BSN
Biological Sales Network

FloMass p-Cresyl Sulfate and Indoxyl Sulfate (total and free) in Serum

Reagents for 200 assays

Instruction Manual

REF **EUM03200**

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
Σ	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

CCL2: Collision Cell Lens 2

CE: Collision energy

CLSI: Clinical and Laboratory Standards Institute

CV: Coefficient of variation

CXP: Collision Cell Exit Potential
DP: Declustering potential
ED: Entrance Deflector
EP: Entrance Potential
ESI: Electrospray Ionization
EV: Entrance Voltage
HPLC-MS/MS: High Performance Liquid chromatography-tandem mass spectrometry
kDa: kiloDalton
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
pCS: p-Cresyl Sulfate
PP: Polypropylene
Q1: Quadrupole 1
Q3: Quadrupole 3
RT: Retention Time
S/N: Signal/Noise ratio

1.3 CLINICAL APPLICATION

FloMass p-Cresyl Sulfate and Indoxyl Sulfate (total and free) in Serum kit is a vitro diagnostic kit intended for the quantitative determination of p-Cresyl Sulfate and Indoxyl Sulfate (total and free) in human serum samples using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).

Along chronic kidney disease, modification of both metabolic processes and urinary excretion ability provoke an increase of uremic toxins into the body [1-4].

Uremic toxins could be classified as three main classes:

1) Free hydro soluble compounds with a molecular weight lower than 500 g/mol, as example Urea
2) Compounds linked to proteins, as example Indoxyl Sulfate and p-Cresyl Sulfate (pCS)
3) Compounds with a molecular weight higher than 500 g/mol, as example ß-2 microglobulin

Indoxyl Sulfate and pCS have revealed important targets for therapeutic removal. They are, in fact, associated with vascular calcification, arterial stiffness and mortality risk in patients with chronic kidney disease [5-10].

These two uremic toxins, synthesized by gut bacteria through tryptophan-phenylalanine-tyrosine pathway, are predominantly linked to albumin.

Since only free uremic toxins can cross the dialytic membranes, their removal is limited in the dialysis therapies currently in use. In fact, hemodialysis, even with a high membrane's potential, cannot effectively remove the uremic toxins linked to proteins.

Increase of pCS and Indoxyl Sulfate can lead to the formation of a reversible bond to albumin in blood and, mainly in dialysis patients, can cause renal inflammation and consequent risk of complications, such as cardiovascular diseases [7-10].
Indoxyl Sulfate plays an important role in many pathological conditions. It is synthesized in the liver from Indole, produced from Tryptophan by gut bacteria [6]. More than 96% of Indoxyl Sulfate is linked to albumin, thus renal excretion occurs through tubular secretion by an organic anions transport system. Many studies showed a link between Indoxyl Sulfate serum level and progression of chronic renal diseases, aortic calcification, vascular stiffness, diabetic nephropathy and cardiovascular events [9].
pCS is synthesized as well at intestinal level from aromatic amino acids metabolism by resident bacterial flora. It is catabolized mainly through the sulfating process that converts p-Cresyl in pCS. Many scientific studies have shown that serum concentration of pCS and particularly free pCS, could be associated with the progression of chronic renal disease, the cardiovascular pathologies and the effectiveness of hemodialysis.

2 PRINCIPLE OF THE METHOD

This kit is intended for the quantitative and simultaneous determination of pCS and Indoxyl Sulfate in human serum samples using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).
pCS and Indoxyl Sulfate are isolated from serum samples as total and free analytes, i.e., not bonded to proteins.
The total amount method provides protein precipitation followed by dilution of the supernatant. Instead, the free-fraction method provides firstly a molecular cut-off filter centrifugation and then protein precipitation. No supernatant dilution is necessary.
At the beginning of the preparatory phase, to normalize the sample preparation and instrumental variability, the internal standards marked with stable isotopes are added (Table 1).

ANALYTE	INTERNAL STANDARD
p-Cresyl Sulfate	p-Cresyl Sulfate $^{2}H_4$
Indoxyl Sulfate	Indoxyl Sulfate $^{13}C_6$

Table 1: Analytes measured by kit EUM03200 and related internal standards

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 defined mass/charge ratio (m/z) are isolated in 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 separation ensures high selective and sensitive analyte identification and quantification.

