

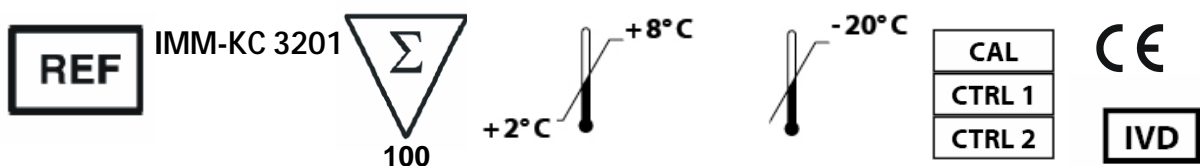


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HPLC Kit Hydroxy-Pyridinium-Crosslinks

For the determination of Hydroxy-Pyridinium-Crosslinks in urine

Valid from 15.11.2006



1. INTENDED USE

The *Immundiagnostik* Assay is intended for the quantitative determination of Pyridinium crosslinks in urine. This Assay is designed for *in vitro* diagnostic use only.

2. SUMMARY AND EXPLANATION OF THE TEST

The hydroxypyridinium compounds pyridinoline (PYD) and deoxypyridinoline (DPD) are specific constituents of mature skeletal collagens. They are released into the circulation and excreted in the urine. Their measurement in urine is a sensitive index of the extent of ongoing bone resorption. The quantification of collagen Crosslinks in urine is achieved by chromatographic techniques.

Clinical applications of hydroxypyridinium markers include numerous metabolic bone disorders such as osteoporosis, primary hyperparathyroidism, Paget's disease of bone, and metastatic bone disease. Urinary pyridinium Crosslinks of collagen also show great promise as markers of therapeutic efficacy in bone disorders associated with accelerated bone resorption.

3. PRINCIPLE OF THE TEST

The first step in determining the hydroxy pyridinium crosslinks is a hydrolysis of the sample followed by a solid phase extraction on cellulose cartridges. 100 µl of the eluate are injected into the HPLC system.

The separation via HPLC follows an isocratic method at 30°C using a reversed phase column. One run lasts 20 minutes. The chromatograms are recorded by a fluorescence detector. The quantification is performed with the delivered urine calibrator; the concentration is calculated via integration of the peak areas by the external standard method.

Summary

Besides many other parameters, the advantage of HPLC method lies in the simultaneous handling of many analytes in a single test. The HPLC system enables even laboratories without experience in high performance liquid chromatography to use this technique for clinical routine determination in a quick and precise manner. Unlike immuno assays with up to six calibrators per test, a one-point calibration is mostly sufficient to calibrate the test system. It is possible to automate the sample application and calculation of the results so that even higher sample numbers of can be handled nearly without control.

4. MATERIAL SUPPLIED

Cat. No	Content	Kit Components	Quantity
KC 3201LM	MOPHA	Mobile phase	1000 ml
KC 3201KA	CAL	Calibrator, lyophilized	3 vials
KC 3201HY	HYDREA	Hydrolysis reagent	100 ml
KC 3201CS	CELLULOSE	Cellulose	3 x 4 g
KC 3201RB	REABUF	Reaction buffer	
		Reagent I	3 x 22 ml
		Reagent II	3 x 88 ml
KC 3201W1	WASHSOL1	Washing solution I	
		Reagent I	3 x 280 ml
		Reagent II	3 x 560 ml
KC 3201W2	WASHSOL2	Washing solution II	50 ml
KC 3201EL	ELUSOL	Elution solution	50 ml
KC 3201KO	CTRL1	Control 1 and 2; 2.2 ml lyophilized	2 x 3 vials
	CTRL2		

HPLC column (KC 3201RP) as well as individual components can be ordered separately from Immundiagnostik. Please ask for the price list of the individual components.

