FloMass VMA, HVA and 5-HIAA in Urine

Reagents for 100 assays

Instruction Manual

REF **EUM19100**

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

CE

PROVIDE PERSONAL SOLUTIONS

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1 INTRODUCTION

1.1 IVD SYMBOLS

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

Batch code / Code du lot / Chargenbezeichnung / Código de lote / Codice del lotto / Código do lote / Número do lote / Lotnummer

Packing number / Numéro d'emballage / Packnummer / Número de envase / Numero confezioni / Número de embalagem / Número de embalagem / Emballagenummer

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

5-HIAA: 5-Hydroxyindoleacetic Acid
CAD: Collision Gas Pressure
CE: Collision Energy
CLSI: Clinical and Laboratory Standards Institute
CUR: Curtain Gas
CV: Coefficient of Variation
CXP: Collision Exit Potential
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
HVA: Homovanilic Acid
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
PP: Polypropylene
Q1: Quadrupole 1
Q3: Quadrupole 3
RT: Retention Time
S/N: Signal/Noise ratio
SPE: Solid Phase Extraction
TEM: Source Temperature
VMA: Vanillymandelic Acid

1.3 CLINICAL APPLICATION

FloMass VMA, HVA e 5-HIAA in Urine is an in vitro diagnostic kit intended for the quantitative and simultaneous determination of 3 analytes in human urine samples (Table 1) using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).

ANALYTE
VMA
5-HIAA
HVA
Creatinine

Table 1: Analytes measured by FloMass VMA, HVA e 5-HIAA in Urine

Catecholamines (Epinephrine, Norepinephrine and Dopamine) are neurotransmitters produced by adrenal gland. Their release into the body cause physiological changes, including increased heart rate and arterial pressure. Catecholamines metabolism involves conversion to Metanephrines (Metanephrine, Normetanephrine and 3-Methoxytyramine), molecules linked to pheochromocytoma and others neuroendocrine tumors. Further metabolism of Metanephrines lead to formation of: VMA (Vanillymandelic Acid), that at high levels could be a marker of pheochromocytoma, 5-HIAA (5-Hydroxyindoleacetic Acid), possible tumoral marker of carcinoid syndrome, HVA (Homovanillic Acid), useful marker in diagnosis of neuroblastoma and neuroendocrine pathologies [1-3]. Creatinine in a molecule arising from Creatine degradation reaction in muscles, involved in energy-yielding metabolism. It is released into blood flow and then undergoes to glomerular filtration, then it is secreted via urine. 24 h-Creatinine blood/plasma/urine concentration is commonly used as marker of renal function. High Creatinine levels indicate nephrons damage and renal impairment, whereas low levels can occur in case of anemia, muscular atrophy, or debilitating conditions of the organism.
Creatinine levels are higher in men because of higher muscular mass.

2 PRINCIPLE OF THE METHOD

The kit is intended for the quantitative and simultaneous determination of VMA, HVA and 5-HIAA in human urine samples by using high performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS).
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 2).

ANALYTE	INTERNAL STANDARD
VMA	VMA $^{13}C^{2}H_{3}$
5-HIAA	5-HIAA $^{2}H_{5}$
HVA	HVA $^{13}C^{2}H_{3}$
Creatinine	Creatinina $^{13}C^{2}H_{3}$

Table 2: Analytes measured by kit EUM19100 and related internal standards

The urine samples are diluted with a specific solution to match the chromatographic condition. Once diluted, the samples are injected in the HPLC-MS/MS system and analytes are chromatographically separated by a specific reverse phase column. Subsequently, they enter in ESI source where they are transferred into 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 the first quadrupole and subsequently transferred in to 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 by the third quadrupole for subsequent detection.

Measurement in MRM mode with HPLC chromatographic separation ensures high selective and sensitive analytes identification and quantification [4-6].

3 COMPONENTS AND ACCESSORIES

3.1 KIT CONTENTS

Components for sample preparation included in the kit are shown in Table 3.

