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Manual

IDK® Hemoglobin ELISA

For the in vitro determination of hemoglobin in stool

Valid from 2020-02-01

REF **K 7816D** Σ 96

REF **K 7816D.20** Σ 20 x 96

+8 °C / +2 °C

IVD

CE

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1. INTENDED USE

This immunoassay is intended for the quantitative determination of human haemoglobin in stool, e. g. for colorectal cancer screening. For *in-vitro* diagnostic use only.

2. INTRODUCTION

In colon cancer prevention, the detection of haemoglobin in stool can be used as marker for gastrointestinal bleedings. Tumours and polyps are a possible source of blood in stool. After the detection of haemoglobin, a colonoscopy has to be performed to clarify if the blood comes indeed from a tumour or a tumour precursor. In combination with colonoscopy, the hemoglobin stool immunoassay is proven to be able to reduce the risk of mortality due to colon cancer.

The advantage of the *IDK*® hemoglobin ELISA over guaiac-based tests is its sensitive and specific detection of exclusively human hemoglobin. It is not necessary to follow a special diet before drawing a stool sample for the *IDK*® hemoglobin ELISA, as neither raw meat, radish, nor food containing vitamin C have an influence on the test result.

Indications

- Detection of occult blood in stool
- Crohn´s disease; Ulcerative Colitis
- Suspicion of colon carcinoma
- Polyps in the colon

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 7816D	K 7816D.20
K 7816D	PLATE	Microtiter plate, pre-coated	12 x 8 wells	20 x 12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10 x	2 x 100 ml	40 x 100 ml
K 6999.C.100	IDK Extract®	Extraction buffer concentrate *IDK Extract*®, 2.5 x	1 x 100 ml	–
K 7816D	SAMPLE-BUF	Sample dilution buffer, ready-to-use	2 x 15 ml	3 x 100 ml

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 7816D	K 7816D.20
K 7816D	CONJ	Conjugate, (mouse-anti human Hb, peroxidase-labelled), ready-to-use	1 x 15 ml	20 x 15 ml
K 7816D	STD	Standards, lyophilised (50; 10; 3.3; 0.67; 0 µg/g)	2 x 5 vials	25 x 5 vials
K 7816D	CTRL1	Control, lyophilised (see specification for range)	1 x 2 vials	25 vials
K 7816D	CTRL2	Control, lyophilised (see specification for range)	1 x 2 vials	25 vials
K 0002.15	SUB	Substrate (Tetramethyl-benzidine), ready-to-use	1 x 15 ml	20 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml	20 x 15 ml

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultrapure water*
- Stool sample application system such as Cat. No.: K 6998SAS
- Calibrated precision pipettors and 10–1000 µl single-use tips
- Foil to cover the microtiter plate
- Multi-channel pipets or repeater pipets
- Centrifuge, 3000 *g*
- Vortex
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* Immundiagnostik AG recommends the use of ultrapure 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).

5. PREPARATION AND STORAGE 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 4 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 the wash buffer:** The **wash buffer concentrate (WASHBUF)** has to be diluted with ultrapure water **1:10** before use (100 ml WASHBUF + 900 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37 °C. The **WASHBUF** is stable at **2–8 °C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask **for 1 month at 2–8 °C**.
- **Preparation of the extraction buffer:** The **extraction buffer concentrate *IDK Extract*®** has to be diluted with ultrapure water **1:2.5** before use (100 ml *IDK Extract*® + 150 ml ultrapure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at 37°C in a water bath. The ***IDK Extract*®** is stable at **2–8 °C** until the expiry date stated on the label. Extraction buffer (1:2.5 diluted *IDK Extract®)* can be stored in a closed flask **for 4 months at 2–8 °C**.
- The **lyophilised standards** (STD) and **controls** (CTRL) are stable at **2–8 °C** until the expiry date stated on the label. Before use, the STD and CTRL have to be reconstituted with **500 µl of ultrapure water**. Allow the vial content to dissolve for 10 minutes and mix thoroughly to ensure complete reconstitution. **Standards and controls** (reconstituted STD and CTRL) **can be stored at 2–8 °C for 4 weeks.**
- All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at **2–8 °C**.

6. STORAGE AND PREPARATION OF SAMPLES

Sample stability and storage

Due to the degradation of hemoglobin at room temperature, which can amount to 50 % per day, **raw stool** samples should be shipped frozen. If shipment either at -20 °C or cooled is not possible, the samples can be mailed overnight, but this will reduce the sensitivity. Raw stool can be stored at -20 °C for 1 month.

Stool extract is stable at room temperature* (15–30 °C), 2–8 °C as well as at -20 °C for 7 days. Avoid more than three freeze-thaw cycles.