5. MATERIAL REQUIRED BUT NOT SUPPLIED

- Glass tubes for hydrolysis (dark, heat-sealable)
- Vacuum chamber for solid phase extraction
- HPLC-pump (isocratic) with an Fluorescence-detector
- Superspher RP18-column, 4 µm, 125 x 4 mm
- Cryostat or heating block
- Chromatographic-cartridges with discs or glass wool

6. PREPARATION AND STORAGE OF REAGENTS

Preparation of the Calibrator and controls

- Reconstitute the **calibrator** (CAL; urine spiked with defined amounts of pyridinoline and desoxypyridinoline) in 5.5 ml aqua bidest. Divide the **calibrator** (CAL) in several portions and store at –20 °C. Avoid several times thawing and freezing. The contents of Pyridinium- and Deoxypyridinium-Crosslinks might have minor changes from lot to lot. The exact content is given on the label.
- Reconstitute the **controls** (CTRL1, CTRL2) in 2.2 ml aqua bidest.
- All other test reagents are stable at 20-25 °C, up to the date of expiry stated on the label.

Preparation of Reaction buffer

- Add the total content of Reaction buffer (REABUF) reagent I to the bottle of Reaction buffer (REABUF) reagent II and incubate the mixture 12 hours at room temperature. The freshly prepared Reaction buffer is stable for 3 month.

Preparation of Washing solution I

- Add the total content of Washing solution I (WASHSOL1) reagent I to the bottle of Washing solution (WASHSOL1) reagent II and incubate the mixture 12 hours at room temperature. The freshly prepared Washing solution I is stable for 3 month.

Preparation of the Cellulose Slurry

- Add 40 ml of Washing solution I to 4 g Cellulose (CELLULOSE) and mix well. The freshly prepared Cellulose-Slurry is stable for 1 month.

7. PRECAUTIONS

- For *in vitro* diagnostic use only.
- This product contains human source material which was tested and found to be non-reactive to HBsAg, anti-HIV-1/2, and anti-HCV. Since no method can offer complete assurance that hepatitis B virus, HIV-1/2, HVC or other infectious agents are absent, these reagents should be handled as if potentially infectious.
- The hydrolysis reagent (HYDREA), reaction buffer (REABUF) and washing solution I (WASHSOL1) contain acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapors and avoid inhalation.
- Reagents should not be used beyond the expiration date shown on kit label.

8. SPECIMEN COLLECTION AND PREPARATION

Urine is suited for this test system. A two hour urine sampling between 7⁰⁰ and 10⁰⁰ pm correlates well with a 24 h collection. Samples are stable for 24 h at room temperature and up to one week at 2-8 °C. Longer storage should be at -20 °C.

9. ASSAY PROCEDURE

Procedural notes

- The quality control guidelines should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the test results. Immundiagnostik can therefore not be held reliable for any damage resulting from this.
- The assay should always be performed due to the manual which is given in the kit.

Sample and standard preparation

Patient urine, calibrator (CAL) or controls (CTRL1, CTRL2) should be diluted 1+1 (1 ml + 1 ml) with Hydrolysis reagent (**Caution:** Hydrochloride acid concentrate) and hydrolysed for **4 hours** at **120 °C**.

Fill up column with 1 ml Cellulose-Slurry. Mix the Slurry carefully, so that the column could be packed homogeneously.

Soak 5 ml Washing solution I through the column by vacuum.

Add **0.5 ml** Washing solution I to **0.5 ml** hydrolyzed cold urine and **3 ml** Reaction buffer.

Shake very well, pipette in the column and let it soak through by vacuum.

Rinse the column with **2 x 8 ml** Washing solution I under vacuum.

Dry the column by vacuum for 10 min.

Rinse the column with **0.5 ml** Washing solution II (WASHSOL2) under vacuum.

Dry the column by vacuum for 10 min.

Elute the sample with **0.5 ml** Elution solution (ELUSOL) under vacuum.

Centrifuge the sample and inject **100 µl** into the HPLC system.

Chromatographic conditions

Column material:	Merck Supersher, 4 µm	
Column dimension:	125 mm x 4 mm	
Flow rate:	1-1.5 ml/min	
Temperature:	30 °C	
Fluorescence Detection:	Excitation	290 nm
	Emission	400 nm
Injection volume:	100 µl	
Running time:	20 min	

10. TREATMENT OF THE COLUMN

After analysis the column should be flushed with 30 ml aqua bidest (1 ml/min) and stored in 50% methanol in aqua bidest (approx. 30 ml, flow 0.7 ml/min). Before use, the system should be equilibrated with ca. 30 ml eluent.

