RIDASCREEN® Haemoglobin

REF G09030

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Haemoglobin is an enzyme immunoassay for the quantitative determination of human haemoglobin in stool specimens.

2. Summary and explanation of the test

In Germany, about 62,000 people are newly diagnosed with colon cancer each year, and around 35,000 people per year die from the consequences of the disease[1]. This makes colon cancer one of the most common types of cancer and causes of death within Germany. About 20 % of new cases have a family history of this type of cancer and belong to a high-risk group[2]. Colon cancer develops slowly over the course of 10 - 12 years from microscopically visible adenomas that frequently remain unchanged for quite a while. If these types are recognized and removed early on, there is a favorable prognosis of reversal up to complete recovery. Colonoscopy is the gold standard for direct detection. Carcinomas and sometimes large adenomas intermittently release blood and hemoglobin into the lumen of the intestine. Hemoglobin can accordingly be indicative of occult (invisible) blood in the stool. Diagnosis by means of immunological tests generally allows polyps, adenomas, and carcinomas to be removed early on in a subsequent colonoscopy. This offers improved chances of recovery as well as reduced expense for subsequent treatment for patients.

3. Test principle

RIDASCREEN® Haemoglobin employs specific antibodies in a sandwich-type method. The surface of the well of the microtiter plate is coated with polyclonal antibodies against epitopes of human hemoglobin. A suspension of the stool specimen to be tested is pipetted into a well of the microtiter plate and incubated. This is followed by a washing step and a second incubation together with a monoclonal anti-hemoglobin antibody that is conjugated with horseradish peroxidase. In the presence of hemoglobin, the immobilized antibodies, hemoglobin, and conjugated antibody form a sandwich complex. Unbound enzyme-marked antibodies are removed in a subsequent washing step. In positive specimens, after the addition of a substrate, the bound enzyme changes the colorless solution in the wells of the microtiter plate to a blue solution. The addition of a stop solution changes the color from blue to yellow. The measured extinction is proportional to the concentration of hemoglobin found in the specimen.

4. Reagents provided

The reagents in the kit are sufficient for 96 determinations.

Plate	96 det.	Microtiter plate, 12 microtiter strips (breakable) in a strip holder; coated with polyclonal antibodies (from rabbits) against human hemoglobin
Diluent | 3	15 ml	Sample dilution buffer 3 (for final dilution), protein-buffered NaCl solution; contains 0.1 % NaN3; ready for use, dyed red
Wash buffer	100 ml	Wash buffer 10x (10x concentration); phosphate-buffered NaCl solution; contains 0.1 % thimerosal
Calibrator	2 pc.	Calibrator (for standard comparison); 0.5 ml lyophilized
High control	2 pc.	High control; 0.5 ml lyophilized
Low control	2 pc.	Low control; 2 ml lyophilized
Conjugate	12 ml	Conjugate; peroxidase-conjugated, monoclonal antibody against human hemoglobin (from mouse) in stabilized protein solution; ready to use
SeroSC	12 ml	Substrate; hydrogen peroxide/TMB; ready for use
Stop	12 ml	Stop solution; 1 N sulfuric acid; ready for use

Information on hazardous substances complies with the labeling requirement. For further details, see the safety data sheets (SDSs) at www.r-biopharm.com.

5. Storage instructions

All reagents as wells as the lyophilized calibrators, High controls, and Low controls must be stored at 2 - 8 °C and can be used up to the expiration date printed on the label of the reagent concerned. After being reconstituted, the calibrator, High control, and Low control must be used immediately in the test. Discard the remaining solutions of the reconstituted lyophilisates after the assay. Providing the diluted wash buffer is stored at 2 - 8 °C, it can be used for a maximum of 4 weeks. Microbial contamination must be prevented. After the expiration date, the quality guarantee is no longer valid. Scissors must be used to open the aluminum bag containing the microtiter plate in such a way that the clip seal is not torn off. Any microtiter strips which are not required must immediately be returned to the aluminum bag and stored at 2 - 8 °C.
The colorless substrate must also be protected from direct light to prevent it from decomposing or turning blue due to auto-oxidation. Once the substrate has turned blue, it must not be used.