* under the requirements of the G-BA-Beschluss of 21.04.2016

Extraction of the stool samples

We recommend the following sample preparation:

Stool Sample Application System (SAS) (Cat. No.: K 6998SAS)

Stool sample tube – Instructions for use

Please note that the dilution factor of the final stool suspension depends on the amount of stool sample used and the volume of the buffer.

SAS with 1.5 ml extraction buffer:

Applied amount of stool:	15 mg
Buffer Volume:	1.5 ml
Dilution Factor:	1:100

Please follow the instructions for the preparation of stool samples using the SAS as follows:

a) The raw stool sample has to be thawed. For particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, inoculation loop or similar device.

b) **Fill the empty sample tube** with **1.5 ml extraction buffer** (1:2.5 diluted *IDK Extract*®) before using it with the sample. Important: Allow the extraction buffer to reach room temperature.

c) Unscrew the tube (yellow part of cap) to open. Insert the yellow dipstick into the sample. The lower part of the dipstick has notches which need to be covered completely with stool after inserting it into the sample. Place dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off, leaving 15 mg of sample to be diluted. Screw tightly to close the tube.

d) Vortex the tube well until no stool sample remains in the notches. **Important:** Please make sure that you have a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for ~ 10 minutes improves the result.

e) Allow sample to stand for ~10 minutes until sediment has settled. Floating material like shells of grains can be neglected.

f) Carefully unscrew the complete cap of the tube including the blue ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again.

The sample suspension is now ready for use.

The sample can also be used in a pipetting automat. Place the sample in the sample rack according to instrument instructions.

7. ASSAY PROCEDURE

Principle of the test

This ELISA is used for quantitative determination of hemoglobin in stool. The hemoglobin in the sample is bound to anti-hemoglobin antibodies (in excess), which are immobilised on the surface of the microtiter wells. To remove all unbound substances, a washing step is carried out. In a second incubation step an anti-hemoglobin peroxidase labelled antibody is added. After another washing step, to remove all unbound substances, the solid phase is incubated with the substrate, tetramethylbenzidine. An acidic solution is then added to stop the reaction. The colour converts from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of hemoglobin in the sample. A dose response curve of the absorbance unit (optical density, OD) vs. concentration is generated, using the values obtained from standard. Hemoglobin, present in the patient samples, is determined directly from this curve.

Test procedure

Bring all **reagents to room temperature** (15–30 °C) and mix well.

Bring **frozen sample suspensions to room temperature** (15–30 °C) and then vortex. Allow the sample to stand for ~10 minutes until sediment has settled before using the supernatant in the test.

Mark the positions of standards/controls/samples on a protocol sheet.

Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminium packaging at 2–8 ° C. Strips are stable until expiry date stated on the label.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	Wash the pre-coated microtiter plate **5 x with 250 µl wash buffer before use**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
2.	Add **50 µl sample dilution buffer** (SAMPLEBUF) into each well.

3.	Add each **50 µl standards/controls/supernatant** into the respective wells.
4.	**Incubate for 1 hour** at room temperature (15–30 °C).
5.	Discard the contents of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
6.	Add **100 µl conjugate** (CONJ) into each well.
7.	**Incubate for 1 hour** at room temperature (15–30 °C).
8.	Discard the contents of each well and wash **5 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
9.	Add **100 µl substrate** (SUB) into each well.
10.	**Incubate for 10–20 minutes** at room temperature (15–30 °C) in the **dark**.*
11.	Add **100 µl stop solution** (STOP) into each well and mix well.
12.	Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at **405 nm** against 620 nm as a reference.

* The intensity of the colour change is temperature sensitive. We recommend observing the colour change and stopping the reaction upon good differentiation.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using the „4 parameter algorithm".

1. 4 parameter algorithm

It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.001).

2. Point-to-point calculation

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline algorithm

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Stool

Since the sample dilution is already considered in the calibration curve, the dilution factor is **1**.

In case **another dilution factor** has been used, multiply the obtained result with the dilution factor used.

9. LIMITATIONS

Samples with concentrations above the measurement range (see definition below) can be further diluted and re-assayed. Please consider this higher dilution when calculating the results.

Samples with concentrations lower than the measurement range cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:

highest concentration of the standard curve × *sample dilution factor to be used*

The lower limit of the measurement range can be calculated as:

LoB × *sample dilution factor to be used*

LoB see chapter "Performance characteristics".

10. QUALITY CONTROL

Immundiagnostik AG recommends the use of external controls for internal quality control, if possible.

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.

Reference range

We recommend each laboratory to establish its own reference range.

Based on a study from Gies *et al*. of 516 stool samples, the cut-off value was estimated to be 10 µg/g for colorectal cancer screening.