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
EUM03011	Mobile Phase A	700 mL	Room temperature
EUM03012	Mobile Phase B	400 mL	Room temperature
EUM03021	Solution Precipitant	60 mL	Room temperature
EUM03031	Internal Standard Mix	3 x 0.8 mL	-20°C

Table 2: Components, description, quantity and storage of kit EUM03200

The kit consists of reagents for 200 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
EUM03041	6-Levels Calibrators, lyophil.	2 x 6 x 0.5 mL	-20°C
EUM03051	2-Levels Controls, lyophil.	2 x 2 x 0.5 mL	-20°C
EUM00C03	Chromatographic Column	1 pc	Room Temperature
EUM00A01	Column Filters	5 pcs	Room Temperature
EUM00A02	Holder A-316 + filter	1 pc	Room Temperature
EUM00A03	Filters A-102	10 pcs	Room Temperature
EUM03061	Free fraction separation filters	200 pcs	Room Temperature

Table 3: Accessories, description, quantity and storage of kit EUM03200

3.3 CONTROLS AND CALIBRATION OF THE ANALYTICAL SYSTEM

Calibration should be done using 6-Levels Calibrators (EUM03041) containing the analytes. Calibrators should follow patient samples preparation in total fraction (Paragraph 7.1). A new calibration series should be prepared for each analytical run.
BSN supplies quality control sets at two different concentration levels (EUM03051). Lyophilized controls in human serum matrix are useful to verify the accuracy and precision of analytical procedures and to determine the analytes in the matrix.
For analyte concentrations, stability and accessories preparation, refer to package leaflet.

3.4 CHROMATOGRAPHIC SYSTEM

The kit has been validated using analytical column (EUM00C03) coupled to the column filter (EUM00A01) and its holder A-316 (EUM00A02) containing filter A-102 (EUM00A03).
Stress tests on column showed that it is possible to carry out approximately 300 analysis in matrix with a set of filters A-102. It is recommended to perform some blank injections before each run and verify the backpressure values.

4 REQUIRED INSTRUMENTS

The method requires a HPLC system with tandem mass spectrometer and dedicated software. Triple quadrupole mass spectrometer should be either low 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 (40°C)
4. Degasser

4.2 REQUIRED EQUIPMENT AND MATERIALS FOR 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 negative mode
MS/MS: specific MRM
Injection volume: 2 µL (variable according to instrumental sensitivity)
Running Time: 6 min
Column heater: 40°C

Chromatographic gradient

TIME (min)	%MPA	%MPB	FLOW (mL/min)
0.00	80	20	0.35
0.75	80	20	0.35
3.10	0	100	0.35
4.30	0	100	0.35
4.35	80	20	0.35
5.50	80	20	0.35
6.00	Stop	Stop	Stop

Table 4: Chromatographic gradient of kit EUM03200

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

Backpressure: at a flow rate of 0.35 mL/min, chromatographic system backpressure should not exceed 400 bar.