6. Reagents required but not provided

6.1. Reagents

- Distilled or deionized water

6.2. Accessories

- RIDA®TUBE Haemoglobin (Art. No. GZ3012)
- Vortex mixer (optional, see 9.4. and 9.5.)
- Micropipette for 20 - 100 µl and 1000 µl volumes
- Graduated cylinder (1000 ml)
- Stopwatch
- Washing device for microtiter plates or multichannel pipettes (300 µl)
- Microtiter plate photometer (450 nm; reference filter 620 nm)
- Filter paper (lab wipes)
- Waste container containing 0.5 % hypochlorite solution

7. Warnings and precautions for the users

For *in vitro* diagnostic use only.
This test must be carried out only by trained laboratory personnel. The guidelines for working in medical laboratories must be followed. Always adhere strictly to the user instructions for carrying out this test. Do not pipette specimens or reagents using your mouth. Avoid contact with broken skin or mucous membranes. Wear personal protective equipment (appropriate gloves, lab coat, safety glasses) when handling reagents and specimens, and wash hands after completing the test. Do not smoke, eat, or drink in areas where specimens are handled.
For further details, please refer to the Safety Data Sheets (SDS) at www.r-biopharm.com.
The calibrator, High control, and Low control contain human blood which has been tested and found negative for HIV and hepatitis. In spite of this, the calibrator, controls, and the stool specimens must be treated as potentially infectious and handled in accordance with the national safety regulations.
The wash buffer contains 0.1 % thimerosal as preservative. This substance must not be allowed to come into contact with skin or mucous membranes.
The sample dilution buffer contains 0.1 % NaN_3 as a preservative. This substance must not be allowed to come into contact with skin or mucous membranes.
Hydrogen peroxide can cause chemical burns. Handle with care.
The Stop solution contains 1 N sulphuric acid. Avoid contact with the skin and clothing. If the reagent comes into contact with your skin, rinse your skin off with water.
All reagents and materials coming into contact with potentially infectious specimens must be treated with suitable disinfectants or autoclaved at 121 °C for at least one hour. CAUTION: To prevent the formation of poisonous gases, any liquid waste

containing the Stop solution must be neutralized before it is added to hypochlorite solution.
Users are responsible for proper disposal of all reagents and materials after use. For disposal, please adhere to national regulations.

8. Collection and storage of specimens

Collect the stool specimen using the grooves of the sampling rod provided with RIDA®TUBE Haemoglobin, and place it in the buffer in RIDA®TUBE Haemoglobin. The specimen will remain stable in the extraction buffer for five days at 2 - 30 °C.

9. Test procedure

9.1. General information

All reagents and the microtiter plate Plate must be brought to room temperature (20 - 25 °C) before use. Once they have reached room temperature, remove the microtiter strips from the aluminum bag. Mix the reagents well immediately before use. After use, the microtiter strips (placed in sealed bags) and the reagents must be stored again at 2 - 8 °C. Once used, the microtiter strips must not be re-used. Do not use reagents or microtiter strips if the packaging is damaged or the containers are not tightly sealed. In order to prevent cross-contamination, the specimens must be prevented from coming into direct contact with the kit components. The test must not be carried out in direct sunlight. We recommend covering the microtiter plate or sealing with plastic wrap to prevent evaporation losses.

9.2. Preparing the wash buffer

Mix 1 part Wash buffer 10x Wash buffer with 9 parts distilled water (1:10). Any crystals present in the concentrate should be dissolved beforehand through heating (water bath at 37 °C).

9.3. Preparing the calibrator and High control (optional Low control)

The calibrator Calibrator, High control High control, and Low control Low control are lyophilized and must be freshly dissolved for each assay shortly before the test in sample dilution buffer 3 Diluent | 3. The calibrator Calibrator and High control High control are placed in 500 µl, and the Low control Low control is placed in 2 ml sample dilution buffer 3 Diluent | 3. The lyophilisates must be completely dissolved without any residues before use. A vortex mixer can be used for this purpose. Remaining calibrator, High control, or Low control must be discarded.

9.4. Sample preparation and suspension with RIDA®TUBE Haemoglobin (Art. No. GZ3012)

10 mg stool specimen is added to RIDA®TUBE Haemoglobin pre-filled with 2.5 ml ready-to-use extraction buffer. If the stool specimen is liquid, 10 µl of the stool sample can be taken using the pipette and pipetted directly into the extraction buffer. The sample collection and extraction procedure is described in detail in the instruction leaflet, which is enclosed with the RIDA®TUBE Haemoglobin stool sample tube, and can also be downloaded from www.r-biopharm.de.
The stool extracts may not be stored for longer than 5 days at 4 - 30°C, or 2 weeks at 4 °C.

a) Manual sample dilution

As described under point 8 in the instructions for use of GZ3012, 50 µl of the stool suspension obtained using RIDA®TUBE Haemoglobin (Art. No. GZ3012) is added to the well in the microtiter plate and further diluted with 50 µl RIDASCREEN® sample dilution buffer 3 Diluent | 3 directly in the microtiter plate.

b) Specimen dilution in an automated system

If the assay is to be run on the DSX automated ELISA system (Dynex Technologies, Inc.), the specific assay protocol required for this should be requested from R-Biopharm AG and applied to the system.
The required number of microtiter strips are placed in the strip holder for the RIDASCREEN® Haemoglobin microtiter plate Plate. The stool suspension extracted with the stool tube is automatically diluted 1:2 in the ELISA system on the microtiter plate Plate (final dilution 1:500). 50 µl of the stool suspension from RIDA®TUBE Haemoglobin (Art. No. GZ3012) is pipetted into the hemoglobin microtiter plate and further diluted with 50 µl sample dilution buffer Diluent | 3 directly in the microtiter plate.

