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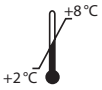
Manual

*IDK*monitor® vedolizumab drug level LC-MS/MS Kit

For the in vitro determination of free vedolizumab concentration (e. g. Entyvio®) in EDTA plasma and serum

Valid from 2016-04-13

REF **KM9600**



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1. INTENDED USE

This LC-MS/MS application is for the quantitative determination of the free human $\alpha_4\beta_7$ integrin therapeutic antibody vedolizumab (e. g. Entyvio®) in EDTA plasma and serum. For research use only. Not for use in diagnostic procedures.

2. INTRODUCTION

Patients with moderately to severely active disease, who have had an inadequate response to conventional therapy or to anti-tumor necrosis factor alpha (TNFα) agents, can also be treated with vedolizumab. The humanized monoclonal therapeutic antibody vedolizumab binds $\alpha_4\beta_7$ integrin on activated lymphocytes and stops them from migrating into the intestinal mucosa. Thus, vedolizumab suppresses the inflammatory response via a different mechanism than anti-TNFα agents and specifically targets inflammation in the gastrointestinal tract.

The clinical efficacy of vedolizumab therapy correlates with the trough level, that is the drug level just before the next application of vedolizumab. Several factors influence the trough level, among them dosage and frequency of vedolizumab infusion, disease activity, individual pharmacokinetics and immune reaction (formation of anti-drug antibodies, ADA). The *IDK*monitor®LC-MS/MS application for the determination of free vedolizumab (e.g. Entyvio®) measures reliably the effective drug concentration, giving the treating physician an opportunity to monitor and optimize the therapy early on.

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
KM9600LA	MOPHA A	Mobile phase A	250 ml
KM9600LB	MOPHA B	Mobile phase B	250 ml
KM9600KA	CAL 1 CAL 2 CAL 3	Calibrator 1 to 3, lyophilised*	5 x 3 vials
KM9600KO	CTRL 1 CTRL 2	Control 1 and 2, lyophilised*	5 x 2 vials
KM9600RE	RECSOL	Reconstitution solution	3 x 15 ml
KM9600AC	ACTSOL	Activation reagent	2 ml
KM9600IS	INT STD	Internal standard, lyophilised*	5 vials

Cat. No.	Label	Kit components	Quantity
KM9600SO	SOL A	Solution A	25 ml
KM9610		Vedolizumab *ImmuTube*® Extraction Kit	1 Kit
KM9620		Vedolizumab *ImmuTube*® Digest Kit	1 Kit

* Concentrations and reconstitution details are given in the specification data sheet.

As a first step for the application of the *IDK*monitor® LC-MS/MS application for the determination of the therapeutic $\alpha_4\beta_7$ integrin antibody vedolizumab, a tuning is necessary to estimate the optimal LC-MS/MS settings as well as to assess the sufficiency of the sensitivity. The UPLC separation column (KM9600RP) can be ordered separately from us. Please ask for our single components price list.

4. MATERIAL SUPPLIED WITH THE VEDOLIZUMAB *ImmuTube*® EXTRACTION KIT

Cat. No.	Label	Kit components	Quantity
KM9610SI	COLUMNS	*ImmuTube*® columns for affinity purification of vedolizumab from the samples	80 columns
KM9610WL	WASHSOL	Wash buffer	300 ml
KM9610EL	ELUREAG	Elution reagent	20 ml

5. MATERIAL SUPPLIED WITH THE VEDOLIZUMAB *ImmuTube*® DIGEST KIT

Cat. No.	Label	Kit components	Quantity
KM9620B	BUF ABC	Buffer ABC concentrate	5 x 1 vial à 2 ml
KM9620D	BUF D	Buffer D, lyophilised	5 vials
KM9620IA	BUF IA	Buffer IA, lyophilised	5 vials
KM9620ER	ENZSOL	Enzyme reagent	1,5 ml
KM9620E	ENZ	Enzyme, lyophilised	1 vial
KM9620SL	STOP	Stop solution	1,5 ml

6. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Glass tubes, suitable for LC-MS/MS
- 500 ml graduated cylinder, suitable for LC-MS/MS
- Calibrated precision pipettors and 10–1000 µl tips
- Multi-channel pipets or repeater pipets
- Centrifuge, 10 000 *g*, for 1,5 ml Eppendorf reaction tubes or 550 *g* for test tubes
- Vortex
- Vacuum centrifuge or nitrogen distributor
- Standard single-use laboratory plastic vials, cups, etc.
- Thermal block, heatable up to at least 60 °C
- Incubator
- Upside-down shaker
- LC-MS/MS equipment
- RP-C18 column for peptide analysis, (e. g. Acquity BEH130 C18, 1,7 µm (2,1 × 50 mm))

* AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≥ 18.2 MΩ cm).

