Vitamin B1 & B6 in whole blood

1020 M VB1B6

Instructions for use, LC-MS/MS assay

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CE IVD Medical Device

Contents

1. General Information

1.1 Information for the Device

1020 M VB1B6 - Vitamin B1 & B6 reagent set
Basic UDI-DI: 0872051431MVB1B68F
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 B (not self-test/near patient test/companion diagnostic), based on rule 6; (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

Vitamin B1
Thiamine pyrophosphate (TPP)
(Thiamine diphosphate)

Vitamin B6
Pyridoxal-5'-phosphate (PLP)

1.3.2 Function

This In Vitro Diagnostics medical device is intended to quantify the concentration of Vitamin B1 (TPP) and/or Vitamin B6 (PLP) to assess whether the patient has a deficiency, sufficiency, or excess of one or both compounds.

1.3.3 Required Specimen

Human whole blood

1.3.3.1 Conditions for collection, handling and preparation of specimen

EDTA tubes are suitable for collecting specimens. The sample needs to be haemolyzed by freezing overnight before analysis. The method of sample collection, transport to the laboratory, and sample storage for preparation must be determined and validated by the user due to potential user-specific conditions.
However, as sample collection, handling and transport conditions may vary between laboratories, end users are responsible for verifying the suitability of their own procedures. This includes validating sample extraction, **collection tube type and brand compatibility**, and transport/storage conditions in accordance with local protocols and quality requirements. The recommendations provided are based on validated use conditions (including the use of EDTA tubes from validated manufacturers) but may not account for all pre-analytical variables encountered by individual users. Validation of specimen integrity and matrix compatibility under local conditions is required before routine use.

1.3.4 Testing Population

Patients suspected of having deviating levels of vitamin B1 or vitamin B6.

1.3.5 Intended User

Laboratory Professional with basic understanding of LC-MS/MS.

1.4 Test Principle

1.4.1 LC-MS by ESI and MRM

Vitamin B1 & B6 are determined from human whole blood by UHPLC with positive ion electrospray LC-MS/MS.

Prior to the LC-MS/MS analysis a sample clean-up is performed to remove the sample matrix and to spike with the internal standard.

After separation by chromatography on an analytical C-18 column, Vitamin B1 and Vitamin B6 are ionized by electrospray ionization (ESI) and detected by LC-MS/MS.

Electrospray ionization is a soft ionization technique where a strong electric field is applied to the liquid passing through the ESI-capillary of the MS-source. The ions are mostly performed in solution before desorption and then transferred into the ion path of the tandem mass spectrometer which consists of three quadrupoles (two mass selectors connected by a collision cell).

Measurement of the analytes is carried out in MRM (Multiple Reaction Monitoring) mode. In this mode only selected ions (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.

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

Water-soluble B vitamins are crucial cofactors in cellular metabolism. Two clinically relevant B vitamins are Vitamin B1 (thiamine) and Vitamin B6 (pyridoxine and related compounds). These vitamins exist in several interconvertible forms in the human body. Vitamin B1 includes thiamine and its mono-, di-, and triphosphate esters, with thiamine pyrophosphate (TPP or TDP) being the biologically active form [1]. TDP is essential for various metabolic processes, and its deficiency can lead to debilitating neurological diseases such as beriberi and Wernicke-Korsakoff syndrome [3,5]. There are two primary clinical forms of beriberi: dry beriberi, characterized by sensorimotor neuropathy and Wernicke-Korsakoff syndrome, and wet beriberi, which involves edema and congestive heart failure with fewer central nervous system manifestations. Wernicke's encephalopathy, with or without Korsakoff syndrome, is especially prevalent among alcoholics but can also result from hyperemesis, dialysis, or gastrointestinal surgery. Vitamin B6 comprises six related compounds: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), their respective 5'-phosphate esters, and the degradation product pyridoxic acid (PA), with pyridoxal-5'-phosphate (PLP) being the metabolically active form [2]. PLP acts as a coenzyme in numerous transamination reactions and plays a crucial role in both chronic disease and pro-inflammatory responses [1F]. Additionally, both Vitamin B6 and B1 have been associated with increased survival rates in elderly populations [2F].