For instructions on how to perform the assay with other ELISA automated pipetting systems, please contact R-Biopharm AG.

9.5. First incubation

After a sufficient number of wells are placed in the strip holder, 100 µl of the dissolved calibrator Calibrator (in duplicate) of the RIDASCREEN® sample dilution buffer 3 Diluent | 3 (= negative control), the High control High control, and the stool specimen suspension to be examined are added to each well. If needed, the Low control Low control can also be used. In this case, 100 µl is likewise pipetted into the corresponding well. Next incubate the plate for one hour at room temperature (20 - 25 °C).

9.6. First washing

a) Washing step with manual processing

Careful washing is important in order to achieve the correct results and should therefore be carried out strictly according to the instructions. The incubated substance in the wells must be emptied into a waste container containing hypochlorite for disinfection. Next tap the plate over absorbable paper to remove the remaining moisture. Then wash the plate five times using 300 µl diluted wash buffer each time (see 9.2). Make sure that the wells are emptied completely by tapping them out after each wash onto a part of the absorbent paper which is still dry and unused.

b) Washing step in an automated system

When using a microplate washer, make sure that the machine is correctly set to the type of microtiter plate being used. Furthermore, a stool suspension which is not completely particle-free before the first wash should be removed manually by centrifuging in order to avoid blocking the wash needles.
Also ensure that all of the liquid is aspirated during each washing stage. After washing for the last time, knock out the plate thoroughly onto clean absorbent paper or laboratory towels in order to remove any residual moisture.

9.7. Second incubation

Add 100 µl conjugate Conjugate to each well. Next incubate the plate for one hour at room temperature (20 - 25 °C).

9.8. Second washing

a) Washing step with manual processing

Following the incubation period, empty the conjugate in the wells into a waste container containing hypochlorite solution for disinfection. Next tap the plate over absorbable paper to remove the remaining moisture. Then wash the plate five times using 300 µl diluted wash buffer each time. Make sure that the wells are emptied completely by tapping them out after each wash onto a part of the absorbent paper which is still dry and unused.

b) Washing step in an automated system

Also ensure that all of the liquid is aspirated during each washing stage.

9.9. Third incubation

Add 100 µl substrate SeroSC to each well. Then incubate the plate for 15 minutes in darkness at room temperature (20 - 25 °C). Subsequently, stop the reaction by adding 50 µl Stop solution Stop to each well.
After mixing carefully (by tapping lightly on the side of the plate), measure the absorbance at 450 nm and at a reference a wavelength of 620 nm.

10. Quality control — Indication of instability or expiration of reagents

In the quality control, the calibrator [Calibrator] (in duplicate), High control [High control] and RIDASCREEN® sample dilution buffer 3 [Diluent | 3] are also included as negative controls for every test in order to demonstrate that the reagent is stable and that the test has been correctly performed.
The test has been performed correctly when the extinction mean (OD) of the negative control at 450 nm/620 nm is less than 0.05, and the averaged extinction mean (OD) of the calibrator is within the range indicated on the batch-specific data sheet. It is also recommended to routinely test the High control to further validate test results. This should lie within the batch-specific concentration range on the data sheet.

When RIDASCREEN® Haemoglobin is processed on open fully automated ELISA systems, the measured OD of the calibrator [Calibrator] may deviate from the range indicated on the batch-specific certificate depending on the system. On fully automated ELISA systems, the High control [High control] is relevant to the validity of the test results and must therefore always be tested. It is not absolutely essential to use the Low control [Low control].
A deviation from the expected values as well as reagent cloudiness or blue coloring of the colorless substrate prior to addition to the wells can be an indication of an expired reagent. If the stipulated values are not met, the following points must be checked before repeating the test:
- Expiration date of the reagents used
- Functional performance of the equipment used (e.g., calibration)
- Correct test procedure
- Visual inspection of the kit components for contamination or leaks; a substrate solution which has turned blue must not be used.

If the conditions are still not fulfilled after the test is repeated, please consult the manufacturer or your local R-Biopharm distributor.

