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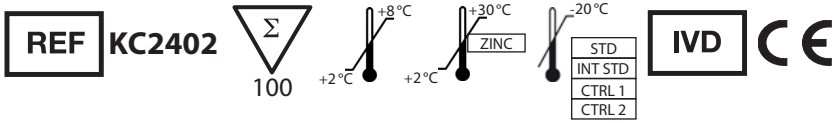
Manual

IDK® Vitamin K_1/K_2 HPLC Kit

For the in vitro determination of Vitamin K_1 and K_2 in plasma and serum

Valid from 2023-06-09



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1. INTENDED USE

The Immundiagnostik AG assay is intended for the quantitative determination of Vitamin K_1 and K_2 in plasma and serum. For *in vitro* diagnostic use only.

2. INTRODUCTION

Vitamin K is metabolized rapidly and is stored in the organism only in small quantities, so that deficiency symptoms become apparent after only a few days. The clinical symptoms are mainly coagulation disorders and are manifested by bleeding in the skin, mucous membranes, muscle tissue and internal organs. In addition, bone metabolism disorders due to deficient modification of osteocalcin have also been-described.

The deficiency symptoms are consequences of a reduced effect of the vitamin as a cofactor of a microsomal, O_2-dependent carboxylase in the carboxylation of specific-glutamic acid residues of the coagulation factors, as well as of osteocalcin. The resulting reduced formation of γ-carboxyglutamic acid residues, which give the coagulation factors the ability to form complexes with Ca^{2+} and membrane phospholipids, from which the active coagulation factors are then released after proteolytic cleavage, ultimately leads to the observed symptoms. Osteocalcin loses the ability to bind to the calcium hydroxyapatite of the bone matrix due to deficient γ-carboxylation. In addition, severe anemia may develop as a result of hypoplastic bone marrow changes in cases of severe vitamin K deficiency.

Indications

- Determination of Vitamin K_1 and K_2 status
- Vitamin K deficiency induced by:
  - obstructive liver disease
  - obstructive icterus
  - malabsorption due to celiac disease,
  - pancreatitis, diarrhea, antibiotic abuse
- Blood clotting disorder
- Bone metabolism disorders
- Haemorrhagic disorders of newborns

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KC2402	MOPHA	Mobile phase (important: do not recirculate)	3 x 1 000 ml
	CAL	Calibrator; lyophilised	4 x
	CTRL1	Control1, lyophilised,	4 x
	CTRL2	Control2, lyophilised,	4 x
KC2400	PREC	Precipitating reagent; ready-to-use	220 ml
	ELUSOL	Elution solution; ready-to-use	420 ml
	STD	Isopropanolic standard; ready-to-use	10 ml
	INTSTD	Internal Standard; ready-to-use	1 ml
	ZINC	Zinc	12 g
	ZUB	Accessories for post-column reduction-reactor	1 x
K 0005.15	RECSOL	Reconstitution solution; ready-to-use	1 x 15 ml

The HPLC column (KC2400RP), the post-column reduction-reactor (KC2400NR) as well as the MERCK column holder "Manu-Kart" (KC2400RK), can be ordered separately from Immundiagnostik. To extend the lifetime of your HPLC column, pre-columns (KC2400VS) should ideally be used. These and also the corresponding pre-column holders (KC2400VH) can also be ordered from Immundiagnostik. In addition to the complete kit, all components can be ordered separately. Please ask for our single component price list.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Centrifuge
- Vortex
- Borosilicate glass tubes for centrifugation, V-bottom (10 ml)
- HPLC with Fluorescence-detector
- Post-column reduction-reactor (KC2400 NR)
- Reversed phase C18-column , Superspher RP 18.4 µm, 125 x 4.6 mm (KC2400 RP)
- Various precision pipettes
- Device for concentrationg the sample (e.g. vacuum centrifuge or evaporator)

5. STORAGE AND PREPARATION OF REAGENTS

Preparation of the calibrator and controls

All reagents are ready-to-use in dissolved form, except for the calibrator (**CAL**) and controls (**CTRL1** and **CTRL2**). Reconstitute calibrator **(CAL)** in x ml (x = see the enclosed product specification for the volume needed) reconstitution solution (**RECSOL**). One vial is for a single use only; discard the material, which has not been used. The content of vitamin K_1 and K_2 varies slightly from batch to batch, please refer to the enclosed product specification sheet for the exact amount.

Reconstitute control 1 and 2 (**CTRL1** and **CTRL2**) in x ml (x = see the enclosed product specification for the volume needed) reconstitution solution (**RECSOL**).

