FloMass Serum Methotrexate and Metabolites

Reagents for 100 assays

Instruction Manual

REF **EUM16100**

IVD **For *in vitro* diagnostic use**

CE

PROVIDE PERSONAL SOLUTIONS

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1 INTRODUCTION

1.1 IVD SYMBOLS

IVD	In vitro diagnostic medical device /Dispositif médical de diagnostique en vitro /In-Vitro-Diagnostikum / Producto sanitario para diagnóstico in vitro / Dispositivo medico-diagnostico in vitro / Dispositivo médico para in til in vitro diagnostik
LOT	Batch code / Code du lot / Chargenbezeichnung / Código de lote / Codice del lotto / Código do lote / Número do lote / Lotnummer
PN	Packing number / Numéro d'emballage / Packnummer / Número de envase / Numero confezioni / Número de embalagem / Número de embalagem / Emballagenummer
REF	Catalog number / Référence du catalogue / Bestellnummer / Número de catálogo / Numero di catalogo / Referência de catálogo / Código / Katalognummer
	Use by / Utiliser jusqu'au / Verwendbar bis / Fecha de caducidad / Utilizzare entro / Prazo de validade / Data limite de utilização / Holdbar til
	Temperature limitation / Limites de température / Temperaturbegrenzung / Limite de temperatura / Limiti di temperatura / Limites de temperatura / Limite de temperatura / Temperaturbegrænsning
	Add liquid / Ajout de liquide / Flüssigkeit zugeben / Añadir líquido / Aggiungi liquido / Adicionar líquido / Adicionar líquido / Tilføj væske
	Store in the dark / Conserver à l'abri de la lumière / Dunkel aufbewahren / Almacenar en ambiente oscuro / Conservare al buio / Armazenar no escuro / Guardar longe da luz / Opbevares mørkt
	Contains sufficient for <n> tests / Contenu suffisant pour "n" tests / Inhalt ausreichend für <n> Prüfungen / Contenido suficiente para <n> ensayos /Contenuto sufficiente per "n" saggi / Conteúdo suficiente para "n" ensaios / Conteúdo suficiente para <n> testes / Indeholder tilstrækkeligt til "n" test
	Consult instructions for use / Consulter les instructions d'utilisation / Gebrauchsanweisung beachten / Consulte las instrucciones de uso / Consultare le istruzioni per l'uso / Consulte as instruções de utilização / Consultar Instruções de uso / Se brugsanvisning
	Manufacturer / Fabricant / Hersteller / Fabricante / Fabbricante / Fabricante / Fabricado por / Producent
	This way up / Haut / Diese Seite oben / Este lado arriba / Questo lato in alto / Este lado para cima / Este lado para cima / Denne side op
	Recyclable / Recyclable / Recyclebar / Reciclable / Riciclabile / Reciclável / Reciclável / Genanvendeligt
	Brittle / Fragile / Zerbrechilich / Fragile / Fragil / Skrøbelig

1.2 ABBREVIATIONS

7-OH-MTX: 7-hydroxy-Methotrexate
CAD: Collision Gas Pressure
CE: Collision energy
CLSI: Clinical and Laboratory Standards Institute
CUR: Curtain Gas
CV: Coefficient of Variation
CXP: Collision Exit Potential
DAMPA: 2, 4-DiAmine-N^{10}-MethylPteroic Acid
DP: Desolvation Potential
EP: Entrance Potential
ESI: Electrospray Ionization
GS1: Gas 1
GS2: Gas 2
HPLC-MS/MS: High Performance Liquid chromatography-tandem mass spectrometry
IS: Ion Spray Voltage
LLOD: Lower Limit Of Detection
LLOQ: Lower Limit Of Quantification
M/Z: Mass/Charge ratio
MPA: Mobile Phase A
MPB: Mobile Phase B
MRM: Multiple Reaction Monitoring
MTX: Methotrexate
PP: Polypropylene
Q1: Quadrupole 1
Q3: Quadrupole 3
RT: Retention Time
S/N: Signal/Noise ratio
TEM: Source temperature of gas

1.3 CLINICAL APPLICATION

FloMass Serum Methotrexate and Metabolites is an in vitro diagnostic kit intended for the quantitative determination and simultaneous of Methotrexate (MTX) and its two main metabolites, 7-hydroxy-Methotrexate (7-OH-MTX) and 2,4-Diamine-N^{10}-Methylpteroic Acid (DAMPA) in human serum samples using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).
The cytotoxic action of MTX is related to the cell cycle. It inhibits the synthesis of DNA, RNA, thymidylates and
proteins by acting as a competitive antagonist of folic acid.
For this reason, the drug has a greater toxic effect on cells with a high replication rate, such as malignant tumor cells [1].