Pyridoxine deficiency, though rare, has been implicated as a cause of severe neonatal and infantile convulsions. Pyridoxine dependency is a genetic condition that manifests in foetal life and results in both intrauterine and postnatal seizures. Neurologic disorders may also arise from

pyridoxine toxicity, where overdose causes sensory neuronopathy and sensory ataxia, whereas deficiency affects both motor and sensory axons [4].

Both TPP and PLP are indispensable cofactors in a variety of enzymatic reactions, especially those involved in carbohydrate and amino acid metabolism. Approximately 80% of vitamin B1 in the human body is located in erythrocytes, predominantly as TPP. Plasma primarily contains unphosphorylated thiamine in low concentrations, making it a less reliable matrix for quantitative assessment [5,6]. The TPP concentration in erythrocytes reflects tissue stores and is considered an accurate marker for vitamin B1 status, as depletion rates mirror those in major organs [7]. Furthermore, whole blood TPP levels strongly correlate with those in washed erythrocytes, rendering whole blood a practical matrix for assessment [8].

Vitamin B6 circulates in plasma mainly as PLP and PL, while PN and PM are largely absent in individuals with sufficient intake [9]. Erythrocytes predominantly contain PLP and pyridoxamine-5′-phosphate (PMP), with approximately 60% of total PLP in whole blood residing within red blood cells [10]. PLP is the preferred biomarker for assessing vitamin B6 status, though other markers such as plasma or urinary PA, PL levels, and functional indicators may also be used [11,12]. In healthy individuals, plasma and erythrocyte PLP levels are typically well-correlated. However, in critically ill patients, PLP may redistribute from plasma to erythrocytes, which supports using whole blood as a more stable matrix in these cases [13].

Mass spectrometry-based methods have been tested for determining vitamins B1 and B6. Chromatographic separation is essential but challenging for these analytes. LC–MS/MS methods have been developed for simultaneous quantification of TPP and PLP in whole blood, offering a valuable alternative to traditional HPLC with fluorescence detection [15,16]. Compared to HPLC, LC–MS/MS methods have the advantage of requiring only simple protein precipitation under acidic conditions, bypassing the need for derivatization due to the lack of fluorophores in target compounds [8,13]. The method is robust and adaptable across different laboratory configurations and has been successfully implemented using various chromatographic and mass spectrometric conditions [15–20].

The method is well-suited for routine analysis. Sample preparation is straightforward and rapid, applicable to multiple biological matrices. A six-point lyophilized whole blood calibrator at clinically relevant concentrations is included in the diagnostic kit. Lyophilized whole blood controls are also available for quality assurance.

For enhanced accuracy and reproducibility, two isotope-labelled internal standards—Pyridoxal-5′-phosphate (methyl D3) and Vitamin B1 pyrophosphate (methyl D3)—are added to each sample to compensate for matrix effects and measurement variability. Analysis is performed using positive ion electrospray in Multiple Reaction Monitoring (MRM) mode, maximizing both sensitivity and selectivity.

In the Netherlands, the demand for vitamin B1 and B6 testing has significantly increased following their inclusion in national general practitioner guidelines for the differential diagnosis of dementia [14]. Since the vitamins are often requested together, a rapid, combined analytical approach such as LC–MS/MS is increasingly favoured.

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

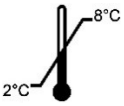
Order Number

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 Description of 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.

2.3.1 Calibrators and Controls

1022a CAL M VB1B6 | Vitamin B1 & B6 Calibrator Set
UDI: 8720514310076

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

1021a CON M VB1B6 | Vitamin B1 & B6 Control Set
UDI: 8720514310083
1033a M VB1B6 | Vitamin B1 & B6 Control I
UDI: 8720514310168
1033a.10 M VB1B6 | Vitamin B1 & B6 Control I (10-pack)
UDI: 8720514313510
1034a M VB1B6 | Vitamin B1 & B6 Control II
UDI: 8720514310175
1034a.10 M VB1B6 | Vitamin B1 & B6 Control II (10-pack)
UDI: 8720514313503
1035a M VB1B6 | Vitamin B1 & B6 Control III
UDI: 8720514310182
1035a M VB1B6 | Vitamin B1 & B6 Control III (10-pack)
UDI: 8720514313527

