AMANITIN

ELISA

This product is for research use only
It is not intended for use in diagnostic procedures

EK-AM1-U 96 tests

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ENGLISH

INTENDED USE

The BÜHLMANN Amanitin ELISA kit is intented for the direct and quantitative determination of α- and γ-Amanitin present in human urine, serum and plasma. This product is for research use only. It is not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The BÜHLMANN Amanitin ELISA is a competitive immunoassay. A polyclonal antibody (Ab) specific for α- and γ-Amanitin (1,2) has been coated onto the wells of the Microtiter Plate. During the first incubation, Amanitin present in the prediluted urine samples and the calibrators, respectively, competes with biotinylated Amanitin for the binding sites of the specific rabbit anti-Amanitin antibody. After washing, streptavidin conjugated to horseradish peroxidase (HRP) is added, which binds during a second incubation step to the Biotin-Ab complexes. Unbound enzyme label is removed by a second washing step and tetramethylbenzidin (TMB) Substrate Solution is added to the wells. During the following incubation step, a colored product is formed in inverse proportion to the amount of Amanitin present in the sample. Upon addition of acidic Stop Solution the color changes from blue to yellow and can be measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate precoated with anti-α-Amanitin polyclonal Ab.	12 x 8 wells	B-EKAM1-MP	Ready to use
Plate Sealer	3 pieces		
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 ml	B-EKAM1-WB	Dilute with 900 ml of deionized water
Incubation Buffer with preservatives	1 bottle 100 ml	B-EKAM1-IB	Ready to use
Blanking Reagent *) lyophilized amanitin in a buffer matrix with preservatives	1 vial lyophilized	B-EKAM1-BR	Add 1 ml of Incubation Buffer
Calibrators A to E **) lyophilized α-amanitin in a buffer matrix with preservatives	5 vials	B-EKAM1-CASET	Add 1 ml of Incubation Buffer
Control Low / High ***) lyophilized amanitin within human urine	2 vials	B-EKAM1-CONSET	Add 1 ml of Incubation Buffer
Biotin Conjugate amanitin conjugated to biotin in a buffer matrix with preservatives	1 vial 5.5 ml	B-EKAM1-BC	Ready to use
Enzyme Label streptavidin conjugated HRP in a protein-based buffer with preservatives	1 vial 11 ml	B-EKAM1-EL	Ready to use
TMB Substrate TMB in citrate buffer with H_2O_2	1 vial 11 ml	B-TMB	Ready to use
Stop Solution 0.25 M sulfuric acid	1 vial 11 ml	B-STS	Ready to use **Corrosive agent**

Table 1

*) The Blanking Reagent contains 100 µg/ml α-Amanitin.

**) After reconstitution the Calibrators A ,B, C, D and E contain 1, 3, 10, 30 and 100 ng/ml of α-Amanitin, respectively.

***) The Controls contain lot-specific amounts of α-Amanitin. Refer to the additional QC Data Sheet for actual concentrations.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
Lyophilized Calibrators, Controls and Blanking Solution must be stored at -20°C. The other unopened kit components are stable at 2-8°C. Do not use past kit expiration date.	
Opened / Reconstituted Reagents	
Microtiter Plate	Return unused strips immediately to the aluminum pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store for up to 2 months at 2-8°C.
Diluted Wash Buffer	Store for up to 4 months at 2-8°C.
Controls	Stable at -20°C for at least 3 months
Calibrators	
Blanking Solution	
Incubation Buffer	Stable at 2-8°C until expiration date marked on the vial
Biotin Conjugate	
Enzyme Label	
Substrate Solution	Store in darkness at 2-8°C until expiration date.
Stop Solution	Store at 18-28°C.

Table 2

WARNINGS AND PRECAUTIONS

The Microtiter Plate and the controls of this kit contain components of human origin. Each serum donor unit used in the preparation of the kit components was tested by an FDA approved method and found negative for HBV surface antigen, so as for HCV and HIV1/2 antibodies. Although these methods are highly accurate, there is no guarantee that this material cannot transmit Hepatitis or AIDS. *Therefore, all individual's specimens and kit components should be handled as if capable of transmitting infections.* All products containing human source material should be handled in accordance with good laboratory practice using appropriate precautions.