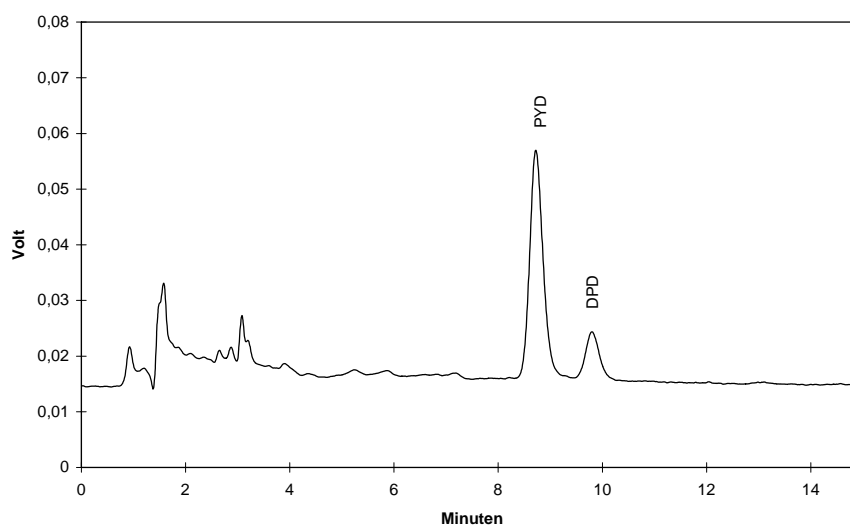
11. RESULTS

Calculation

$$\text{Conc. patient sample (pmol/ml)} = \frac{\text{peakheight patient} * \text{conc. calibrator (pmol/ml)}}{\text{peakheight calibrator}}$$

For calculation better use the peak area instead of the peak height, if possible.

Typical chromatogram



12. LIMITATIONS

Don't use serum, plasma or whole blood.

13. QUALITY CONTROL

Expected values

38.4 ± 18.4 nmol PYD/mmol Creatinin

10.3 ± 5.3 nmol DPD/mmol Creatinin

We recommend that each laboratory should develop it's own normal range. The values mentioned above are only for orientation and can deviate from other published data.

Controls

Control samples or urine pools should be analyzed 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. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the patient sample may not be valid.

14. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay CV:	PYD	4.5 % (958 pmol/ml)	[n = 10]
	DPD	6.2 % (238 pmol/ml)	[n = 10]
Inter-Assay CV:	PYD	8.9 % (1051 pmol/ml)	[n = 10]
	PYD	7.9 % (177 pmol/ml)	[n = 10]
	DPD	8.6 % (254 pmol/ml)	[n = 10]
	DPD	8.2 % (45 pmol/ml)	[n = 10]

Linearity

PYD up to 2500 pmol/ml

DPD up to 1000 pmol/ml

Detection limit

PYD 7.3 pmol/ml

PYD 7.3 pmol/ml

Recovery

PYD 97.4 %

DPD 94.4 %

15. DISPOSAL

The mobile phase (MOPHA), washing solution I and II (WASHSOL1, WASHSOL2), reaction buffer (REABUF) and elution solution (ELUSOL) must be disposed as non-halogenated solvent. The hydrolysis solution (HYDREA) can be neutralized with NaOH. If the pH value is neutral, it can be disposed as salt solution.

Important: Reaction will produce extreme heat, be careful!

Please refer to the appropriate national guidelines.

16. TROUBLESHOOTING

Problem	Possible reason	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 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 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

17. REFERENCES

Seibel et al. (1992). TEM, Vol. 3, No. 7, 263-270.

Robbins et al. (1991). Europ. J. of Clin. Invest., Vol. 21, 310-315.

Seibel et al. (1992). J. of Clin. Endocrin. and Metabol., Vol. 74, No. 3, 481-486.

18. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- The test components contain organic solvents. Contact with skin or mucous membranes has to be avoided.
- All reagents in the test package are to be used for research only.
- The reagents should not be used after the date of expiry stated on the label.
- Single components with different lot numbers should not be mixed or exchanged.
- The guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature and pipetting volumes of the different components are defined by the producer. Any variations of the test procedure, that are not coordinated with the producer, may influence the results of the test. Immundiagnostik AG can therefore not be held reliable for any damage resulting from this.