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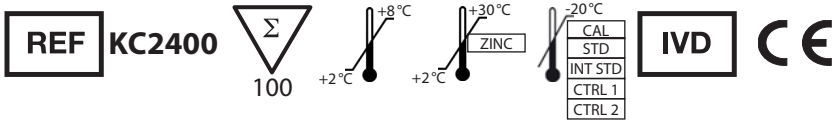
**Manual**

# Vitamin $K_1$ HPLC Kit

***For the in vitro determination of Vitamin $K_1$ in plasma and serum***

Valid from 2022-09-06

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# 1. INTENDED USE

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

# 2. INTRODUCTION

Vitamin $K_1$ is a derivative of 2-methyl-1,4-naphtochinone and is found in green plants. Vitamin $K_1$ is insoluble in water and readily soluble in ether, n-hexane and chloroform.

It`s an essential co-factor in the posttranslational carboxylation reaction of glutamic acid residues (GLU) to γ-carboxyglutamic acid residues (GLA) in a number of blood clotting factors and also in some other proteins e.g. osteocalcin. The adjacent carboxyl groups of the GLA-residues provide the Vitamin K dependent proteins with characteristic calcium- and phospholipid-binding properties that are essential for their activation and function. A decrease in Vitamin $K_1$ is reported in osteoporotic patients. Other clinical symptoms of Vitamin $K_1$ deficiency are clotting disorders, which manifest themselves as bleeding in the skin, in the mucous membranes, in muscles and in internal organs. Symptoms of deficiencies normally appear within few days. Vitamin $K_1$ is rapidly metabolised and only minor amounts are stored in the organism.

**Indications**

- Determination of Vitamin $K_1$ 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 |
|---|---|---|---|
| KC2400 | MOPHA | Mobile phase (important: do not recirculate) | 2 x 1 000 ml |
| | CAL | Calibrator; (lyophilised, see specification data sheet for concentration) | 4 x |
| | STD | Isopropanolic standard; ready-to-use | 10 ml |
| | INTSTD | Internal Standard; ready-to-use | 1 ml |
| | PREC | Precipitating reagent; ready-to-use | 500 ml |
| | ELUSOL | Elution solution; ready-to-use | 4 x 25 ml |
| | ZINC | Zinc | 20 g |
| | ZUB | Accessories for post-column reduction-reactor | 5 x |
| | CTRL1 | Control1 (lyophilised, see specification data sheet for concentration) | 4 x |
| | CTRL2 | Control2 (lyophilised, see specification data sheet for concentration) | 4 x |
| | WASHSOL | Wash solution; ready-to-use | 300 ml |
| K 0005.15 | RECSOL | Reconstitution solution; ready-to-use | 2 x 15 ml |

The HPLC column (KC2400RP), the solid phase extraction cartridges (KC2400CK), 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

- Borosilicate glass tubes for centrifugation, V-bottom (10 ml)
- SPE cartridges, C18 (KC2400 CK)
- Evaporator
- Centrifuge
- Various precision pipettes
- HPLC with Fluorescence-detector
- Reversed phase C18-column – Superspher RP 18,4 µm, 125 x 4,6 mm (KC2400 RP)
- Vortex mixer
- Post-column reduction-reactor (KC2400 NR)

## 5. STORAGE AND PREPARATION OF REAGENTS

**Preparation of the calibrator and controls**

Reconstitute calibrator **(CAL)** in reconstitution solution (**RECSOL**). The volume is given on the label. One vial is for a single use only; discard the material, which has not been used. The content of Vitamin $K_1$ might have minor changes from lot to lot.

Reconstitute control 1 and 2 (**CTRL1** and **CTRL2**) in 1.1 ml reconstitution solution (**RECSOL**).

All other reagents are delivered solved and ready-to-use except calibrator (CAL) and controls (CTRL 1 and CTRL 2).

Test reagents are stable at room temperature, calibrator (CAL), internal standard (INT STD), controls (CTRL 1 and CTRL 2) and standard (STD) at -20 °C, up to the date of expiry stated on the label.