Test reagents are stable at room temperature, calibrator (**CAL**), internal standard (**INTSTD**), controls (**CTRL1** and **CTRL2**) and standard (**STD**) at -20 °C, up to the date of expiry stated on the label. The other components, with the exception of zinc and the filters/seals for the reactor, must be stored at 2–8 °C.

Preparation of the mobile phase

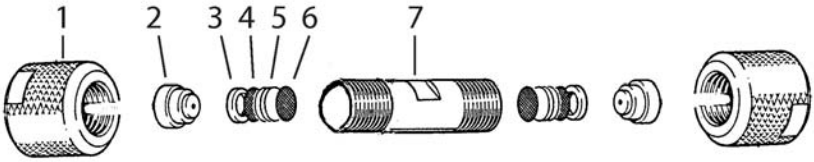
If the used HPLC system does not have an in-line degasser, the mobile phase is degassed in an ultrasonic bath for 10–15 min. Since the mobile phase is an organic solvent, care must be taken to seal the vessels for injection into the HPLC system with a septum in order to avoid evaporation and to ensure the consistent quality of the measurement.

Preparation of the post-column reduction reactor

Vitamin K_1 is reduced in the post-column reduction reactor and thus made accessible for detection in the fluorescence detector. In order to obtain a constant detector signal quality, it is necessary to freshly fill the post-column reduction reactor before each series of samples, as the surface of the zinc particles is oxidised after 12 h of running time. The filling process is very simple and takes only about 10 min.

For assembling the column see the following figure.

1. Cap nut
2. Stainless steel inlet
3. PTFE seal
4. Stainless steel sieve (grey)
5. Glass fiber sieve (3 pieces, white)
6. Stainless steel sieve (grey)
7. Column tube



1. Close one side of the column according to the figure above.
2. Fill in the zinc-particles with a funnel or spatula while knocking the column slightly on the table, so that the packing will not show any cavities.
3. Close the upper side of the column.

The post-column reduction reactor should be mounted in the HPLC-system as described by the following picture:

Note: The performance of the reactor must be checked (see chapter 7 "Assay procedure" - *Test procedure*).

6. STORAGE AND PREPARATION OF SAMPLES

Plasma and serum can both be used for analysis. The samples must be centrifuged and frozen immediately.

The samples are stable at -20° C and can be stored for a longer time.

7. ASSAY PROCEDURE

Principle of the test

The first step in the determination of Vitamin K_1 and K_2 is the sample preparation. A precipitation reagent is added to the plasma or serum samples for separation of higher molecular weight substances. Elution solution is then added to the sample to transfer the vitamins K_1 and K_2 to the organic phase. After resuspension the sample is measured in an isocratic HPLC-system. A post-column reduction reactor reduces vitamin K_1 and K_2 and enables the measurement of these vitamins with a fluorescence detector. An internal standard is added before the precipitation to ensure the high quality of the measurement.

Summary:

The application of vitamin K_1 and K_2 for HPLC makes it possible to determine the vitamin in an easy, fast and precise way. The kit includes all reagents in ready-to-use form for preparation and separation of the samples with exception of the columns. As with many other parameters the advantage of HPLC measurements is the simultaneous handling of many analytes in a single test. The „complete HPLC-system" enables even laboratories without experience in „high performance liquid chromatography" to use this technique for clinical chemical routines quickly and precisely. Mostly a one- point calibration is sufficient for calibrating the test system - unlike immunoassays with up to 6 calibrators per test. It is possible to automate the sample application and calculation of the results so that even a higher number of samples can be handled nearly without the need of control. (With short test runs the one-point calibration is much more economic than 6-point calibration for immunoassays).

Test procedure

1.	Pipette 500 µl sample, calibrator (**CAL**) and control 1 and 2 (**CTRL1** and **2**) in borosilicate glass tubes, add 10 µl internal standard (**INTSTD**) and mix
2.	Add 2 ml precipitation reagent (**PREC**), and vortex
3.	Add 4 ml elution solution **(ELUSOL)**, vortex and centrifuge for 5 min at 3 000 *g*
4.	Pipette 3.2 ml of the organic phase into a new borosilicate glass tube
5.	Evaporate eluate until dryness for 2 h or over night in vacuum centrifuge) or for 20 min in an evaporator
6.	Connect the post-column reduction reactor in the HPLC-system, as described above and wait for equilibration (20 min)
7.	Check the performance of the reactor by the injection of 100 µl of isopropanolic standard (**STD**) and determine the signal to noise ratio, which should be > 25
8.	Add 200 µl mobile phase (**MOPHA**) to the dried sample, vortex and inject 100 µl supernatant in the HPLC-system

Chromatographic conditions

For determination of the retention time, inject **100 µl** of the **isopropanolic standard** (**STD**) into the HPLC-system.

