were measured two days apart in duplicate. From this the linearity was verified and the upper limit of detection was determined with EP Evaluator.

Analyte	**Linearity (nmol/l)**

3-MT	18
Metanephrine	25
Normetanephrine	25

6.3 Limit of Blank

The Limit of Blank (LOB) was analysed by measuring a zero sample (calibrator 1) twentyfold and a non-zero sample (calibrator 2) fivefold. The LOB was calculated from the responses with EP Evaluator.

Analyte	**Limit of quantification (nmol/l)**
3-MT	0.0257
Metanephrine	0.0034
Normetanephrine	0.00966

6.4 Limit of Quantification

The Limit of Quantification (LOQ) was analysed by preparing a series of incrementally decreasing known metanephrines concentrations. The samples for metanephrine and normetanephrine were measured fifteen times and the 3-MT was measured nineteen times. The sample preparation had a variance (different analist, pipet, day, reagent temperature and/or calibration) each time to simulate different days in a laboratory. The limit of quantification was calculated with EP Evauator.

Analyte	**Limit of quantification (nmol/l)**
3-MT	0.032
Metanephrine	0.041
Normetanephrine	0.093

6.5 Correlation (Comparison)

For the correlation forty patient samples were measured with the Diagnotix method and the method of another established method. The datasets were analyzed in EP Evaluator.

Analyte	**Passes**	**Correlation Coeff**

3-MT	Yes	0.9994
Metanephrine	Yes	0.9996
Normetanephrine	Yes	0.9989

6.6 Carryover

To verify that there is no carryover two calibrators were prepared. Calibrator 2 and calibrator 6. These samples were measured in a particular order after which the data was analyzed with EP Evaluator.

Analyte	**Passes**
3-MT	Yes
Metanephrine	Yes
Normetanephrine	Yes

6.7 Accuracy

The accuracy of the method was determined by participating in the subscription schemes from the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML). This organization gathers results from all contributing laboratories and establishes a consensus or average. This in turn is compared to the results from Diagnotix.

Analyte	**Accuracy**
3-MT	Pass
Metanephrine	Pass
Normetanephrine	Pass

6.8 Matrix effect

Matrix effects are addressed through use of stable isotope-labelled internal standards and matrix-matched calibrators and controls. No separate matrix effect study is therefore required.

6.9 Interferences

Molecular interferences are rare when using LC-MS/MS. The only molecules that can interfere are isobaric compounds, which share both the precursor and product masses. MS/MS detection cannot differentiate between these molecules as they exhibit identical mass transitions. Therefore, chromatographic separation is essential to distinguish these components before MS/MS analysis.

Molecules suspected of having isobaric characteristics relative to the target analytes must be chromatographically separated to prevent simultaneous detection by the MS/MS system.

Davison, *et al.* published an overview of possible interferences for the measurement of metanephrines with several chromatographic techniques.[6]

In this review Davison, *et al.* list all published interferences that were found by the writers with the following table.

Agent causing increase in plasma or urine metadrenalines	**Cause of increase**	**Plasma NMA**	**Plasma MA**	**Plasma 3-MT**	**Urine NMA**	**Urine MA**	**Urine 3-MT**
Phenoxybenzamine	Pharmacological	Y			Y		
Amitriptyline, venlafaxine	Pharmacological	Y			Y		
Moclobemide, phenelzine	Pharmacological		Y			Y	
Amphetamines, ephedrine, cocaine	Pharmacological			Y			Y
L-DOPA	Pharmacological		Y			Y	
Banana, pineapple, shelled walnuts	Pharmacological			Y			Y
Coffee, nicotine	Pharmacological			Y			Y
Curry leaves (methoxyhydroxybenzylamine)	Analytical[a]				Y		
Buspirone	Analytical[a]				Y		
Paracetamol	Analytical[a]		Y		Y		
Sulphasalazine	Analytical[a]				Y		
Mesalamine	Analytical[a]				Y		
Methenamine	Analytical[a]				Y		
Amoxicillin	Analytical[a]				Y		
L-DOPA	Analytical[a]			Y			Y
L-DOPA	Analytical[b]		Y			Y	
Isoproterenol	Analytical[b]						Y
MDMA	Analytical[b]						Y
HMMA	Analytical[b]						Y
Isoetharine	Analytical[b]						Y
MDA	Analytical[b]						Y

Only compounds specified as Analytical[b] are suspected to interfere in LC-MS analyses. Compounds specified as Analytical[a] are suspected to interfere with LC-methods coupled with non-MS detection (for example UV). The numbers in the reference section refer to the references listed in the discussed review paper and can be found at https://doi.org/10.1177/0004563217739931 .

Based on the information summed, the following molecules are suspected of possible interferants in the detection of Metanephrines:

- L-DOPA

[6] Davidson *et al.* Annals of Clinical Biochemistry: International Journal of Laboratory Medicine, Vol 55, Issue 1, 2018, https://doi.org/10.1177/0004563217739931

- Isoproterenol
- MDMA
- HMMA
- Isoetharine
- MDA

It is important to recognise that chromatographic separation must be demonstrated, or alternatively, one must consider that elevated concentrations may have been caused by isobaric compounds present in the background. To rule out xenobiotic isobars, complementary research can be carried out into the patient's intake prior to sample extraction. In cases of potential endogenous isobaric interferences, it is important to evaluate elevated results to determine whether there is reason to suspect an increased concentration of endogenous isobaric compounds. For borderline results, it is advisable to recollect samples under standardised low-stress conditions.