MTX compromises tumor growth without causing irreversible damage to normal tissues. However, the inhibition of the development and proliferation of non-cancerous cells can lead to a series of unexpected side effects.
Low doses of MTX are effective against rheumatoid arthritis, Crohn's disease and psoriasis [2,3].
Due to the narrow therapeutic index of this drug, it is necessary to determine the MTX concentration in serum of patient in order to avoid intoxication (if elevated) or therapeutic failure (if low).
The determination of the two main inactive metabolism is useful to monitor the pharmacokinetic trend of the molecule.
7-OH-MTX, the main metabolite of the drug, has been recognized as the main cause of nephrotoxicity because of its precipitation in the renal tubules causing kidney damage [4-6].
DAMPA has a lower toxicity than 7OH-MTX. Although clinically less interesting, DAMPA is to be considered as an analytical problem because of its cross-reactivity in the immunochemical dosage of MTX, leading to an overdose of the drug.
Differently, HPLC-MS/MS methods are not affected by this problem, allowing to determine the two molecules individually.

2 PRINCIPLE OF THE METHOD

The kit is intended for the quantitative and simultaneous determination of MTX and its metabolites using high performance liquid chromatography technique coupled with tandem mass spectrometry (HPLC-MS/MS).
At the beginning of the preparatory phase, to normalize sample preparation and instrumental variability, the internal standard marked with stable isotopes is added (Table 1).

ANALYTE	INTERNAL STANDARD
MTX	Methotrexate $^{13}C^{2}H_{3}$
7-OH-MTX	Methotrexate $^{13}C^{2}H_{3}$
DAMPA	Methotrexate $^{13}C^{2}H_{3}$

Table 1: Analytes measured by kit EUM16100 and related internal standard

Serum sample is precipitated to eliminate proteins and subsequently the supernatant obtained by centrifugation is diluted.
Once extracted, analytes are chromatographically separated by a specific reverse phase column. Subsequently, they enter in ESI source where they are transferred to the gas phase and ionized. Then ions enter in the triple quadrupole mass spectrometer, where they are measured in MRM mode.
Thus, only selected ions with a defined mass/charge ratio (m/z) are isolated in the first quadrupole and subsequently transferred into the collision cell where they are fragmented by impact with an inert gas (nitrogen or argon). Among the fragments, only those with a defined m/z ratio are isolated in the third quadrupole for subsequent detection.

Measurement in MRM mode with HPLC chromatographic separation ensures high selective and sensitive analytes identification and quantification [4,7,8].

3 COMPONENTS AND ACCESSORIES

3.1 KIT CONTENTS

Components for sample preparation included in the kit are shown in Table 2.

CATALOG NUMBER	DESCRIPTION	QUANTITY	STORAGE
EUM16011	Mobile Phase A	500 mL	Room Temperature
EUM16012	Mobile Phase B	400 mL	Room Temperature
EUM16021	Precipitant Solution	10 mL	Room Temperature
EUM16031	Internal Standard	1 x 1.5 mL	-20°C

Table 2: Components, description, quantity and storage of the kit EUM16100

The kit consists of reagents for 100 assays.
The expiry date of the intact kit is shown on the external product label. Follow storage conditions given on the product label of each component of the kit and keep it away from light and/or heat.

3.2 KIT SUPPORT ACCESSORIES

CATALOG NUMBER	DESCRIPTION	QUANTITY	STORAGE
EUM16041	7-Levels Calibrators, lyophil.	2 x 7 x 0.5 mL	-20°C
EUM16051	3-Levels Controls, lyophil.	2 x 3 x 0.5 mL	-20°C
EUM00C16	Chromatographic Column	1 pc	Room Temperature

Table 3: Accessories, description, quantity and storage of kit EUM16100

3.3 CONTROLS AND CALIBRATION OF ANALYTICAL SYSTEM

Calibration should be done using 7-Levels Calibrators (EUM16041) containing the analytes. Calibrators should follow patient samples preparation (Chapter 7). A new calibrator series should be prepared for each analytical run.
BSN supplies quality control sets at three different concentration levels (EUM16051).
Lyophilized controls in serum matrix are useful to verify the accuracy and precision of analytical procedures and to determine the analysis in the matrix.
For analytes concentrations, stability and accessories preparation, refer to package leaflets.