CATALOG NUMBER	DESCRIPTION	QUANTITY	STORAGE
EUM17011	Mobile Phase A	500 mL	Room temperature
EUM17012	Mobile Phase B	500 mL	Room temperature
EUM19021	Diluting Solution	110 mL	Room temperature
EUM19031	Internal Standard Mix	2 x 1.0 mL	-20°C

Table 3: Components, description, quantity and storage of kit EUM19100

The kit consists of reagents for 100 assays.
The expiry date of the intact kit is shown on the external product label. Follow the 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
EUM19041	7-Levels Calibrators, lyophil.	2 x 7 x 0.5 mL	-20°C
EUM17051	2-Levels Controls, lyophil.	2 x 2 x 0.5 mL	-20°C
EUM00C17	Chromatographic Column	1 pc	Room temperature
EUM00A14	Precolumn	4 pcs	Room temperature
EUM00A15	Holder + precolumn	1 pc	Room temperature

Table 4: Accessories, description, quantity and storage of kit EUM19100

3.3 CONTROLS AND CALIBRATION OF ANALYTICAL SYSTEM

Calibration should be done using 7-Levels Calibrators (EUM19041) 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 two different concentration levels (EUM17051). Urinary matrix Quality Lyophilized Controls are useful to verify the accuracy and precision of the analytical procedures.
For analytes concentrations, stability and accessories preparation, refer to package leaflet.

3.4 CHROMATOGRAPHIC SYSTEM

The kit has been validated using analytical column (EUM00C17) coupled to the precolumn (EUM00A14) and its holder (EUM00A15).
Stress tests on column showed that is possible to carry out approximately 200-250 analyses in matrix with a single precolumn. It is recommended to perform some blank 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-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 oven (50°C)
4. Degasser

4.2 REQUIRED EQUIPMENT AND MATERIALS FOR SAMPLE PREPARATION

1. Centrifuge (13000 rpm) for 1.5- or 2.0-mL vials
2. Vortex for vials
3. Pipettes and tips
4. 1.5- or 2.0-mL 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: MRM specific
Injection Volume: 1 µL (variable according to the instrumental sensitivity)
Running Time: 3.2 min
Column Oven: 45°C

Chromatographic gradient

TIME (min)	%MPA	%MPB	FLOW (mL/min)
0	98	2	1.20
1.80	5	95	1.20
2.15	5	95	1.20
2.16	98	2	1.20
3.20	98	2	1.20
3.21	Stop	Stop	Stop

Table 5: Chromatographic gradient of kit EUM19100

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

Backpressure: using a flow rate of 1.2 mL/min, chromatographic system backpressure should not exceed 200 bar.

Column storage: if it is required to disconnect column, in order to preserve it, it's recommended to store it in the starting condition of the gradient program and to close it tightly.