7 General guidelines for using LC-MS/MS

7.1 Maintenance

LC-MS/MS devices are delicate systems that can easily being contaminated. Some parts of the device needs to be replaced or can damage over time. These factors can influence your obtained results. Therefore it is recommended to keep a tight LC-MS/MS cleaning schedule and consult a professional for general maintenance.

Injecting samples derived from biological matrices (e.g., whole blood, serum, plasma, urine, saliva) can lead to contamination of the U(HPLC) system, the analytical column, and the ion source over time. There is a significant risk that contamination will progressively spread further into the LC-MS/MS system. Therefore, general maintenance and preventive measures are essential to ensure long-term performance and accuracy.

Consult your LC-MS/MS supplier for the recommended cleaning procedures.

7.2 Optimalisation of your LC-MS/MS device

Every system, even those of the same type from the same manufacturer, can behave differently. Therefore, copying U(H)PLC and MS settings from this manual or any other system to your own may result in suboptimal measurements.

It is advisable to use the settings provided in this manual as a starting point and optimize the UHPLC settings accordingly. For MS settings, we recommend tuning your system, as fragmentation can vary depending on your specific system and environment. Please refer to section 7.2.1 for general tuning guidelines.

7.2.1 General guidelines for tuning

Note: Please refer to guidelines and settings provided to your specific instrumentation. Ensure you are completely aware of the tuning procedures of your system. If our guidelines are conflicting guidelines of your device, please follow the guidelines of your device. The information giving in this manual has to been seen as purely informative.

1. Use Diagnotix 10 µg/ml solutions or a single pure molecule solution. Tuning mixes of different compounds only allow you the possibility of checking given conditions, but avoid you possibly the change of finding optimal conditions per single compound.
2. Use only LC-MS grade solvents to prevent contamination and background noise.
3. Check the instructions of your LC-MS device for the optimal tuning range (typically in the range of 50 to 500 ng/ml).
4. Prepare a dilution in a separate clean vial with a solvent mixture of 50% methanol / 50% water or what is likely the dissolving mixture wherein the compound will dissolve and precipitate from the analytical column.
5. Ensure the LC-MS/MS device is thoroughly cleaned prior to use and is functioning properly.
6. Start with a highly diluted sample to avoid contamination due to overloading. MS/MS systems can contaminate quickly when injecting too concentrated tuning solvents. You may increase the concentration in small steps if the signal is too low.
7. Rinse the source extensively with methanol or acetonitrile before and after each measurement.

7.2.2 Event window recommendations

Best practice is to make your event window as close as possible to avoid any contamination into the MS/MS. Therefore it is advisable reduce the window time as much as possible.

Set the concentration of the internal standard at 1.

Select the most abundant transition for the calculation, and the second most abundant transition as control.

8. Change Log

Version Change	Section changed	What has been changed	Date
3.5 → 4.00	Complete revision	Complete rework of manual for IVDR compliance	12-09-2025

9. References

1 Weismann D, et al. (2015). *Measurements of plasma metanephrines by immunoassay vs liquid chromatography with tandem mass spectrometry for diagnosis of pheochromocytoma*. European Journal of Endocrinology, 172(3), 251–260. https://doi.org/10.1530/EJE-14-0892

2 De Jong WHA, et al. (2007). *Plasma free metanephrine measurement using automated online solid-phase extraction HPLC–tandem mass spectrometry*. Clinical Chemistry, 53(9), 1684–1693. https://doi.org/10.1373/clinchem.2007.088401

3 Davidson DF, et al. (2018). *Clinical evaluation and treatment of phaeochromocytoma*. Annals of Clinical Biochemistry, 55(1), 34–48. https://doi.org/10.1177/0004563217739931

4 Lenders JWM, Eisenhofer G, Mannelli M, Pacak K. (2005). *Phaeochromocytoma*. The Lancet, 366(9486), 665–675. https://doi.org/10.1016/S0140-6736(05)67139-5

5 Lenders JWM, Duh QY, Eisenhofer G, Gimenez-Roqueplo AP, Grebe SKG, Murad MH, Naruse M, Pacak K, Young WF Jr. (2014). *Pheochromocytoma and paraganglioma: An Endocrine Society clinical practice guideline*. Journal of Clinical Endocrinology & Metabolism, 99(6), 1915–1942. https://doi.org/10.1210/jc.2014-1498

6 Darr R, Pamporaki C, Peitzsch M, Miehle K, Prejbisz A, Peczkowska M, Weismann D, Beuschlein F, Sinnott R, Bornstein SR, et al. (2014). *Biochemical diagnosis of phaeochromocytoma using plasma-free normetanephrine, metanephrine and methoxytyramine: Importance of supine sampling under fasting conditions*. Clinical Endocrinology, 80(4), 478–486. https://doi.org/10.1111/cen.12319

7 Boot C, Toole B, Johnson SJ, Ball S, Neely D. (2017). *Single-centre study of the diagnostic performance of plasma metanephrines with seated sampling for the diagnosis of phaeochromocytoma/paraganglioma*. Annals of Clinical Biochemistry, 54(1), 143–148. https://doi.org/10.1177/0004563216650463