7. STORAGE AND PREPARATION OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** The kit can be used up to 5 times within the expiry date stated on the label.
- Reagents with a volume less than **100 µl** should be centrifuged before use to avoid loss of volume.
- **Preparation of solution A and mobile phases:** The **mobile phases (MOPHA A, MOPHA B) and solution A (SOL A)** have to be spiked with **0.2 % activation reagent (ACTSOL)** before use (e. g. 250 ml MOPHA A/MOPHA B + 0,5 ml ACTSOL; e. g. 25 ml SOL a + 50 µl ACTSOL), mix well. The **mobile phases and solution A** are stable at **2–8 °C** until the expiry date stated on the label. **Mobile phases and solution A spiked with activation reagent** can be stored for four weeks in a closed flask at **2–8 °C**.

  Attention: The **activation reagent** must be added under the **fume hood**. All vials to be used must be absolutely clean, detergent-free and preferably made of glass suitable for LC-MS/MS.

- The **lyophilised controls** (CTRL) and **calibrators** (CAL) are stable at **-20 °C** until the expiry date stated on the label. Before use, the standards and controls have to be reconstituted with each **100 µl of reconstitution solution (RECSOL)**. Allow the vial content to dissolve for 10 minutes and mix well to ensure complete reconstitution. **Controls and calibrators** (reconstituted CTRL and CAL) **are not stable and cannot be stored.**
- The **lyophilised internal standard** (INT STD) is stable at **-20 °C** until the expiry date stated on the label. Before use, the INT STD has to be reconstituted with **2 ml of activated solution A** (SOL A with 2 % ACTSOL). Allow the vial content to dissolve for 10 minutes and mix well to ensure complete reconstitution. **Internal standard** (reconstituted INT STD) **is not stable and cannot be stored.**

  Attention: Do not prepare internal standard until directly before use!
- **Preparation of buffers**:
  - The **buffer concentrate ABC** (BUF ABC) is stable at **4 °C** until the expiry date stated on the label. Before use, the BUF ABC has to be diluted **1:20 with reconstitution solution** (RECSOL), mix well.
  - The **lyophilised buffers D** (BUF D) and **IA** (BUF IA) are stable at **-20 °C** until the expiry date stated on the label. Before use, they have to be reconstituted with each **500 µl of buffer ABC**, mix well. **Buffers D and IA** (reconstituted BUF D and BUF IA) **are not stable and cannot be stored.**
- The **lyophilised enzyme (ENZ)** is stable at **-20 °C** until the expiry date stated on the label. **Directly before use**, it is reconstituted with **1 ml of enzyme reagent** (ENZSOL). **Enzyme** (reconstituted ENZ) **can be stored at 2–8 °C for 6 months.**
- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2–8 °C**.

8. SAMPLE PREPARATION WITH VEDOLIZUMAB *ImmuTube*® EXTRACTION KIT FOR 80 SAMPLES

Serum and plasma samples can be used with this assay. All samples must be vortexed before use in the test.

Quality control samples should be analysed with each run.

1.	Bring **all reagents and samples** to **room temperature** (18–26 °C) and mix well.
2.	Put the *ImmuTubes*® into an appropriate rack. Assure that the included suspension does not stick to the lid. Label the lids of the *ImmuTubes*®.
3.	Unscrew the lids of the *ImmuTubes*® and **open** the outlet by breaking the endpiece at the predetermined breaking point. Put the *ImmuTubes*® into appropriate tubes. **Wash** the matrix (add 500 µl wash buffer (WASHSOL) and centrifuge for 30–60 s at 1000 *g*). **Repeat** the washing step **twice**.
4.	Put **500 µl wash buffer** (WASHSOL) into the *ImmuTubes*®. **Close** the *ImmuTubes*® firmly by recapping the outlet with the upside-down endpiece. Speedily add **25 µl calibrator/sample/control** (CAL/SAMPLE/CTRL). **Close** the *ImmuTubes*® by screwing the lid on and **mix** the content carefully by gentle inversion.
5.	Mix for **1 hour** by slow **end-over-end rotation** at room temperature. After the incubation, shortly centrifuge the columns to collect matrix remains in the lid.
6.	Unscrew the lid of the *ImmuTubes*® and open the outlet. Put the *ImmuTubes*® into plastic test tubes and centrifuge for 30–60 s at 1000 *g*. Add **500 µl wash buffer** (WASHSOL) and centrifuge for 30–60 s at 1000 *g*. **Repeat** this step **once with wash buffer** and **three times with ultra pure water**. Discard flow-through if necessary.
7.	Label new 1.5 ml test tubes (AG recommends Protein LowBind Tubes from Eppendorf) and insert *ImmuTubes*®. Add **100 µl ELUREAG** (elution reagent for *ImmuTubes*®), centrifuge for 30–60 s at 1000 *g* and collect the eluate containing the vedolizumab in the test tube. **Repeat** this step once with **50 µl ELUREAG**, collect the eluate in the same test tube.
8.	Dry the **eluate** under a nitrogen stream at 37 °C or in a vacuum centrifuge.