Three levels of lyophilized whole 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 Vitamin B1 & B6 Calibrator Set and Controls with exactly 250 µl distilled or deionised water using a volumetric pipette.
3. Re-place 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 dissolved, do not shake. Avoid foaming.
5. Let stand for 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	5 days
After reconstitution:	- 20 °C	8 weeks with the maximum of 1 freeze-thaw cycle

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

2.3.1.3 Metrological Traceability

Metrological traceability is established by comparing each batch to the highest available order of reference material, as well as the last batch produced before the current batch.

For Vitamin B1 & B6 the highest available order of reference material has been established to be the reference laboratory network of the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML).

Refer to the Metrological traceability sheet for the specific batch for more information.

2.3.2 Internal Standard

1029 M VB1B6 | Vitamin B1 & B6 Internal Standard D3
UDI: 8720514310007
1029.10 M VB1B6 | Vitamin B1 & B6 Internal Standard D3 (10-pack)
UDI: 8720514313480

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 ingredient(s): Thiamine pyrophosphate-D3 & Pyridoxal-5'-phosphate-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 Vitamin B1 & B6 Internal Standard D3 with exactly 6.0 ml distilled or deionised water using a volumetric pipette.
3. Re-place 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 dissolved, do not shake. Avoid foaming.

5. Let stand for 15 minutes at room temperature.
6. Swirl the vial carefully, do not shake. Avoid foaming.

2.3.2.2 Stability and Storage

The stability of the internal standard is:

Before reconstitution:	2 - 8 °C	Until expiry date printed on the product label
After reconstitution:	2 - 8 °C	14 days *
After reconstitution:	- 20 °C	1 month *

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

* Over time a decrease in peak area may be observed, but until the peak area is between standard 2 and 3 the product is still usable.

2.3.3 Deproteinization Solutions

1030 M VB1B6 | Vitamin B1 & B6 Deproteinization Solution
UDI: 8720514310014
1030.10 M VB1B6 | Vitamin B1 & B6 Deproteinization Solution (10-pack)
UDI: 8720514313497

A solution provided to deproteinize the sample with trichloric acid (TCA) in order to prevent pollution in the LC-MS.

Active ingredient(s): Trichloric Acid (5% - <20%)

2.3.3.1 Handling

The Reagent is liquid and ready for use.

2.3.3.2 Storage and Stability

Store at 2 - 8 °C	After first opening the Reagent can be used for 3 weeks if closed and stored at 2 - 8 °C

2.3.4 Mobile Phases

1031 M VB1B6 | Vitamin B1 & B6 Mobile Phase I
UDI: 8720514310144
1032 M VB1B6 | Vitamin B1 & B6 Mobile Phase II
UDI: 8720514310151

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 column at differing speeds.

Active ingredient(s):

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

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 or 2 weeks on the UHPLC
Store at RT	Before first opening the Reagent can be stored for 6 weeks at Room Temperature

2.3.5 Autosampler Washing Solution

10201 M VB1B6 | Vitamin B1 & B6 Autosampler Washing Solution
UDI: 8720514310199

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

Active ingredient(s): Methanol 10% - <25%

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 or 2 weeks on the UHPLC

2.4 List of components provided

1020 KIT M VB1B6 - Vitamin B1 & B6 Reagent Set
UDI: 8720514311707
EMDN code: W01019099

Contents (for 300 assays):

Description	Part number	Content	EMDN Code
Vitamin B1 & B6 Calibrator Set (Calibrator 1 – 6)	1022a CAL M VB1B6	6 x 2 x 250 µl	W0101050301
Vitamin B1 & B6 Internal Standard D3	1029 M VB1B6	3 x 6 ml	W0101050303
Vitamin B1 & B6 Deproteinization Solution	1030 M VB1B6	3 x 46 ml	W01019099
Vitamin B1 & B6 Mobile Phase I	1031 M VB1B6	1 x 500 ml	W01019099
Vitamin B1 & B6 Mobile Phase II	1032 M VB1B6	1 x 250 ml	W01019099
Vitamin B1 & B6 Autosampler washing solution	10201 M VB1B6	1 x 1000 ml	W01019099
Vitamin B1 & B6			