11. Evaluation and interpretation

11.1. Single-point quantification according to the 4-parameter logistic log model

RIDASCREEN® Haemoglobin uses the 4-parameter logistic log model (4PL) to determine the concentration of hemoglobin in a stool specimen in µg/g.
The RIDA®SOFT Win.net evaluation software is required to calculate the results. RIDA®SOFT Win.NET or updates can be obtained on request by contacting R-Biopharm AG or your local R-Biopharm distributor.
Other evaluation software that provides the 4-parameter logistic log model can also be used instead of RIDA®SOFT Win.net.
The parameters (A - D) required for the 4PL calculation of the standard curve and the target value for the calibrator, High control, and Low control can be found in the

batch-specific data sheet that comes with the test kit, and they must be compared with the values in the evaluation software before measurement.
R-Biopharm AG calculates the standard curve (including parameters A - D) under optimum test conditions for each kit batch, as well as a target value and a permissible range for the standard deviation for the calibrator, High control, and Low control.
The calibrator Calibrator is also tested to compensate for test fluctuations and to check the quality of the test procedure. The High control is only for laboratory-internal test validation for manual processing. If the test is performed on an automated ELISA system, the High control also needs to be tested.
RIDA®SOFT Win.net calculates correction factor F internally from the mean of the calibrator duplicate analysis and its target value. This correction factor is then reconciled with the absorbances for the stool specimens. The test results can be confidently and reliably evaluated within the limits of the standard curve.

11.2. Test result

The cutoff values for the evaluation of a positive test in national colorectal cancer screening are set by each state. In Germany, there is currently no official cutoff value in µg hemoglobin/g stool for defining a positive test result. We advise each laboratory to establish its own standard value range.
With reference to the study by *Gies et al.* [3], we recommend a cut off of 12.27 µg/g.

12. Limitations of the method

The RIDASCREEN® Haemoglobin kit detects epitopes of human hemoglobin in stool specimens. Blood in the stool can also be caused by other factors beyond colorectal cancer. A correlation between the level of the determined extinction mean with the severity of clinical symptoms cannot be derived from such. The obtained results must always be interpreted in connection with the clinical signs and symptoms.
A positive result is a clear indication of bleeding in the intestine which, however, may also result from nonspecific patient factors (such as menstrual blood in female patients or hemorrhoids).
A negative result and does not exclude potential hemorrhagic changes in the intestinal wall. This can be caused by intermittent excretion of blood and the associated insufficient antigen level in the specimen, or by an inhomogeneous distribution of hemoglobin in the stool specimen.

13. Performance characteristics

13.1. Analytical sensitivity

To determine the analytical sensitivity of the RIDASCREEN® Haemoglobin ELISA, the limit of blank (LoB) was determined with 207 assays of negative specimens. The provisional limit of detection (LoD) was then determined by a serial dilution series with 10 dilution steps. To confirm the LoD, 3 specimens were also tested from the provisional LoD in 10 replicates each with a kit batch from the RIDASCREEN® Haemoglobin kit. The results are shown in the following table.

	MV [OD 450/620 nm]	µg/g
LoB	0.007	-
LoD	0.021	0.28

The detection limit is 0.28 µg/g hemoglobin.

13.2. Specificity - interference

To identify potential interfering substances, mucin, stearin/palmitic acid, diclofenac and a cyclamate/saccharin mixture was added in the following concentrations to stool specimens:

Substance	**concentration**
Mucin	5%
Stearin/palmitic acid	40%
Diclofenac	0.1%
Cyclamate/saccharin mixture	1.3%

None of the substances had an effect on the test results at the indicated concentrations.

13.3. Analyte stability

To determine the stability of the analyte hemoglobin in RIDA®TUBE Haemoglobin, 20 specimens were added to the extraction buffer using the RIDA®TUBE Haemoglobin sampling rod. The tubes with the extracts were stored at 4 °C, 23 °C, and 30 °C. The extracts were stable over five days.
Five additional extracts were aliquoted and measured after two weeks while being stored at 4 °C and -20 °C. The results revealed that the extracts can be stored for two weeks at both the 4 °C and -20 °C.

14. Version history

Version number	Chapter and description
2018-08-23	General revision
2018-08-23	13. Performance characteristics 13.1. Analytical sensitivity 13.2. Specificity—interference 13.3. Analyte stability 14. Version history 15. Overview of symbols 16. References

15. Explanation of symbols

General symbols

IVD	For *in vitro* diagnostic use
	Consult instructions for use
LOT	Lot number
	Expiry
	Store at
REF	Article number
	Number of tests
	Date of manufacture
	Manufacturer

Test-specific symbols

Plate	Microtiter plate
Diluent | 3	Sample dilution buffer
Wash buffer	Wash buffer 10x
Calibrator	Calibrator
High control	High control
Low control	Low control
Conjugate	Conjugate
SeroSC	Substrate
Stop	Stop solution

16. References

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