9. PEPTIDE PREPARATION WITH VEDOLIZUMAB *ImmuTube*® DIGEST KIT FOR 80 SAMPLES

1.	All solutions for the enzymatic reaction should always be **prepared directly before use**.
2.	Reconstitute the dried eluate in **20 µl buffer D** (BUF D) by intense vortexing for 1 min. If necessary, incubate for 5 min in an ultrasonic bath.
3.	Icubate for **1 hour** in a thermal block at **60 °C**.
4.	Add **20 µl buffer IA** (BUF IA), vortex shortly and **shake** for **30 min** at room temperature **in the dark**.
5.	Add **100 µl buffer ABC** (BUF B) to the samples.
6.	Now **reconstitute** the **lyophilised enzyme** in **1 ml enzyme reagent** (ENZ-SOL) at **2–8 °C**.
7.	**Preparation of the enzyme solution:** For each sample, fill the reconstituted **enzyme** up to a **final volume of 50 µl** with **buffer ABC** (BUF ABC), e. g. per sample 12.5 µl enzyme solution + 37.5 µl buffer ABC (BUF ABC). Aliquot the remaining enzyme and store it immediately at -20 °C until the next usage.
8.	Add **50 µl enzyme solution** to each sample and incubate in an incubator or thermal block at **37 °C over night** (~ 16 h).
9.	Stop the reaction by adding **10 µl stop solution** (STOP).
10.	Reconstitute lyophilised **internal standard** (INT STD) in solution A and add **200 µl** to each sample (final volume: 400 µl). Vortex shortly and put the samples in vials suitable for LC-MS/MS. **Inject 25 µl** into the HPLC system.

10. CHROMATOGRAPHIC CONDITIONS

Example of a comparable UPLC method

Column material: e. g. Acquity UPLC ® BEH130 C18 (Waters); 1.7 µm

Column dimension: 2,1 × 50 mm

Flow rate: 0,1 ml/min

Column temperature: 45 °C

Injection volume: 25 µl

Running time: 25 min

Gradient:

Time	Mobile phase A	Mobile phase B
0 min	97 % A	3 % B
5.1 min	97 % A	3 % B
15.1 min	55 % A	45 %B
17.0 min	20 % A	80 %B
20.0 min	20 % A	80 %B
20.1 min	97 % A	3 % B
25.0 min	97 % A	3 % B

It is recommended to use a guard column/filter to extend the column's life.

After the analysis, the separation column can be washed with ~ 50 ml MOPHA A using the above mentioned gradient. The column can be stored in 80 % acetonitrile.

11. MS/MS METHOD

The MS/MS method shown below is an example for a TSQ Vantage Triple Quadrupol-mass spectrometer (Thermo Fisher Scientific)*.

Mode:	SRM
Polarity:	ESI^+
Capillary temperature [°C]:	200
Vaporizer temperature [°C]:	378
Sheath gas pressure [Arb]:	60
Aux gas pressure [Arb]:	5
Spray voltage [V]:	4000
Resolution in 1st and 3rd Quadrupol [FWHM]:	0,7
Collision gas [mTorr]:	1,5
Cycle time [s]:	1

* These settings are sample settings an can be different depending on the manufacturer and the machine used. Before analysis, tuning is necessary to estimate the optimal LC-MS/MS settings as well as to assess the sufficiency of the sensitivity.

SRM transitions (m/z)

Vedolizumab, peptide for quantification: MH1+: 2237.0357 (MH2+: 1119.022)

Transitions			
Precursor ion	**Fragment ion**	**S-LENS voltage**	**Collision energy**
1119,022	429,213	231	37
1119,022	956,472	231	28
1119,022	1001,965	231	26
1119,022	1281,571	231	33

Internal Standard (isotopic labelled peptide: MH1+: 2245.28 (MH2+: 1123.029)

Transitions			
Precursor ion	**Fragment ion**	**S-LENS voltage**	**Collision energy**
1123,029	429,213	231	37
1123,029	956,472	231	28
1123,029	1001,965	231	26
1123,029	1289,585	231	33

12. CALCULATION

Linear regression can be used as model for evaluation of the results. The three calibrator concentration points are connected by a straight line. The samples can be calculated using this obtained line.

13. QUALITY CONTROL

Control samples should be analysed with each run. Results, generated from the analysis of 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.

14. PRECAUTIONS

- For research use only
- Quality control samples should be analyzed with each run.
- 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.

15. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay.
- Reagents should not be used beyond the expiration date stated on kit label.
- The assay should always be performed according the enclosed manual.

16. DISPOSAL

Mobile phase (MOPHA A, MOPHA B), solution A (SOL A), activation reagent (ACTSOL), reconstitution solution (RECSOL) and stop solution (STOP) have to be disposed as non-halogenated solvents.

17. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- All reagents in the kit package are for research use only.

- The guidelines for laboratories should be followed.
- *IDK*monitor® and *ImmuTube*® are trademarks of Immundiagnostik AG.
- 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 AG along with a written complaint.

Used symbols:

 Temperature limitation

REF Catalogue Number

RUO For research use only

→REF To be used with

 Manufacturer

 Contains sufficient for <n> tests

LOT Lot number

 Use by

 Attention

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