Instructions for Use			

2.5 Separately available materials and components

Vitamin B1 & B6 Calibrator Set (Calibrator 1 – 6)	1022a CAL M VB1B6	6 x 2 x 250 µl	W0101050301
Vitamin B1 & B6 Internal standard D3	1029.10 M VB1B6	10 x 6 ml	W0101050303
Vitamin B1 & B6 Deproteinization Solution	1030.10 M VB1B6	10 x 46 ml	W01019099
Vitamin B1 & B6 Mobile Phase I	1031 M VB1B6	1 x 500 ml	W01019099
Vitamin B1 & B6 Mobile Phase II	1032 M VB1B6	1 x 250 ml	W01019099
Vitamin B1 & B6 Autosampler washing solution	10201 M VB1B6	1 x 1000 ml	W01019099

Vitamin B1 & B6 Control I	1033.10 M VB1B6	10 x 250 µl	W0101050101
Vitamin B1 & B6 Control II	1034.10 M VB1B6	10 x 250 µl	W0101050101
Vitamin B1 & B6 Control III	1035.10 M VB1B6	10 x 250 µl	W0101050101
Vitamin B1 & B6 Control Set (Control I – III)	1021a CON M VB1B6	3 x 3 x 250 µl	W0101050101
10 µg/ml solution Thiamine Pyrophosphate (RUO)	TS-1001	1 ml	W0101050399
10 µg/ml solution Thiamine Pyrophosphate-D3 (RUO)	TS-1001-I	1 ml	W0101050399
10 µg/ml solution Pyridoxal 5'-Phosphate (RUO)	TS-1002	1 ml	W0101050399
10 µg/ml solution Pyridoxal 5'-Phosphate-D3 (RUO)	TS-1002-I	1 ml	W0101050399

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
General laboratory material	Sample tubes	

	Autosampler vials, inserts, caps or well plates	
	Pipette tips 20-200 µl	
	Pipette tips 100-1000 µl	
	Analytical column	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

Trichloric acid (TCA) is present in the deproteinization solution. TCA has been classified as a Category 1B reprotoxic substance according to Annex VI of Regulation (EC) No. 1272/2008.

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 required. However, in most cases, the optimal injection volume is 20 µL; therefore, a loop with a minimum volume of 20 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 conditioned column prior to use (see 4.1.5)
- A minimum retention time of 1 column volume.
- A resolution of at least 1 between TPP and PLP, but advised is at least 1.5 (baseline separation)
- Repeatable retention times for both TPP and PLP 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.

4.1.5 Column conditioning and maintenance

Before use, condition the analytical column by repeatedly injecting a prepared patient sample, as outlined in sections 4.2.2 or 4.2.3. Calibrators and controls must not be used.

The column is fully conditioned when the retention times of both compounds stabilise. Section 4.1.6 provides a list of suitable columns that have been successfully tested with this method. However, users are free to select other columns, provided that the minimum requirements described in section 4.1.4 (Minimum Requirements Chromatography) are met.

Not every column will be suitable, as some may fail to maintain stable retention times over time, even after conditioning. This stability is required, as specified in section 4.1.4.
The optimal column choice may also depend on the (U)HPLC system and configuration used. In some cases, better results may be achieved with an alternative column. For technical support, please contact us at **techsupport@diagnotix.com**.

Minor batch-to-batch variations may affect retention time or column lifespan. Ensure all runs comply with the criteria in section 4.1.4.

After conditioning, flush the column only with Mobile Phase I (1030 M VB1B6) and Mobile Phase II (1031 M VB1B6). If retention or resolution is lost after thorough flushing, recondition the column as described. If that fails, replace it.