Example of chromatogram

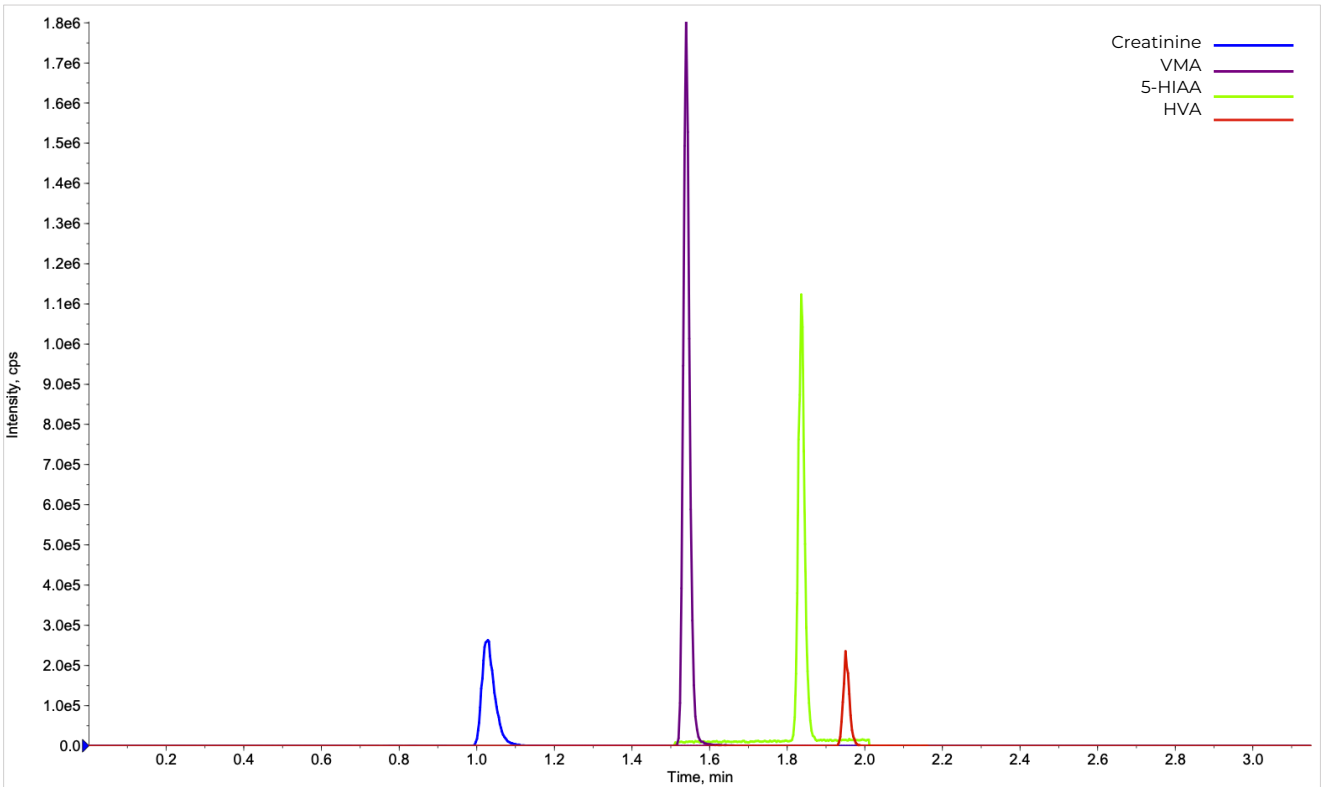

Figure 1: Example of chromatogram identified using kit EUM19100

6 SOURCE PARAMETERS AND TRANSITIONS

6.1 SOURCE PARAMETERS

Source parameters used in MS Method of kit EUM19100 with a Sciex series X500 QTrap mass spectrometer are shown below.

Curtain Gas (CUR): 25 psi
Collision Gas Pressure (CAD): Medium
Ion Spray Voltage (IS): -4500 V
Temperature (TEM): 500°C
Gas 1 (GS1): 45 psi
Gas 2 (GS2): 50 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 6. ESI negative mode.

ANALYTE	RT	Q1	Q3	DP	EP	CE	CXP
Creatinine 1	1.0	112.1	68.0	-50	-10	-20	-12
Creatinine 2	1.0	112.1	83.0	-50	-10	-25	-12
VMA 1	1.5	197.1	137.1	-30	-10	-30	-12
VMA 2	1.5	197.1	108.1	-30	-10	-55	-12
5-HIAA 1	1.8	190.1	144.1	-40	-10	-30	-12
5-HIAA 2	1.8	190.1	146.1	-40	-10	-15	-12
HVA 1	1.9	181.1	122.1	-30	-10	-20	-12
HVA 2	1.9	181.1	105.1	-30	-10	-25	-12
Creatinine IS	1.0	118.1	69.0	-50	-10	-22	-12
VMA IS	1.5	201.2	137.1	-30	-10	-38	-12
5-HIAA IS	1.8	195.1	148.1	-40	-10	-28	-12
HVA IS	1.9	185.1	122.1	-40	-10	-20	-12

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

7 SAMPLE PREPARATION

Calibrators and controls follow the same samples preparation.

7.1 SAMPLE PREPARATION (CALIBRATORS/CONTROLS)

1. Prepare a mix with 20 µL of Internal Standards Mix (EUM19031) + 960 µL of Diluting Solution (EUM19021) sufficient for the number of samples to be analyzed
2. Pipette 20 µL of urine in a vial (if human, preserved with 0.1% HCl)
3. Add 980 µL of the Mix solution obtained in step 1 of the procedure
4. Vortex for 30 sec
5. Centrifuge for 5 min at 12000 rpm
6. Pipette 200 µL of supernatant in an autosampler vial with low volume insert
7. Inject 1-10 µL according to instrumental sensitivity and analyze with HPLC-MS/MS technique

8 COLLECTION AND STORAGE OF SAMPLES

The kit is intended for the analysis of human urine samples collected with standard methods, such as those described in document GP16-A3 of the Clinical and Laboratory Standards Institute (CLSI) [7]. The measurement should be performed on 24' hours urine samples without using any preserving agent.

Stability of the samples: urinary Catecholamines and their metabolites are stable at acidic pH values. In untreated urine samples (pH between 5-8), acidic metabolites are stable until 2 weeks if stored at room temperature and until 1 month if stored at 2-8°C or at -20°C [3].

8.1 EXPECTED VALUES AND RESULTS INTERPRETATION

Each laboratory should conduct a pilot study in order to determine the distribution of analytes in relation to its population. In order to establish the population dimension study, it is recommended to check CLSI document EP28-A3C [8].
Reference values and normal ranges are set according to the distribution.

8.2 REFERENCES RANGES

Acidic metabolites and Creatinine ranges in adults are listed in Table 7, classified by gender.