Column material :	Superspher 100 RP 18; 4 µm
Column dimension:	125 mm x 4.6 mm
Temperature:	32 °C
Fluorescence detector:	ex.: 246 nm em.: 430 nm
Injection volume:	100 µl
Running time:	approx. 16.5 min
Standby flow:	0.8 ml/min
Flow gradient:	

Time [min]	Flow rate [ml/min]
0	0.8
6	0.8
13	2.2
15.5	2.2
16.5	0.8

8. TREATMENT OF THE COLUMN

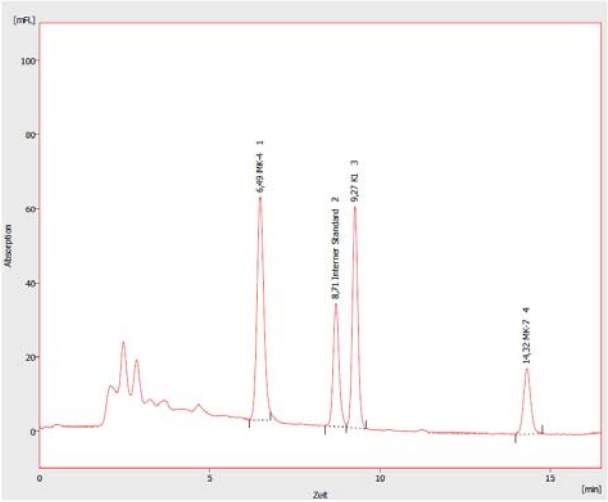
Immundiagnostik AG recommends to use a guard-column to enlarge lifetime of the column.

After analysis the column should be flushed with 30 ml aqua bidest (1.0 ml/min) and stored in 50 % methanol in aqua bidest (approx. 30 ml, flow 0.5 ml/min). Before use, the system should be equilibrated with ca. 30 ml mobile phase (**MOPHA**).

Important: Do not re-circulate the mobile phase (MOPHA) in this test system.

9. RESULTS

Typical chromatogram



Calculation

$$\frac{\text{Peak height sample x Concentration of the calibrator x F}}{\text{Peak height internal standard in the sample}} = \text{Concentration sample}$$

$$F = \frac{\text{Peak height internal standard in the calibrator}}{\text{Peak height calibrator}}$$

10. LIMITATIONS

We recommend not to measure hemolytic and lipaemic patient samples.

11. QUALITY CONTROL

Reference value

On the basis of an internal laboratory study with samples from apparently healthy persons (n = 100), the following values were determined:

Vitamin K_1 :	0.1–2.66 ng/ml
MK-4:	0.1–0.42 ng/ml
MK-7:	0.13–1.47 ng/ml

We recommend each laboratory to establish its own reference range as they are strongly dependent on the selection of the patient collective. The reference range for vitamin K_1 and K_2 is given for guidance only and may differ from other published data. The values mentioned above are only for orientation and can deviate from other published data.

Controls

Control samples should be analysed with each run of calibrators and patient samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

12. PERFORMANCE CHARACTERISTICS

Intra-Assay

The repeatability was assessed with 3 control samples under **constant** parameters (same operator, measurement system, day and kit lot).

Vitamin K_1 (n = 9)

Sample	Mean value [ng/ml]	CV [%]
1	1.051	2.8
2	0.556	3.5
3	1.530	9.0

MK-4 (n = 9)

Sample	Mean value [ng/ml]	CV [%]
1	0.904	6.6
2	0.583	10.8
3	1.222	15.7

MK-7 (n = 9)

Sample	Mean value [ng/ml]	CV [%]
1	0.899	8.0
2	0.596	11.5
3	1.185	7.9

Inter-Assay

The reproducibility was assessed with 3 control samples under **varying** parameters (different operators, measurement systems, days and kit lots).

Vitamin K_1 (n = 27)

Sample	Mean value [ng/ml]	CV [%]
1	1.051	3.1
2	0.543	6.0
3	1.450	9.0

MK-4 (n = 27)

Sample	Mean value [ng/ml]	CV [%]
1	0.886	5.6
2	0.560	5.4
3	1.193	9.8

MK-7 (n = 27)

Sample	Mean value [ng/ml]	CV [%]
1	0.907	9.0
2	0.623	9.2
3	1.207	7.6

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, serum samples were spiked with known amounts of vitamin K_1, MK-4 and MK-7.

The recovery for Vitamin K_1 was found between 98 and 101 %.