Blanking Reagent, Calibrators, Controls, and Biotin Conjugate: Contains α-amanitin. Avoid intake of these reagents

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips for 40 µl, 50 µl, 100 µl and 1 ml.
- Multishot pipettes with disposable tips for 50 µl and 100 µl.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 ml cylinder for the dilution of the Wash Buffer concentrate.
- Blotting Paper
- Microtiter Plate washer or squeeze bottle for the Wash Buffer.
- Microtiter Plate rotator.
- Microtiter Plate reader for measurement of absorbance at 450 nm.

SPECIMEN COLLECTION AND STORAGE

- The assay procedure calls for less than 50 µl of urine, serum or plasma.
- Collect individual urine, serum or plasma and keep the specimen refrigerated. Aliquots may be stored for up to 7 days at 2-8°C or for at least 6 months frozen at –20 °C.
- To reach highest sensitivity, urinary sample collection must be within 36 hours after mushroom ingestion (see Chapter Normal Values and Interpretation of Results)

PROCEDURAL NOTES

- Repeated freezing and thawing of specimens and reagents supplied in this kit must be avoided.
- The enzyme (HRP) used as the label is inactivated by oxygen and is highly sensitive to Sodium azide, Thimerosal, Hypochlorous acid and aromatic Chlorohydrocarbons often found in laboratory water supplies. Therefore, use only deionized or distilled high quality water.
- If the initial concentration of an unknown sample reads greater than the highest calibrator (Calibrator E), the sample should be further diluted with incubation buffer and assayed again according to the assay procedure. The resulting dilution factor must be accounted for final calculations.

ASSAY PROCEDURE

1. Dilute all samples 1:25 with Incubation Buffer (e.g. 40 µl of urine + 960 µl of Incubation Buffer).
2. Prepare a plate with sufficient strips to test the desired number of calibrators and samples. Remove excess strips from the holder and reseal them in the foil pouch together with the desiccant packs **without delay**. Store refrigerated.
   Important: Allow the reagents to come to 18-28 °C before setting up the assay.
3. Wash the coated wells twice using at least 300 µl of Wash Buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

4a. Pipet 50 µl of Blanking Solution (Blank) in duplicate into wells A1+A2.
Pipet 50 µl of Incubation Buffer (Zero Calibrator) in duplicate into wells B1+B2.
Pipet 50 µl of Calibrator A in duplicate into wells C1+C2.
Pipet 50 µl of Calibrator B in duplicate into wells D1+D2.
Pipet 50 µl of Calibrator C in duplicate into wells E1+E2.
Pipet 50 µl of Calibrator D in duplicate into wells F1+F2.
Pipet 50 µl of Calibrator E in duplicate into wells G1+G2.

4b. Pipet 50 µl of the Low Control in duplicate into wells H1+H2.
Pipet 50 µl of the High Control in duplicate into wells A3+A4.

4c. Pipet 50 µl of each diluted sample (1:25) in duplicate into the subsequent wells.

5. Pipet 50 µl Biotin Conjugate to all wells.
6. Cover the plate with a Plate Sealer, place the plate on a plate rotator set at 400-600 rpm and incubate for 30 ± 5 minutes at 18-28 °C.
7. Remove and discard the Plate Sealer. Empty the wells and wash three times using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
8. Pipet 100 µl of Enzyme Label into all wells.
9. Cover the plate with a Plate Sealer, place the plate on a plate shaker set at 400-600 rpm and incubate for 15 ± 5 minutes at 18-28 °C.
10. Remove and discard the Plate Sealer. Empty the wells and wash three times using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
11. Pipet 100 µl of the TMB Substrate Solution to all wells.
12. Cover the plate with a Plate Sealer, place the plate on a plate shaker set at 400-600 rpm, protect the plate from direct light and incubate for 15 ± 5 minutes at 18-28 °C.
13. Pipet 100 µl of Stop Solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 14 within 30 minutes.
14. Read the absorbance at 450 nm in a microtiter plate reader.

RESULTS

Standard Curve

Record the absorbance at 450 nm for B_0, each calibrator, and blank (NSB) well. Average the values, subtract the average of the blank wells (NSB) and record averages (=corrected average absorbance). Calculate the binding (B) of each calibrator as the percentage of B_0, with the NSB-corrected absorbance of the B_0 taken as 100 %.

$$B/B_0\ (\%) = \text{percent bound} = \frac{\text{net absorbance}}{\text{net absorbance of Zero Calibrator}} \times 100$$

Plot the percent bound (vertical axis) versus the concentration of Amanitin in ng/ml (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four parameter logistic.