4.1.6 Examples of suitable columns

Supplier	**Product name**	**Part number**
Waters	Xbridge BEH C18 XP Column 2.5 µm, 3mm x 75 mm	186006034
Waters	XSelect BEH C18 2.5 µm 2.1mm x 75 mm	186006102
Phenomenex	Kinetex EVO C18 75mm x 2 mm	00C-4725-AN

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 Condition

Please note that the provided LC-MS/MS Parameters and Conditions are derived from the system used by Diagnotix to perform the validation of the analytical performance of this assay kit. Conditions may vary between LC-MS/MS, even between systems of the same type from the same manufacturer. End-user systems used to perform this assay may require optimization.

4.3.1 LC Parameters

UHPLC pump	Flow rate 0.6 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 backpressure should not exceed 800 bar. 1 bar = 14.5 PSI

4.3.2 Autosampler Conditions

Injection volume:	10-20 µL
Sample temperature:	10 °C

Runtime:	2.5 min
Column temperature:	30 °C ± 2 °C alarm
Needle wash:	wash twice for 6 seconds
Seal Wash:	10:90 ACN:H2O
Wash Solvent:	Autosampler Washing Solution; 90:10 H2O:MeOH
Analytical column:	Waters Xbridge BEH C18 XP Column 2.5 µm, 3mm x 75 mm

4.3.3 Gradient

Time (min)	**Flow Rate (mL/min)**	**%A**	**%B**	**Curve**
0.00	0.60	95	5	Initial
0.60	0.60	70	30	6
1.20	0.80	3	97	11
1.70	0.80	95	5	11
1.90	0.60	95	5	11

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)

MS System:	(Waters Xevo TQS)
Ion mode:	Electrospray
Capillary voltage:	1.0 kV
Polarity:	positive
Source temperature:	150 °C
Desolvation temperature:	600°C
Desolvation gas flow:	1000L/hr
Detection mode:	MRM
Dwell time:	0.019 sec
Collision gas:	Argon / Nitrogen

Substance	**Precursor**	**Product**
Vitamin B6	248.00	94.00
Vitamin B6	248.00	150.00
Vitamin B6 D3	251.00	153.00

Substance	**Precursor**	**Product**
Vitamin B1	425.20	122.45
Vitamin B1	425.02	304.00
Vitamin B1 D3	428.20	125.45

These conditions are an indication, optimal values can differ slightly between different LC-MS/MS systems. To optimize the MS conditions one can use the 10 µg/ml solutions that are mentioned in sector 2.5. Note: these solutions are strongly concentrated. See section 7.2 for more guidance.

5. Sample

5.1 Sample material

Use whole blood (EDTA- tubes)

Because sample collection, transport and storage conditions may differ per end-user**. End users are responsible for their own sample extraction and storage validation.**

5.2 Sample preparation

Please note: as reagent temperature can negatively impact the effectiveness of the Deproteinization Solution, Diagnotix strongly recommends using this reagent at the indicated temperatures and to not let the reagent warm up to room temperature before use.

5.2.1 Reconstitution of the lyophilised Calibrators / Controls.

See 2.3.1.1 and the product data sheets.

5.2.2 Sample preparation (whole blood, calibrator or control)

1. 50 µl Vitamin B1 & B6 Internal Standard D3.
2. Add 50 µl sample (Calibrator, Control, Patient sample).
3. Mix immediately and add 400 µl Vitamin B1 & B6 Deproteinization Solution in the tubes using a vortex mixer for 30 seconds.
4. After mixing on a vortex set the tube immediately in a shaker for another 30 minutes.
5. Make sure that all the tubes have been shaken for at least 30 minutes.
6. Centrifuge (5 min, 10000 x g or more).
7. Use 200 µl centrifuged supernatant to a vial or 96 well plate, which is suitable for the auto sampler in use and Inject 10-20 µl in the LC-MS/MS.