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

Example of chromatogram

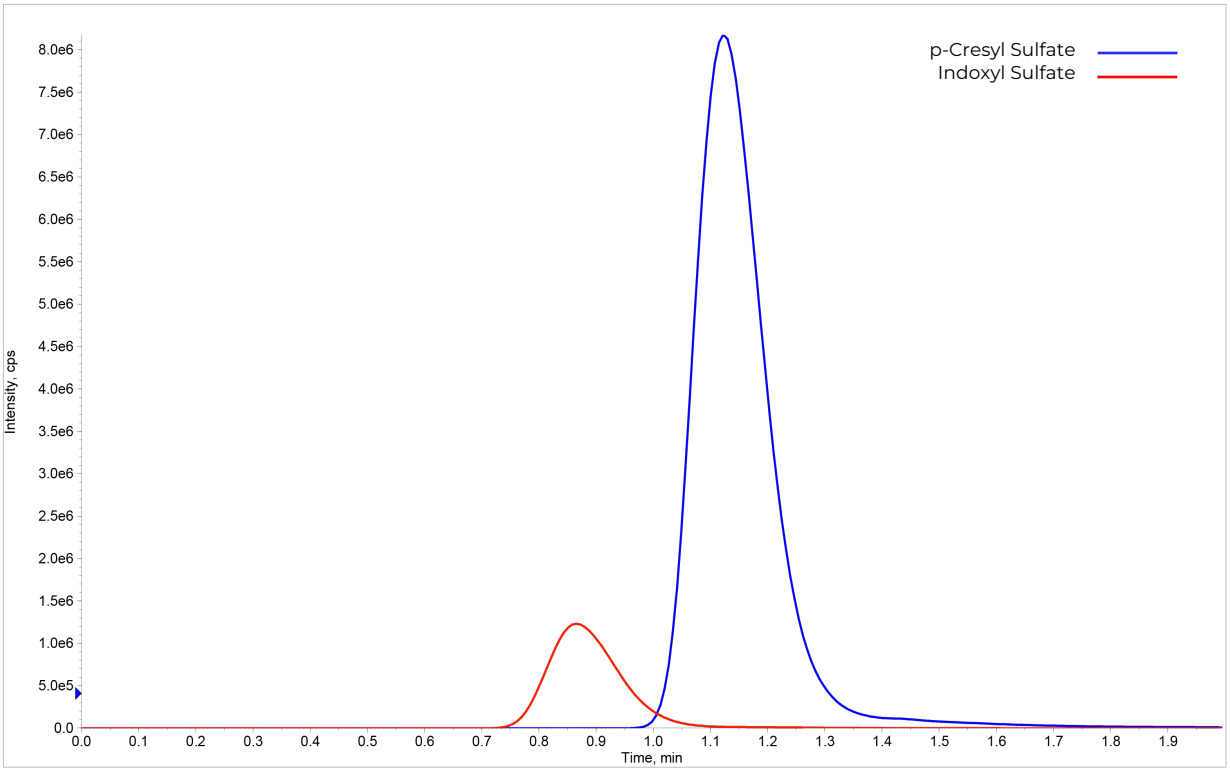

Figure 1: Example of chromatogram identified using kit EUM03200

6 SOURCE PARAMETERS AND TRANSITIONS

6.1 SOURCE PARAMETERS

Source parameters used in the MS method of kit EUM03200 with an Ionics 3Q-120 triple quadrupole mass spectrometer are shown below.

Drying gas: 60°C
Nebulizer gas: 310°C
Source temp: 350°C
HSID temp: 250°C
Electrospray V1: - 5000 V

Source parameters used in the MS method of kit EUM03200 with an Ionics 3Q-120 are shown below.

Curtain gas (CUR): 20 psi
Collision gas (CAD): Medium
IonSpray Voltage: -3500 V
Temperature (TEM): 500°C
Ion Source Gas (GS1): 30 psi
Ion Source Gas (GS2): 40 psi

6.2 TRANSITIONS

Monitored transitions and the MS parameters for each analyte using HPLC Shimadzu Nexera combined with the Ionics 3Q-120 triple quadrupole mass spectrometer are shown in Table 5. ESI negative mode.

ANALYTE	RT	Q1	Q2	CE	CCL2	ED	EV
p-Cresyl Sulfate 1	1.1	186.6	106.9	33	60	-62	-63
p-Cresyl Sulfate 2	1.1	186.6	79.9	26	30	-62	-63
p-Cresyl Sulfate IS 1	1.1	190.6	106.9	33	60	-62	-63
p-Cresyl Sulfate IS 2	1.1	190.6	79.9	26	30	-62	-63
Indoxyl Sulfate 1	0.8	211.5	80.0	52	55	-68	-71
Indoxyl Sulfate 2	0.8	211.5	77.0	50	35	-68	-71
Indoxyl Sulfate IS	0.8	217.5	80.0	52	55	-68	-71

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

Monitored transitions and the MS parameters for each analyte using HPLC Shimadzu Nexera combined with Sciex series X500 triple quadrupole mass spectrometer are shown in Table 6. ESI negative mode.