3.4 CHROMATOGRAPHIC SYSTEM

The kit has been validated using analytical column (EUM00C16).
Stress test on column showed that it is possible to carry out approximately 1000 analysis in matrix with a single column. It is recommended to perform some blanks injections before each run and verify the backpressure values.

4 REQUIRED INSTRUMENTS

The kit requires a HPLC system with a tandem mass spectrometer and dedicated software. Triple quadrupole mass spectrometer should be of medium or medium-high level.

4.1 REQUIRED HPLC MODULES

1. Binary pump able to support a backpressure of 400 bar or more
2. Autosampler with cooling function (10°C)
3. Column Heater (50°C)
4. Degasser

4.2 REQUIRED EQUIPMENT AND MATERIALS FOR THE SAMPLE PREPARATION

1. Centrifuge (10000-13000 rpm) for 1.5- or 2-mL vials
2. Vortex for vials
3. Pipettes and tips
4. 1.5- or 2-mL PP vials
5. Autosampler vials with plastic adapter for 200 µL
6. Chemical hood

5 HPLC-MS/MS SYSTEM CONDITIONS

Ionization: ESI positive mode
MS/MS: specific MRM
Injection volume: 20 µL (variable according to instrumental sensitivity)
Running time: 3.5 min
Column heater: 50°C

Chromatographic gradient

TIME (min)	%MPA	%MPB	FLOW (mL/min)
0.00	95	5	0.40
0.20	95	5	0.40
1.50	0	100	0.40
2.50	0	100	0.40
2.51	95	5	0.40
3.50	95	5	0.40
3.51	Stop	Stop	Stop0

Table 4: Chromatographic gradient of kit EUM16100

Column conditioning: column should be conditioned for 5 min at the chromatography gradient initial condition. Then run 3 blank injections (MPA only) using the gradient as above.

Backpressure: at a flow rate of 0.4 mL/min, chromatographic system backpressure should not exceed 200 bar.

Column storage: in order to preserve the column once detached from the instrument, it is necessary to leave it in the initial conditions of the chromatographic gradient and insert it in the suitable package closing firmly with caps.

Example of chromatogram

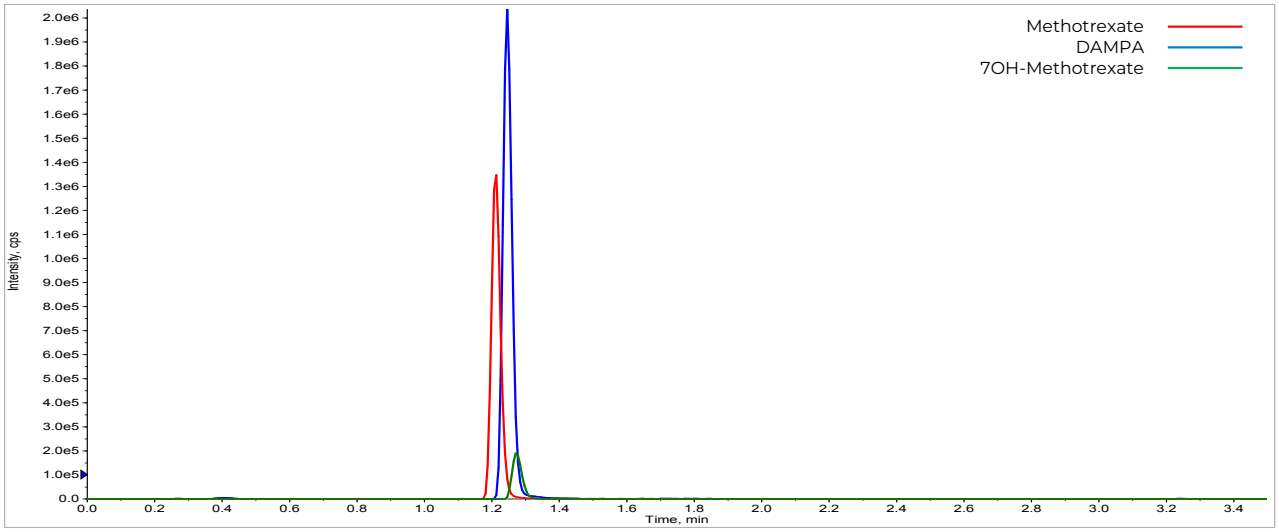

Figure 1: Example of chromatogram identified using kit EUM16100

6 SOURCE PARAMETERS AND TRANSITIONS

6.1 SOURCE PARAMETERS

Source parameters used in the MS method of the EUM16100 with a Sciex series X500 triple quadrupole mass spectrometer are given below.