5.2.3 Sample Preparation with pipette robot

Into a 2 ml 96 well plate:

1. 50 µl Vitamin B1 & B6 Internal Standard D3.
2. Whilst mixing the plate, add 50 µl sample (Calibrator, Control, Patient sample), and leave mixing for 15 minutes.
3. Whilst continuing to mix the plate, add 400 µl Vitamin B1 & B6 Deproteinization Solution and leave mixing for at least 30 minutes.
4. Once mixing is complete, centrifuge (5 min , 10000x g or more).
5. Transfer the samples into a 1 ml 96 well collection plate for injection on the UHPLC/MS/MS system (Needle placement 2 mm) or inject directly off the pellet (Needle placement 10 mm) 10-20 µl in the LC-MS/MS.

Please note: If, in step 3, the Vitamin B1 & B6 Deproteinization Solution is not added during mixing, it may lead to false negative results.

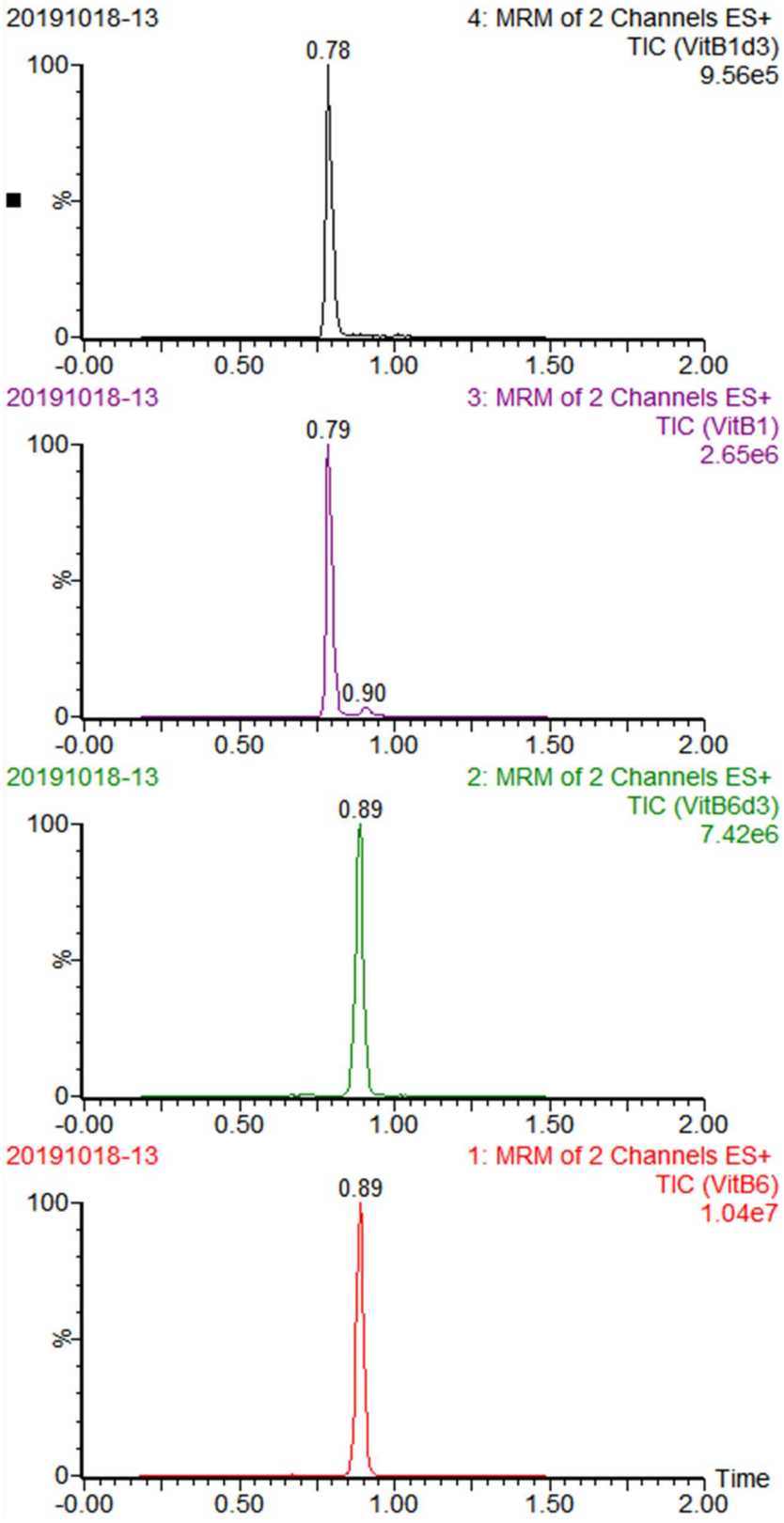
When using a pipette robot, mixing can be briefly paused to facilitate injection, ensuring that sample movement continues during the process. However, before implementing this method for clinical diagnostics, a comparison study with manual preparation must first be conducted to verify that deproteinization has been performed correctly.