ANALYTE	RT	Q1	Q3	DP	EP	CE	CXP
p-Cresyl Sulfate 1	1.1	186.9	106.9	-50	-10	-25	-10
p-Cresyl Sulfate 2	1.1	186.9	79.9	-50	-10	-22	-10
p-Cresyl Sulfate IS 1	1.1	190.9	110.9	-50	-10	-25	-10
p-Cresyl Sulfate IS 2	1.1	190.9	79.9	-50	-10	-22	-10
Indoxyl Sulfate 1	0.8	211.9	131.9	-50	-10	-25	-10
Indoxyl Sulfate 2	0.8	211.9	80.0	-50	-10	-40	-10
Indoxyl Sulfate IS	0.8	217.9	137.9	-50	-10	-25	-10

Table 6: Detected transitions, retention times and potentials using HPLC Shimadzu + Sciex X500 mass spectrometer

7 SAMPLE PREPARATION

Calibrators and controls follow the same sample preparation in total fraction.

7.1 SAMPLE PREPARATION (CALIBRATOR/CONTROL) IN TOTAL FRACTION

1. Pipette 50 µL of serum (samples_T, control/calibrator) in 1.5- or 2-mL PP vial
2. Prepare Mix-T solution, according to the number of samples: for each sample use 10 µL of Internal Standards Mix (EUM03031) + 90 µL of Solution Precipitant (EUM03021). Vortex it
3. Add to each sample 100 µL of Mix-T solution prepared in step 2 of the procedure
4. Vortex for 30 sec
5. Centrifuge for 5 min at 10000 rpm
6. Transfer 100 µL of supernatant in a new 1.5- or 2-mL PP vial*
7. Add to each sample 900 µL of H_2O. Vortex it
8. Transfer 100 µL in autosampler vials with plastic adapter and analyze with HPLC-MS/MS technique

*In case of high sensitivity instruments a different dilution is required:

6'. Transfer 10 µL of supernatant in a new 1.5- or 2-mL PP vials
7'. Add to each sample 900 µL of H_2O. Vortex it
8'. Transfer 100 µL in autosampler vials with plastic adapter and analyze with HPLC-MS/MS technique

7.2 SAMPLE PREPARATION: FREE FRACTION

1. Pipette 300 µL of serum (samples_L) in vials with 10 kDa filter
2. Centrifuge for 7 min at 10000 rpm
3. Transfer 50 µL of ultra-filtrated in a new 1.5- or 2-mL PP vial
4. Prepare Mix-L solution, according to the number of samples: for each sample add 1 µL of Internal Standards Mix (EUM03031) + 100 µL Solution Precipitant (EUM03021). Vortex it
5. Add to each sample 100 µL of Mix-L solution prepared in step 4 of the procedure
6. Vortex for 30 sec
7. Centrifuge for 5 min at 10000 rpm
8. Transfer 100 µL of supernatant in autosampler vials with plastic adapter and analyze with HPLC-MS/MS technique*

* In case of high sensitivity instruments a different dilution is required, following these steps:

8'. Transfer 10 µL of supernatant in a new 1.5- or 2-mL PP vial
9'. Add to each sample 90 µL of H_2O. Vortex it
10'. Transfer 100 µL in autosampler vials with plastic adapter 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 e H1-A5 of the Clinical and Laboratory Standards Institute (CLSI) [11,12]. It is recommended to avoid using serum separator tubes because they can cause significant interferences with dosage system.

Stability of the samples: pCS and Indoxyl Sulfate, as other proteins-bonded uremic toxins, are stable in human serum [4].

8.1 EXPECTED VALUES AND RESULTS INTERPRETATION

pCS and Indoxyl Sulfate reference ranges, free and total, observed in adults are listed in Table 7 [13-15].

FRACTION	p-CRESYL SULFATE		INDOXYL SULFATE	
	µmol/L	µg/mL	µmol/L	µg/mL
Free	0.100 – 2.40	0.020 – 0.450	0.100 – 2.40	0.020 – 0.450
Total	0.00 – 38.4	0.00 – 7.23	0.00– 38.4	0.00 – 7.23

Table 7: Analytes reference values

Note: reference ranges are taken from selected and updated scientific literature. Their update corresponds to the date of revision of this document.
Reference ranges values are not a recommendation by the manufacturer, but they can be used as guideline for own reference ranges of each clinical laboratory.