11. PERFORMANCE CHARACTERISTICS

Accuracy – Precision

Repeatability (Intra-Assay); n = 42

The repeatability was assessed with 2 stool samples under **constant** parameters (same operator, instrument, day and kit lot).

Sample	Mean value [µg/g]	CV [%]
1	7.00	2.6
2	3.99	4.8

Reproducibility (Inter-Assay); n = 68

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

Sample	Mean value [µg/g]	CV [%]
1	0.87	8.6
2	3.51	4.8

Clinical sensitivity and specifity

Based on a study of Gies et al. [6], the *IDK*® hemoglobin ELISA results in a clinical specificity of 95.0 % and a clinical sensitivity of 27.3 % for advanced neoplasms (colorectal cancer [CRC] and large adenoma) with a cut-off of 10 µg hemoglobin/g stool. The expected positivity rate for corresponding samples in an organised colorectal cancer screening is 8.1 % as calculated from a collection of representative stool samples.

In a study of Hoepffner et al. [5], a cut-off value of 2.0 µg hemoglobin/g stool for the *IDK®* Hemoglobin ELISA resulted in a highest clinical sensitivity of 63.8 % for CRC and large adenoma. The respective clinical specificity was 96.3 %.

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, hemoglobin-spikes with known concentrations were added to 3 different stool samples.

Sample	Spike [µg/g]	Obtained [µg/g]	Expected [µg/g]	Recovery [%]
A	unspiked	19.23	–	–
	1.53	20.96	20.76	100.96
	3.06	22.74	22.29	102.02
	7.65	27.67	26.88	102.94
	15.30	34.35	34.53	99.47
B	unspiked	11.03	–	–
	1.53	12.18	12.56	96.93
	3.06	13.36	14.09	94.76
	7.65	17.77	18.69	95.11
	15.30	24.64	26.34	93.57
C	unspiked	1.42	–	–
	1.53	2.76	2.95	93.55
	3.06	4.47	4.48	99.83
	7.65	9.54	9.07	105.16
	15.30	17.44	16.72	104.27

Analytical sensitivity

The following values have been estimated based on the concentrations of the standard without considering possibly used sample dilution factors

Limit of blank, LoB	0.086 µg/g
Limit of detection, LoD	0.152 µg/g
Limit of quantitation, LoQ	0.177 µg/g

The evaluation was performed according to the CLSI guideline EP17-A2. The specified accuracy goal for the LoQ was 20 % CV.

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP6-A by serial dilution of 4 different stool samples. In the table below, the dilution of 2 exemplary samples is shown.

For hemoglobin in stool, the method has been demonstrated to be linear from 0.44 to 23.75 µg/g, showing a non-linear behaviour of less than ± 20 % in this interval.

Sample	Dilution	Expected [µg/g]	Obtained [µg/g]	Recovery [%]
A	undiluted	23.75	23.75	–
	1:2	11.87	13.83	116.49
	1:4	5.94	6.74	113.61
	1:8	2.97	3.52	118.49
	1:16	1.48	1.61	108.42
	1:32	0.74	0.68	91.37
	1:64	0.37	0.30	80.32
B	undiluted	16.56	16.56	–
	1:2	8.28	7.48	90.31
	1:4	4.14	3.95	95.31
	1:8	2.07	1.80	87.05
	1:16	1.04	0.91	87.44
	1:32	0.52	0.44	84.44

Analytical specificity

The specificity of the antibody was tested by measuring the cross-reactivity against 6 compounds with structural similarity to hemoglobin. There was no cross-reactivity observed.

Substance tested	Concentration added	Concentration obtained [µg/g]	Conclusion
Pankreatic amylase	2 800 mU/l	0.026	< LoB
Myeloperoxidase	315.5 ng/ml	0.038	< LoB
Lysozyme	30 ng/ml	0.021	< LoB
Chymotrypsin	1 000 ng/ml	0.028	< LoB

Substance tested	Concentration added	Concentration obtained [µg/g]	Conclusion
sIgA	600 ng/ml	0.023	< LoB
Albumin	6 250 ng/ml	0.001	< LoB

12. 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.
- Kit reagents contain sodium azide or ProClin as bactericides. Sodium azide and ProClin are toxic. Substrates for the enzymatic colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The stop solution consists of diluted sulphuric acid, a strong 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 up immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

13. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not assembling wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analysed with each run.
- Reagents should not be used beyond the expiration date stated on the kit label.
- Substrate solution should remain colourless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according to the enclosed manual.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- The guidelines for medical laboratories should be followed.
- *IDK*® and *IDK Extract*® 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 Immundiagnostik AG along with a written complaint.

15. REFERENCES

General literature

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