5.2.4 Autosampler stability

Prepared calibrators, controls, and patient samples can be stored in an autosampler at a constant temperature of 15 °C for a maximum of 72 hours. However, absolute peak areas may decrease over time. Therefore, we recommend that the end user verifies stability on their own LC-MS/MS system before relying on this guideline.
Additionally, it is important to recognize that if calibrators, controls, or internal standards have already been in use (i.e., previously used or partially consumed), this may affect the autosampler stability time of prepared samples containing these components.

5.3 Examples of chromatograms

Example chromatogram of a Patient sample, recorded with the Waters LC-MS/MS 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.

5.4.2 Reference values

The following reference intervals are provided as indicative values only:

- **Vitamin B1**: 70–140 nmol/L (after one freeze–thaw cycle)
- **Vitamin B1**: 100–220 nmol/L (after two freeze–thaw cycles)
- **Vitamin B6**: 35–110 nmol/L

These intervals are based on Dutch comparison studies. Reference values for vitamins can vary depending on local factors such as population characteristics, diet, pre-analytical handling, and laboratory conditions.

Each laboratory is therefore required to establish or verify its own reference intervals under routine operating conditions before applying these values in clinical practice.
Interpretation of patient results must always be performed by a qualified medical professional with expertise in the relevant field, taking into account the patient's clinical presentation and other diagnostic findings.

The inclusion of this information is required under Annex I, Section 20.4.1 (v) of the IVDR. Diagnotix does not employ medically trained professionals and can only suggest possible approaches to interpreting results.

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.

Repeatability (Simple Precision)

The repeatability, or simple precision, was analysed by measuring a patient sample, Vitamin B1 & B6 control I and Vitamin B1 & B6 control III twenty times from one sample within two hours from each other. 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.

Sample	**Simple precision Vitamin B1 (CV%)**	**Simple precision Vitamin B6 (CV%)**
1035 M VB1B6 Vitamin B1 & B6 Control III	1.9	2.1
Patient sample Whole blood	1.9	2.2

Reproducibility (Complex Precision)

The Reproducibility, or Complex Precision, was analysed by measuring a patient sample, Vitamin B1 & B6 control I and Vitamin B1 & B6 control III in triplicate twice a day over the course of ten days. 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.

Sample	**Complex precision Vitamin B1 (CV%)**	**Complex precision Vitamin B6 (CV%)**
1035 M VB1B6 Vitamin B1 & B6 Control III	2.6	2.1
Patient sample Whole blood	3.5	4.9

Linearity

The linearity was analysed by preparing a series of incrementally increasing vitamin B1 and B6 concentrations. These samples were measured in triplicate from which the linearity was verified and upper limed of detection was calculated.

Analyte	**Linearity (nmol/l)**
Vitamin B1	➢ 8000
Vitamin B6	➢ 3000

Limit of Quantification

The Limit of Quantification (LOQ) was analysed by preparing a series of incrementally decreasing vitamin B1 and B6 concentrations. These samples were measured fifteen times and the limit of quantification was calculated.

Analyte	Limit of quantification (nmol/l)
Vitamin B1	9.4
Vitamin B6	8.0

Correlation (Comparison)

For the correlation forty patient samples were measured by Diagnotix and a well-established hospital in the Netherlands Diagnotix collaborates with. The datasets were analysed in EP Evaluator.