Sample	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
1.05	0	1.05	1.07	101
	0.5	1.55	1.51	98
	1.0	2.05	2.06	101
	1.5	2.55	2.56	101
	2.0	3.05	3.05	100
	2.5	3.55	3.52	99
	3.0	4.05	4.06	100

The recovery for MK-4 was found between 95 and 104 %.

Sample	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
1.04	0	1.04	1.04	100
	0.5	1.54	1.55	101
	1.0	2.04	2.08	102
	1.5	2.54	2.49	98
	2.0	3.04	2.89	95
	2.5	3.54	3.68	104
	3.0	4.04	4.02	100

The recovery for Vitamin MK-7 was found between 95 and 102 %.

Sample	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
1.52	0	1.52	1.15	100
	0.39	1.91	1.95	102
	0.88	2.40	2.28	95
	1.44	2.96	3.04	103
	2.14	3.66	3.63	99
	3.36	4.88	4.88	100

Lower detection limit

The limit of detection (LoD) is definded as 3 times the background noise. It is calculated by the formula below:

$$\frac{(3 \text{ x peak height background noise}) \text{ x concentration calibrator [ng/ml]}}{\text{Peak height calibrator}} = \text{LoD [ng/ml]}$$

LoD Vitamin K_1: 0.000103 ng/ml

LoD MK-4: 0.000836 ng/ml

LoD MK-7: 0.000491 ng/ml

Upper detection limit & linearity

Both native and spiked samples were diluted with high vitamin K1, MK-4 and MK-7 concentrations, respectively. A linear range of 0.05–6.25 ng/ml was detected. The nonlinearity was evaluated as follows:

Vitamin K_1: -2.2 to 2.7 %

MK-4: -6.4 to 0.7 %

MK-7: -3.1 to 2.8 %

Analytical specificity

No interferences from other blood components were found.

13. DISPOSAL

Mobile phase (**MOPHA**), isopropanolic standard (**STD**), internal standard (**INTSTD**), elution solution (**ELUSOL**) and precipitating reagent (**PREC**) must be disposed of as non-halogenated solvent. Please refer to the appropriate national guidelines.

14. TROUBLESHOOTING

Problem	Possible reasons	Solution
No signal	No or defect connection to evaluation system.	Check signal cord and connection.
	Detector lamp is too old	Change lamp
No peaks	Injector is congested	Check Injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol

Problem	Possible reasons	Solution
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump conveys inaccurately	Check pump, degas the system
	System is not in steady state yet	Rinse system with mobile phase for 15 min
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
	System is not in steady state yet	Rinse system with mobile phase for 15 min
	Pump conveys inaccurately	Check pump, degas the system
Baseline is not smooth	Pump conveys inaccurately	Check pump, degas the system
	Detector flow cell is dirty	Clean flow cell

15. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- The supplied reagents contain organic solvents. Although diluted, they still should be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Do not breath vapour and avoid inhalation.
- As a precaution, it is recommended that the human material used is always considered potentially infectious.

16. GENERAL NOTES ON THE TEST

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- All reagents in the kit package are for *in vitro* diagnostic use only.
- Do not use reagents beyond the expiration date stated on the kit label.
- Do not interchange different lot numbers of any kit component within the same assay.
- Do not mix plugs and caps from different reagents.
- Follow the guidelines for medical laboratories.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which has not been consulted with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held responsible for any damage resulting from incorrect use.
- Warranty claims and complaints regarding deficiencies must be lodged within 14 days after receipt of the product. The product should be sent to Immundiagnostik AG along with a written complaint.
- Analyze controls with each run.
- Always perform the assay according to the enclosed manual.
- Serious incidents are to be reported to Immundiagnostik AG and the national regulatory authorities.

17. REFERENCES

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3. Hodges S.J., Akesson K., Vergnaud P., Obrant K., Delmas P.D. (1993). Circulating levels of vitamin K1 and K2 decreased in elderly women with hip fracture. J Bone Miner Res 8:1241-45
4. Haroon Y, Bacon DS, Sadowski JA. (1986) Liquid-chromatographic determination of vitamin K1 in plasma, with fluorometric detection. Clin Chem. Oct;32(10):1925-9.

Symbols:

	Temperature limitation		Catalogue number
	In Vitro Diagnostic Medical Device		To be used with
	Manufacturer		Contains sufficient for <n> tests
	Lot number		Use by
	Contains plasma derivatives or human blood		Consult instructions for use
	Consult specification data sheet		Do not re-use
	Unique Device Identification		Contains material of animal origin
	Medicinal substance		Contains material of human origin