Curtain Gas (CUR): 30 psi
Collision Gas Pressure (CAD): Medium
Ion Spray Voltage (IS): 5500 V
Temperature (TEM): 550°C
Gas 1 (GS1): 50 psi
Gas 2 (GS2): 60 psi

6.2 TRANSITIONS

Monitored transitions and the MS parameters for each analyte using a HPLC Shimadzu Nexera combined with the Sciex 6500 QTrap mass spectrometer are shown in Table 5. ESI positive mode.

ANALYTE	RT	Q1	Q3	DP	EP	CE	CXP
MTX 1	1.2	455.2	308.2	70	10	28	9
MTX 2	1.2	455.2	175.2	70	10	51	9
DAMPA 1	1.2	326.1	175.1	80	10	27	12
DAMPA 2	1.2	326.1	160.1	80	10	45	12
7-OH-MTX 1	1.2	471.1	324.1	50	10	19	12
7-OH-MTX 2	1.2	471.1	191.1	50	10	38	10
Internal Standard	1.2	459.2	312.2	70	10	28	9

Table 5: Detected transitions, retention times and potentials using HPLC Shimadzu + Sciex mass spectrometer

7 SAMPLE PREPARATION

Calibrators and controls follow the same sample preparation.

7.1 SAMPLE PREPARATION (CALIBRATOR/CONTROL)

1. Prepare a mix with 90 µL of Precipitant Solution (EUM16021) + 10 µL of Internal Standard (EUM16031) sufficient for the number of samples to be analyzed
2. Add 50 µL of serum in a vial
3. Add 100 µL of Mix Solution obtained in the step 1 of the procedure
4. Vortex for 30 sec

5. Centrifuge for 5 min at 10000-12000 rpm
6. Transfer 20 µL of supernatant and dispense in vial
7. Add 80 µL of MPA (EUM16011) in vial
8. Inject 5-20 µL and analyze with HPLC-MS/MS technique

8 COLLECTION AND STORAGE OF THE SAMPLES

The kit is indicated for the analysis of human serum samples collected following standard methods, such as those described in documents H18-A3 and H01-A5 of the Clinical and Laboratory Standards Institute (CLSI) [9-11].

Stability of the samples: in serum samples, MTX and metabolites maintain an unchanged concentration if kept at room temperature for 48 h, either stored up to 5 days at 2-8°C or up to 9 months at -80°C [5].

8.1 EXPECTED VALUES AND RESULTS INTERPRETATION

Acute toxicity of the drug may occur above the values are listed in Table 6, according to scientific literature [1-5,7,8].

ANALYTE	UNIT OF MEASURE	24 h	48 h	72 h
MTX	µmol/L	> 10	> 1	> 0.1
	ng/mL	> 4500	> 450	> 45

Table 6: Toxic levels of Methotrexate

9 VALIDATION DATA

Validation data have been obtained with an HPLC-MS/MS system consisting of a HPLC Shimadzu Nexera coupled with a Sciex 6500 triple quadrupole mass spectrometer.
Refer to Paragraph 4.2 for materials and equipment used in the sample preparation.

9.1 LINEARITY, DETECTION LIMITS AND QUANTIFICATION

A linear regression analysis of real values concentration has been completed to evaluate linearity of calibration curve for each analytic session.
Linearity range of acceptability corresponds to $R^2 \geq 0.98$. All values obtained are higher than the above-mentioned value.

Detection limit (LLOD) and quantification limit (LLOQ), which concentration provide a peak with S/N>3 and S/N>10 respectively, are reported in the table below (Table 7).

ANALYTE	LLOD (ng/mL)	LLOQ (ng/mL)	LINEARITY (ng/mL)
MTX	0.050	0.160	0.160 - 5000
7-OH-MTX	0.410	1.38	1.38 - 10000
DAMPA	0.090	0.290	0.290 - 4000

Table 7: LLOD, LLOQ and linearity

9.2 RECOVERY

Increasing amount of standard has been added to 3 blank extracted matrix pools to evaluate the analytical recovery characteristics. Three different levels of enriched urine (low, medium and high level) have been obtained.