**Preparation of the mobile phase**

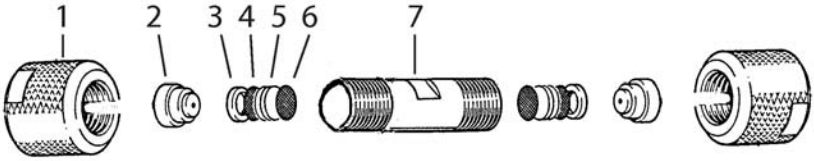
If the used HPLC system does not have an in-line degasser, the mobile phase is degassed in an ultrasonic bath for 10 - 15 min. Since the mobile phase is an organic solvent, care must be taken to seal the vessels for injection into the HPLC system with a septum in order to avoid evaporation and to ensure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

**Preparation of the post-column reduction reactor**

For getting well detectable peaks it is necessary to exchange the zinc particles in the post-column reduction reactor each day. The zinc particles are used up after 12 h by oxidation. Filling the column is very easy and takes just 10 min of time.

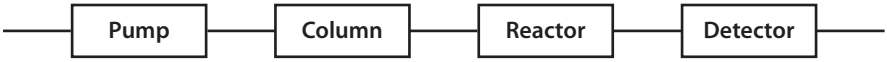
For assembling the column see the following figure.

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 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 cooled immediately.

The samples are stable at 2–8° C for 1 week. For longer storage samples should be frozen at -20 °C.

# 7. ASSAY PROCEDURE

## *Principle of the test*

After a precipitation, serum or plasma samples are prewashed during a solid phase extraction on SPE-cartridges. The eluate contains Vitamin $K_1$ and is then evaporated. After resuspension the sample is measured in an isocratic HPLC-system. A post-column reduction reactor reduces Vitamin $K_1$ and enables the measurement of Vitamin $K_1$ 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 $K_1$ for HPLC makes it possible to determine the Vitamin in an easy, fast and precise way. The kits 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 are 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 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 immuno assays).

## *Test procedure*

| | |
|---|---|
| 1. | Pipette 500 µl sample, calibrator (**CAL**) or control 1 and 2 (**CTRL 1** and **2**) in borosilicate glass tubes, add 10 µl internal standard (**INT STD**) and mix |
| 2. | Add 2 ml precipitation reagent (**PREC**), vortex for 30 s, incubate for 15 min at room temperature and centrifuge for 10 min at 3 000 *g* |
| 3. | Solid phase extraction in the centrifuge:<br>• Place the SPE cartridge into a suitable tube (e.g. conical centrifuge tubes 15 ml)<br>• **Conditioning:** 3 ml **PREC** (100 *g*, 2 min)<br>• **Sample application:** Add supernatant of the centrifugation on the SPE cartridge (100 *g*, 2 min)<br>• **Wash:** 3 ml wash solution (**WASHSOL**) (100 *g*, 5 min), followed by 5 min at 3 000 *g* to dry the bed to the cartridge<br>• **Elution:** 1 ml elution solution (**ELUSOL**) (100 *g*, 4 min), collect eluate in borosilicate glass tubes |
| 4. | Evaporate eluate at 39 °C until dryness (e.g. in vacuum centrifuge) |
| 5. | The dried sample is stable for 8 days at 4–8 °C |
| 6. | Connect the post-column reduction reactor in the HPLC-system, as described above and wait for equilibration (30–45 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 for 5 min, centrifuge for 2 min at 3 000 *g* 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) in the HPLC-system.

| | |
|---|---|
| Column material : | Superspher 100 RP 18; 4 µm |
| Column dimension: | 125 mm x 4.6 mm |
| Flow rate: | 0.9 ml/min |
| Fluorescence detector: | ex.: 248 nm<br>em.: 418 nm |
| Temperature: | 30 °C |
| Running time: | approx. 10 min |