9 VALIDATION DATA

Validation data have been obtained with a HPLC-MS/MS system consisting of a HPLC Shimadzu Nexera coupled with an Ionics 3Q-120 triple quadrupole mass spectrometer.
Refer to Paragraph 4.2 for materials and instruments used in sample preparation.

9.1 LINEARITY, DETECTION LIMITS AND QUANTIFICATION

A linear regression analysis of real values concentration has been completed in order 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 8).

ANALYTE	LLOD (ng/mL)	LLOQ (ng/mL)	LINEARITY (ng/mL)
p-Cresyl Sulfate	0.060	0.200	0.200 – 450
Indoxyl Sulfate	0.090	0.310	0.310 – 500

Table 8: LLOD, LLOQ and linearity

9.2 RECOVERY

Increasing amount of standard has been added to 3 real human serum pools in order to evaluate the analytical recovery characteristics. Three different levels of enriched serum (low, medium, 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 (%)
p-Cresyl Sulfate (total)	94.2	92	108
p-Cresyl Sulfate (free)	102.4	101	118
Indoxyl Sulfate (total)	97	93	115
Indoxyl Sulfate (free)	102.9	97	117

Table 9: Average, minimum and maximum recovery values

9.3 PRECISION

Average concentration values (µg/mL) measured in the real human serum pool (endogenous) and in 2 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) of 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. (µg/mL)			CV% INTRA			CV% INTER			CV% TOTAL		
	Endo	Medium	high	Endo	Medium	high	Endo	Medium	high	Endo	Medium	high
p-Cresol Solfato (total)	3.96	15.8	42.4	1.91%	1.63%	2.53%	3.98%	3.04%	3.37%	5.61%	3.50%	4.56%
p-Cresol Solfato (free)	1.22	14.5	43.0	5.82%	2.09%	1.76%	4.75%	2.34%	2.46%	7.38%	2.54%	2.64%
Indoxyl Solfato (total)	1.43	17.5	49.4	12.8%	6.99%	8.62%	5.33%	6.35%	3.78%	7.22%	6.82%	3.92%
Indoxyl Solfato (free)	0.420	17.4	50.7	9.84%	4.14%	7.75%	6.90%	3.40%	4.62%	7.09%	3.78%	9.90%

Table 10: Intra-assay, inter-assay and total precision

10 GENERAL LIMITATIONS

- Kit must be used with calibrators and internal standard indicated in the kit instructions. The use of other standards or materials with this kit has not been validated.
- The use of different mobile phases, solutions or reagents other than those indicated in Paragraph 3.1 “KIT CONTENTS” has not been validated.
- This kit has been validated with configuration described in Chapter 9 “VALIDATION DATA”.
  The use of other triple quadrupole system, HPLC system and columns, which may require further development of the method, has not been validated.
- Do not use the kit after 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 p-Cresyl Sulfate and Indoxyl Sulfate (Free and Total) in Serum	EUM03200	CE-IVD marked medical device according to Annex III
Mobile Phase A	EUM03011	CE-IVD marked medical device according to Annex III
Mobile Phase B	EUM03012	CE-IVD marked medical device according to Annex III
Solution 1 Precipitant	EUM03021	CE-IVD marked medical device according to Annex III
Internal Standards Mix	EUM03031	CE-IVD marked medical device according to Annex III
6-Levels Calibrators, lyophil.	EUM03041	CE-IVD marked medical device according to Annex III
2-Levels Controls, lyophil.	EUM03051	CE-IVD marked medical device according to Annex III
Chromatographic Column	EUM00C03	CE-IVD marked medical device according to Annex III
Column Filters	EUM00A01	CE-IVD marked medical device according to Annex III
Holder A-316 + filter	EUM00A02	CE-IVD marked medical device according to Annex III
Filters A-102	EUM00A03	CE-IVD marked medical device according to Annex III
Free fraction separation filters	EUM03061	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), 29 April 2022 Director