Samples and Controls

Record the absorbance at 450 nm for each sample well. Average the values, subtract the average of the blank and record the averages (=corrected average absorbance). Calculate, as described above, the binding of each sample wells as a percent of B_0, with the NSB-corrected absorbance of the B_0 taken as 100%. Locate the B/B_0 value of the samples on the vertical axis, draw a horizontal line intersecting the standard curve and read the Amanitin concentration (ng/ml) from the horizontal axis.

See Table 3 and Figure 1 for examples of results and standard curves. *These results and standard curves are for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.*

QUALITY CONTROL

A thorough understanding of this package insert is necessary for the successful use of the product. Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this package insert.

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and printed on the additional QC data sheet.

If the precision of the assay does not correlate with the established limits and repetition excludes errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices ii) ELISA reader settings iii) expiration dates of reagents iv) storage and incubation conditions v) TMB Substrate Solution should be colorless vi) purity of water.

LIMITATIONS

- The reagents supplied with this kit are optimized to measure α- and γ-Amanitin in human urine, serum and plasma.
- **AMANITIN CONCENTRATIONS** in urine does strongly depend on the time after mushroom ingestion at which the urine sample was collected.

PERFORMANCE CHARACTERISTICS

Intra-Assay Precision (Within-Run): 6.3% (urine). The intra-assay precision was calculated from the results of 20 pairs of values from four spiked urine samples obtained in a single run. The mean values (ng/ml) are presented in Table 4.

Inter-Assay Precision (Run-to-Run): 7.3% (urine). The inter-assay precision was calculated from the results of 3 spiked urine samples obtained in 20 different runs. The mean values (ng/ml) are presented in Table 5.

Dilution Linearity/Parallelism: 97.8% (urine). Three human urine samples spiked with a high concentration of α-Amanitin was diluted (1:25 to 1:800) with Incubation Buffer and assayed according to the assay procedure. The mean values (ng/ml) are presented in Table 6.

Spiking Recovery in Urine: 99.9%. One urine sample was spiked with increasing amounts of α-Amanitin and assayed four times according to the assay procedure. The resulting mean values (ng/ml) are presented in Table 7.

Spiking Recovery in Plasma and Serum: 111.2% (plasma), 104.6% (serum). One human plasma and serum sample, each was spiked with increasing amounts of α-Amanitin and assayed according to the assay procedure. The values (ng/ml) are presented in Table 8.

Sensitivity:

Functional Sensitivity: **1.5 ng/ml.** The functional least detectable dose (FLDD) was calculated to be 1.5 ng/ml (cut-off of intra assay CV= 15%).

Analytical Sensitivity: **0.22 ng/ml.** Twenty duplicates of Incubation Buffer were assayed in a single run. Mean and standard deviation were calculated for the absorbance values. The minimum detectable dose of Amanitin was calculated to be at 0.22 ng/ml by adding two standard deviations to the mean absorbance of the reagent blank (Incubation Buffer) and intersecting this value with the standard curve obtained in the same run.

Specificity: The following cross-reactions of the polyclonal rabbit anti-α-Amanitin antibody with different Amatoxins and Phallotoxins have been determined at 50% binding.

α-Amanitin :	100.0 %	ε-Amanitin:	0.1 %
β-Amanitin :	0.1 %	Phalloidin:	not detectable
γ-Amanitin :	90.0 %	Phallacidine:	not detectable

Table 3: **Example of Results**

	Absorb. (OD)	CV (%)	B/B_0 (%)	Conc. (ng/ml)
Blank	**0.120**			
B0	2.301		101.2	0
B0	2.246		98.8	
B0 Avg.	**2.273**	1.7	**100.0**	
Cal A	2.048		90.1	1.0
Cal A	1.946		85.6	
Cal A Avg.	**1.997**	3.6	**87.8**	
Cal B	1.567		68.9	3.0
Cal B	1.491		65.6	
Cal B Avg.	1.529	3.5	**67.2**	
Cal C	0.787		34.6	10.0
Cal C	0.783		34.4	
Cal C Avg.	**0.785**	0.4	34.5	
Cal D	0.218		9.6	30.0
Cal D	0.202		8.9	
Cal D Avg.	**0.210**	5.4	**9.2**	
Cal E	0.073		3.2	100.0
Cal E	0.069		3.0	
Cal E Avg.	**0.071**	4.0	**3.1**	
Control low	1.316		57.9	4.3
Control low	1.293		56.9	4.4
Control low Avg.	**1.304**	1.2	**57.4**	**4.35**
Control high	0.307		13.5	25.6
Control high	0.295		13.0	26.5
Control high Avg.	0.301	2.8	**13.3**	**26.1**