## 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.**

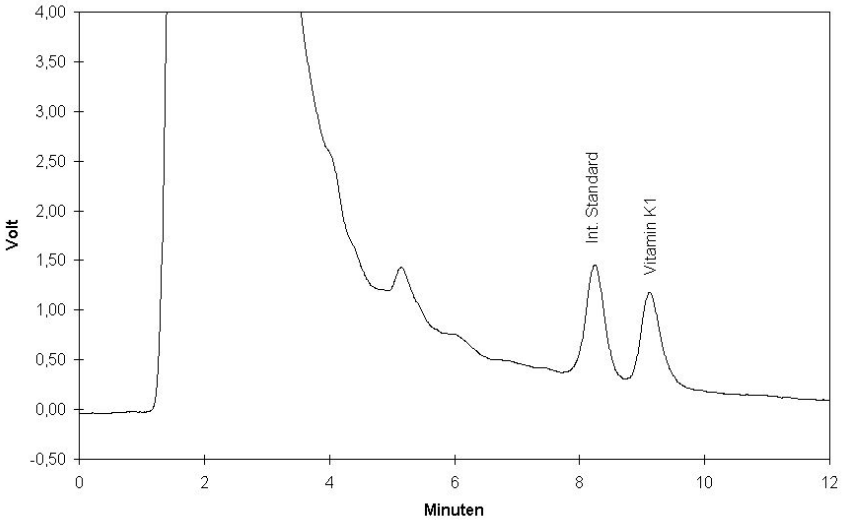
## 9. RESULTS

### *Calculation*

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

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

*Typical chromatogram*




## 10. LIMITATIONS

We recommend not to measure hemolytic and lipaemic patient samples.

## 11. QUALITY CONTROL

### *Reference value*

**0.22–2.28 ng/ml (Mean value = 1.29 ng/ml) (n = 19)**

We recommend each laboratory to establish its own reference range. The values mentioned above are only for orientation and can deviate from other published data.

### *Controls*

Control samples or serum pools 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*

**n = 10**

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

| Mean value [nmol/l] | CV [%] |
|---|---|
| 2.28 | 4.1 |

### *Inter-Assay*

**n = 18**

The reproducibility was assessed with 2 control samples under **varying** parameters (different operators, measurement systems, days and kit lots).

| Sample | Mean value [nmol/l] | CV [%] |
|---|---|---|
| 1 | 0.739 | 8.3 |
| 2 | 2.501 | 8.4 |

### *Accuracy – Trueness*

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, two serum samples were spiked with known amounts of Vitamin $K_1$.

The recovery for Vitamin $K_1$ was found between 94.4 and 103.0 %.

| Sample | Spike [ng/ml] | Expected [ng/ml] | Obtained [ng/ml] | Recovery [%] |
|---|---|---|---|---|
| 1.11 | 2 | 3.11 | 3.14 | 100.96 |
| | 20 | 21.11 | 21.75 | 103.03 |
| | 50 | 51.11 | 52.58 | 102.88 |
| | 100 | 101.11 | 95.48 | 94.43 |
| 1.99 | 2.75 | 4.74 | 4.62 | 97.47 |

### *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 $K_1$: 0.094 ng/ml

### *Upper detection limit & linearity*

The upper limit of detection states up to which concentration a method results in a linear signal. Between the range from 0.26 to 49.15 ng/ml the non-linearity was found below 20 %.

| Expected [ng/ml] | Obtained [ng/ml] | Recovery [%] |
|---|---|---|
| 0.22 | 0.26 | 116.2 |
| 0.34 | 0.36 | 106.9 |
| 0.67 | 0.66 | 99.2 |
| 3.81 | 3.62 | 94.9 |
| 25.46 | 25.07 | 98.5 |
| 50.63 | 49.15 | 97.1 |

It should be noted that the detection limits depend on the instrument as well as on the application.

### *Analytical specificity*

There were found no interferences to other blood components.

## 13. DISPOSAL

Mobile phase (**MOPHA**), **i**sopropanolic standard (**STD**), internal standard (**INTSTD**), elution solution (**ELUSOL**), wash solution (**WASHSOL**) and precipitating reagent (**PREC**) must be disposed 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 altered | 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 |
| Broad peaks, tailing | Precolumn / column exhausted | Use new precolumn / column |
| Variable retention times | Drift in temperature | Use a column oven |
| | Pump delivers imprecise | 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 delivers imprecise | Check pump, degas the system |
| Baseline is not smooth | Pump delivers imprecise | 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.
- The guidelines for medical laboratories should be followed.
- Control samples should be analysed with each run.
- Reagents should not be used beyond the expiration date.
- Do not mix plugs and caps from different reagents.
- Do not interchange different lot numbers of any kit component within the same assay.
- The assay should always be performed according to the enclosed manual.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated 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 logged within 14 days after receipt of the product. The product should be send to Immundiagnostik AG along with a written complaint.
- Serious incidents are to be reported to Immundiagnostik AG and the national regulatory authorities.

## 17. REFERENCES

1. Saupe J. (1992). Vitamin K und Indikationen zur Bestimmung im Plasma. Klin. Lab. 38: 51-54
2. Szulc P., Arlot M., Chapuy M.C., Duboef F., Meunier P.J., Delmas P.D. (1994). Serum undercarboxylated osteocalcin correlates with hip bone mineral density in elderly woman. J Bone Miner Res 9:1591-95
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.

***Used symbols:***

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