ANALYTE	ADULT MALES (µg/24 h)	ADULT WOMEN (µg/24 h)
VMA	< 8.00	< 8.00
5-HIAA	< 8.50	< 10.0
HVA	< 8.00	< 8.00
Creatinine	955 - 2936	601 - 1689

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 [2,3].
Reference ranges 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 an HPLC-MS/MS system consisting of a HPLC Shimadzu Nexera coupled to a Sciex 6500 QTrap triple quadrupole mass spectrometer.
Refer to Paragraph 4.2 for the 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 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)
VMA	0.1000	0.330	0.330 – 750
5-HIAA	0.140	0.460	0.460 – 750
HVA	0.150	0.490	0.490 – 750
Creatinine	3.50	11.7	11.7 – 7500

Table 8: LLOD, LLOQ and linearity

9.2 PRECISION

Average concentration values (ng/mL) measured in the real urine pool (endogenous) and in 2 pools enriched with increasing concentrations of analytes (medium and high levels) are reported in Table 9.
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) for 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		
	Endo	Medium	High	Endo	Medium	High	Endo	Medium	High	Endo	Medium	High
VMA	5.46	25.0	100	5.1	5.2	2.6	5.0	2.7	3.3	7.18	5.81	4.21
5-HIAA	5.22	22.9	92.6	6.1	2.8	4.7	5.7	5.7	4.5	8.34	6.33	6.53
HVA	5.70	24.0	94.2	4.6	5.9	2.8	6.0	4.0	4.6	7.55	7.09	5.34
Creatinine	291	480	1230	4.7	4.3	3.2	5.9	2.8	4.0	7.54	5.09	5.07

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 manual. 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 system, HPLC systems and columns, which may require further development of the method, has not been validated.
- Do not use the kit after expiration date of its components.

11 REFERENCES

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[2] Hsu W., Chen C., Tsai, F. Lai C. (2012): **Simultaneous detection of diagnostic biomarkers of alkaptonuria, ornithine carbamoyltransferase deficiency, and neuroblastoma disease by high-performance liquid chromatography/tandem mass spectrometry.** *Clinica Chimica Acta*

[3] Shen Y., Li H., Lu J., Luo X., Guan Q., Cheng L. (2019): **Analytical validation and clinical application of urinary vanillylmandelic acid and homovanillic acid by LC–MS/MS for diagnosis of neuroblastoma.** *Biomedical Chromatography* 33, 4484

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[5] Magera M.J., Thompson A.L., Matern D., Rinaldo P. (1003): **Liquid chromatography – Tandem Spectrometry method for the determination of Vanillymandelic Acid in Urine.** *Clinical Chemistry* 49:5 825-826

[6] Côté L. (2016): **Urinary Vanillylmandelic, Homovanillic, and 5-Hydroxyindoleacetic Acids by LC/MS/MS.** *Application Agilent Technologies Canada*

[7] Clinical and Laboratory Standards Institute (CLSI) (2009*):* **Urinalysis; Approved Guideline – Third Edition.** *CLSI document GP16-A3.* CLSI, Wayne, Pennsylvania 19087-1898, USA

[8] Clinical and Laboratory Standards Institute (2010): ***Defining, Establishing and verifying reference intervals in the clinical laboratory – Approved Guideline Third Edition*** CLSI Document EP28-A3C

ANNEX 1: EC DECLARATION OF CONFORMITY

B.S.N. 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 point 9 of Legislative Decree 332/2000 and subsequent amendments, the in vitro diagnostic medical device belongs to the fourth category of devices, that is GENERIC IN VITRO MEDICAL-DIAGNOSTIC DEVICES.

COMPONENT	CODE	CERTIFICATION
Mobile Phase A	EUM17011	CE-IVD marked medical device according to Annex III
Mobile Phase B	EUM17012	CE-IVD marked medical device according to Annex III
Diluting Solution	EUM19021	CE-IVD marked medical device according to Annex III
Internal Standards Mix	EUM19031	CE-IVD marked medical device according to Annex III
Calibrators in Urine, for VMA, HVA e 5-HIAA, lyophil.	EUM19041	CE-IVD marked medical device according to Annex III
Controls in Urine for biogenic amines, lyophil.	EUM17051	CE-IVD marked medical device according to Annex III
Chromatographic Column	EUM00C17	CE-IVD marked medical device according to Annex III
Precolumn	EUM00A14	CE-IVD marked medical device according to Annex III
Holder + precolumn	EUM00A15	CE-IVD marked medical device according to Annex III

Quality assurance system complying following directives:

- ✓ 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 the 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 products 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.
This declaration is valid five years from the date of issue.

Castelleone (CR), 29/04/2022 Director