ED-20 = 17.6 ng/ml ED-50 = 5.6 ng/ml ED-80 = 1.7 ng/ml

Figure 1: **Example of Standard Curve**

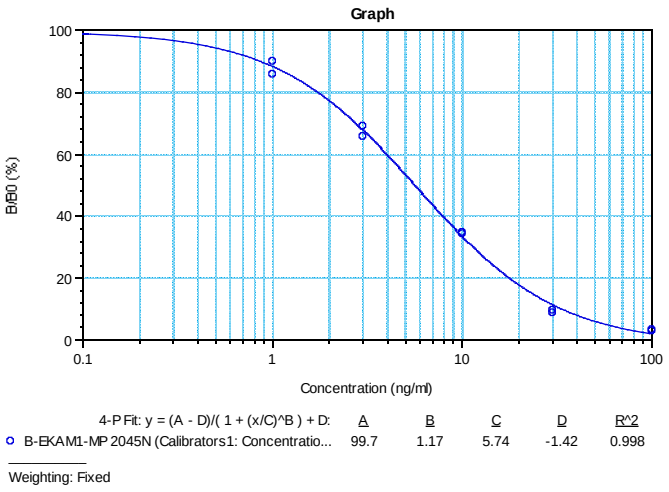

Table 4: **Intra-Assay Precision**

Sample type (spiked)	n	Sample range (mean)	C.V. (%) Range	Mean of C.V. (%)
Urine	4	3.1 – 90.8	2.8 – 14.0	6.3
EDTA-plasma	4	4.6 – 103.0	4.5 – 12.6	7.5
Serum	3	9.0 – 81.5	5.0 – 17.6	13.1

Table 5: **Inter-Assay Precision**

Sample type (spiked)	n	Sample range (mean)	C.V. (%) Range	Mean of C.V. (%)
Urine	3	6.7 – 81.8	5.3 – 10.6	7.3
EDTA-plasma	4	3.2 – 76.6	9.3 – 14.0	11.6
Serum	4	2.6 – 81.4	9.9 – 21.4	14.0

Table 6: **Dilution Linearity/Parallelism**

Samples	Dilution	Observed	Expected	Recovery O/E (%)
Urines (n=3)	1:25	106.95		
	1:50	42.28	53.50	79.0
	1:100	24.09	26.80	89.9
	1:200	12.29	13.40	91.7
	1:400	7.09	6.70	105.8
	1:800	3.97	3.34	119.0
Mean				97.8

Table 7: **Spiking Recovery in Urine**

Samples	Spiked with	Observed	Expected	Recovery O/E (%)
Basic Value: 0.2 ng/ml (n=4)	2	1.8	2.2	80.0
	5	4.9	5.2	94.4
	10	9.5	10.2	92.5
	20	19.8	20.2	97.9
	50	53.7	50.2	106.9
	80	102.3	80.2	127.5
Mean				99.9

Table 8: **Spiking Recovery in Plasma and Serum**

Sample	Spiked with	Observed	Expected	Recovery (O/E) (%)
Plasma Basic Value: 0.8 ng/ml	2	3.2	2.8	113.8
	5	6.6	5.8	112.8
	10	12.4	10.8	114.7
	20	23.6	20.8	113.1
	50	50.1	50.8	98.6
	80	92.4	80.8	114.3
Mean				111.2
Serum Basic Value: 0.8 ng/ml	2	3.2	2.4	132.5
	5	5.7	5.4	105.5
	10	10.3	10.4	98.7
	20	20.6	20.4	101.2
	50	44.7	50.4	88.6
	80	81.0	80.4	100.8
Mean				104.6

Table 9: **Normal Values**

	Urine ng/ml	Serum ng/ml
Total (n)	**75**	**100**
Median	**0.4**	**0.8**
95th Quantile	**0.8**	**1.3**
99th Quantile	**1.1**	**1.8**
Min./Max	**0/1.3**	**0.2/2.3**
Mean	**0.4**	**0.9**
SD	**0.2**	**0.3**
Mean+3SD	**1.1**	**1.8**

Table description: cf. “Results” (page 3), “Performance Characteristics”(page 4).