Analyte	Passes
Vitamin B1	Yes
Vitamin B6	Yes

Carryover

To verify that there is no carryover two samples were prepared. One low (38 nmol/l) and one high (1500 nmol/l). The samples were divided into eleven low samples and ten high samples. The samples were measured in a particular order after which the datasets were analysed.

Analyte	Passes
Vitamin B1	Yes
Vitamin B6	Yes

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
Vitamin B1	Pass
Vitamin B6	Pass

Matrix effect

Matrix effects are addressed through the use of isotope-labelled internal standards and matrix-matched calibrators and controls. Validation has demonstrated no measurable matrix effect from the type or brand of specimen collection tube (e.g., EDTA) under the recommended use

conditions.
However, as pre-analytical conditions may vary between laboratories, end users must confirm matrix compatibility under their own conditions before routine use. This ensures that any unforeseen influences from local collection tubes, including brand-specific differences, sample handling, or storage conditions are appropriately controlled.

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. To the best of our knowledge, no isobaric interferences have been identified for PLP and TPP. However, structurally similar compounds within the same metabolic pathways may be potential candidates, as minor modifications can occur while preserving the core molecular structure.

For PLP, two structurally related vitamin B6 analogs may exhibit similar mass transitions, but the mass difference is at least 1 Da, which is large enough to distinguish between PLP in LC-MS/MS analyses:

Name	Mass	Mass difference to PLP (247.14 m/z):
Pyridoxine 5'-Phosphate	249.16	+ 2 Dalton
Pyridoxamine 5'-Phosphate	248.17	+1 Dalton

For TPP, the most structurally similar metabolites are Thiamine Monophosphate and Thiamine Triphosphate. However, these compounds have significantly different precursor masses and are therefore not relevant for interference studies.

Since the sample consists of homolysed whole blood, there is no risk of icteric or lipemic samples, which could otherwise potentially cause interference.

No additional interference analysis is therefore required.

7. General guidelines for using LC-MS/MS

7.1 Maintenance

LC-MS/MS devices are delicate systems that are prone to contamination. Some components may need replacement or become damaged over time, potentially affecting the accuracy and reliability of results. Therefore, it is recommended to maintain a strict LC-MS/MS cleaning schedule and consult a qualified professional for regular maintenance.

Injecting samples derived from biological matrices (e.g., whole blood, serum, plasma, urine, saliva) can lead to gradual contamination of the U(HPLC) system, the analytical column, and the ion source. If left unchecked, this contamination may spread deeper into the LC-MS/MS system. Preventive measures and regular maintenance are essential to ensure long-term performance and accurate quantification.

When working with biological samples, it is particularly important to pay close attention to the ion source during routine maintenance, as it is highly susceptible to build-up from matrix components and may require more frequent cleaning.

It is also important to ensure that the mass spectrometer (MS) is only active ("open") when the measurand is expected to elute. The MS acquisition window should be kept as narrow as possible, both before and after the elution peak. This is considered good analytical practice to keep the MS system as clean as possible—especially when working with whole blood samples. The samples contain TCA (present in the Deproteinization Solution), which may cause damage to certain MS systems upon repeated exposure. This is an additional reason to minimise the MS detection window wherever possible.

We strongly recommend arranging a comprehensive maintenance contract for your LC-MS/MS system with your supplier or a qualified service provider. This helps ensure that maintenance checks, servicing, and performance monitoring are carried out regularly and professionally, supporting long-term system reliability.

Always consult your LC-MS/MS supplier for the recommended cleaning and maintenance procedures specific to your system.

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
4.0 → 4.1	2.5	10 µg/ml solutions marked as RUO	02-09-2025
	4.1.4	Minimum retention reduced to 1 column volume.	
	4.1.5	More clarity is provided on how to select and handle a column.	
	4.2	The description of the preparation process has been improved to provide greater clarity.	
	5.4.1	Maintenance guidelines have been updated to address the	

	5.4.2	presence of TCA in the samples. The guidance on interpreting reference standards has been clarified and enhanced	
3.15 → 4.0	Complete revision	Complete rework of manual for IVDR compliance	22-05-2025

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