Recovery = (Measured quantity on enriched matrix - Measured quantity on non-enriched matrix) / Added quantity

Average recovery range of acceptability = ±20%, all the values obtained are higher than the above-mentioned value.

ANALYTE	AVERAGE RECOVERY (%)	MIN RECOVERY (%)	MAX RECOVERY (%)
MTX	96.3	88.8	105
7-OH-MTX	103.2	95.7	112.5
DAMPA	101.1	95.5	107.1

Table 8: Average, minimum and maximum recovery values

9.3 PRECISION

Average concentration values (pg/mg) measured in the 3 pools enriched with increasing concentrations of analytes (medium and high level) are reported in Table 10.
Precision has been evaluated as intra-assay, inter-assay and total coefficient of variation.

Intra-assay precision has been determined assaying 10 replicates (n=10) of each sample.
Inter-assay precision has been determined assaying 3 repetitions in 8 analytical series (n=24) for each sample.

Total CV% = $(CV\%Intra^2 + CV\%Inter^{2})^{1/2}$

Range of acceptability used for each variation coefficient are reported below.
Range of acceptability CV% Intra-assay = 10%
Range of acceptability CV% Inter-assay = 20%

Range of acceptability CV% Total = 20%

Obtained results respect the imposed ranges of acceptability.

ANALYTE	AVERAGE CONC. (ng/mL)			CV% INTRA			CV% INTER			CV% TOTAL		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
MTX	48.9	379	2282	2.0%	6.1%	2.1%	6.1%	6.3%	6.0%	6.4%	8.8%	6.4%
7-OH-MTX	108	816	5083	5.6%	6.0%	6.2%	6.8%	6.0%	5.9%	8.8%	8.4%	7.8%
DAMPA	41.2	317.1	1942	2.7%	2.4%	3.8%	4.3%	3.7%	3.1%	5.0%	4.4%	4.9%

Table 9: Intra-assay, inter-assay and total precision

10 GENERAL LIMITATIONS

- Kit must be used with the calibrators and the internal standard indicated in the kit instructions. The use of other standards or materials with this kit has not been validated.
- The use of other mobile phases, solutions or reagents other than those indicated in Paragraph 3.1 “KIT CONTENTS” has not been validated.
- The kit has been validated with the configuration described in Chapter 9 “VALIDATION DATA”.
  The use of other triple quadrupole systems, HPLC systems and columns, which may require further development of the method, has not been validated.
- Do not use the kit after the expiry date of its components.

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ANNEX 1: EC DECLARATION OF CONFORMITY

BSN Srl as Manufacturer and the only responsible for in-vitro diagnostic medical devices placed on the market under his own name, declares that these products meet all the provisions of the Legislative Decree n. 332 of the 8th September 2000, directive of in vitro diagnostic medical device 98/79/EC (in particular with regard to annex I) and subsequent amendments and additions. According to article 9 of the Legislative Decree 332/2000 and similar, this device belongs to the fourth class of devices, GENERIC IN VITRO DIAGNOSTIC MEDICAL DEVICES (all the other in vitro diagnostic medical devices except those in annex II and self-diagnostic tests).

COMPONENT	CODE	CERTIFICATION
FloMass Serum Methotrexate and Metabolites	EUM16100	CE-IVD marked medical device according to Annex III
Mobile Phase A	EUM16011	CE-IVD marked medical device according to Annex III
Mobile Phase B	EUM16012	CE-IVD marked medical device according to Annex III
Precipitant Solution	EUM16021	CE-IVD marked medical device according to Annex III
Internal Standard	EUM16031	CE-IVD marked medical device according to Annex III
7-Levels Calibrators, lyophil.	EUM16041	CE-IVD marked medical device according to Annex III
3-Levels Controls, lyophil.	EUM16051	CE-IVD marked medical device according to Annex III
Chromatographic column	EUM00C16	CE-IVD marked medical device according to Annex III

Quality assurance system complying with the following directive:
UNI CEI EN ISO 13485:2016
UNI EN ISO 9001:2015

This declaration becomes invalid if modifications are introduced without B.S.N. Srl consent.
It is declared that the product is placed on the market in non-sterile package.
It is declared that B.S.N. Srl will keep all documents referred to in Annex III of the European Directive 98/79/EC at the disposal of the competent authorities for a 5-year period from the last date of production of the kit.
After the placing on the market of the product in question, it is declared that the Manufacturer has notified the competent authority of the application of post-market surveillance as requested from the European Directive 98/79/CE.

Castelleone (CR), 22 april 2022

Director