APPENDIX II
REFERENCES/ LITERATURREFERENZEN/ REFERENCES/ RIFERIMENTI/ REFERENCIAS

1. Andres RY et al.: *Radioimmunoassay for amatoxins by use of a rapid, 125I-tracer-based system.* Clin Chem. **32**, 1751-5 (1986).

2. Andres RY et al.: *(I125)amatoxin and anti-amatoxin for radioimmunoassay prepared by a novel approach: chemical and structural considerations.* Toxicon **25**, 915-22 (1987).

3. Piqueras J: *Hepatotoxic mushroom poisoning: diagnosis and management.* Mycopathologia **105**, 99-110 (1989).

4. Rabe C et al.: *Vorgehen bei Knollenblätterpilzvergift-ungen.* Dtsch med Wschr. **124**, 1073-76 (1999).

5. Piering WF and Bratanow N: *Role of the clinical laboratory in guiding treatment of Amanita virosa mushroom poisoning: a report of two cases.* Clin Chem. **36**, 571-4 (1990).

6. Staack RF and Maurer HH: *New Bühlmann ELISA for determination of Amanitins in urine – Are there false positive results due to interferences with urine matrix, drugs or their metabolits* Toxichem + Krimtech. **68**, (2) 65-70 (2000)

7. Butera R et al.: Diagnostic *accuracy of urinary amanitin in suspected mushroom poisoning: a pilot study*. J Tox Clin Tox (2004) **42,6** 901-12.

AMANITIN ELISA

Precoated Microtiter Plate

wash 2 x

50 µl Calibrators, Controls
or Samples (1:25)
+
50 µl Amanitin-Biotin-Conjugate

incubate 30 minutes at 18-28°C
on a plate rotator

wash 3 x

add 100 µl Enzyme Label

incubate 15 minutes at 18-28°C
on a plate rotator

wash 3 x

add 100 µl TMB Substrate

incubate 15 minutes at 18-28°C
on a plate rotator

add 100 µl Stop Solution

➡ Read absorbance at 450 nm (within 30 minutes)

TIME TO RESULT: 1 HOUR

APPENDIX IV
SYMBOLS/ SYMBOLE/ SYMBOLES/ SIMBOLI/ SIMBOLOS

Symbol	Explanation
	Use By Verwendbar bis Utiliser jusqu'au Utilizzare entro Fecha de caducidad
REF	Catalogue number Bestellnummer Référence du catalogue Numero di catalogo Número de catálogo
LOT	Batch code Chargenbezeichnung Code du lot Codice del lotto Codigo de lote
Σ	Contains sufficient for <n> tests Ausreichend für "n" Ansätze Contenu suffisant pour „n" tests Contenuto sufficiente per „n" saggi Contenudo sufficiente para <n> ensayos

	Consult Instructions for Use- Gebrauchsanweisung beachten Consulter le mode d'emploi Consultare le istruzioni per l'uso Consulte las instrucciones de uso
	Temperature limitation Zulässiger Temperaturbereich Limites de température Limiti di temperatura Limite de temperatura
	Upper limit of temperature Temperaturobergrenze Limite supérieure de température Limite superiore di temperatura Límite superior de temperatura
MP	Microtiterplate Mikrotiter-Platte Microplaque Micropiastra Microplaca
BUF WASH 10X	Wash Bufer Concentrate (10x) Wasch-Puffer Konzentrat (10x) Concentré de tampon de lavage (10x) Tampone di lavaggio concentrato (10x) Tampón de lavado concentrado (x10)

Symbol	Explanation
BUF INC	Incubation Buffer Inkubations-Puffer Tampon d'incubation Tampone d'incubazione Tampón de incubación
REAG BLANK	Blanking Reagent Nullwert-Reagenz Réactif blanc Reagente bianco Reactivo blanco
CAL A - CAL E	Calibrator A -E Kalibrator A -E Calibrateur A -E Calibratore A - E Calibrador A - E
CONTROL L	Control Low Kontrolle tief Contrôle bas Controllo basso Control bajo
CONTROL H	Control High Kontrolle hoch Contrôle élevé Controllo alto Control alto
BC	Biotin Conjugate Biotin-Konjugat Conjugué Biotine Coniugato biotinilato Conjugado de Biotina
EL	Enzyme Label Enzym-Marker Marqueur enzymatique Marcato enzimatico Marcador enzimático
SUBS TMB	TMB Substrate TMB-Substrat Substrat TMB Substrato di TMB Substrato de TMB
SOLN STOP	Stop Solution Stopp-Lösung Solution stop Soluzione stoppante Solución de parada

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