RIDASCREEN®Haemo-/Haptoglobin Complex

Art. No: G09031

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Haemo-/Haptoglobin Complex is an enzyme immunoassay for the quantitative determination of human haemoglobin/haptoglobin complexes in stool specimens.

2. Summary and explanation of the test

In Germany, there are about 66,000 new cases of intestinal cancer every year and approximately 30,000 people die annually of the consequences of the disease. Thus colon carcinoma is one of the most frequent forms of cancer and causes of death throughout the nation. Approximately 10 % of new cases are part of a high-risk group. Because of the high incidence of the disease, the Bundesärztekammer (German Medical Association) recommends regular preliminary examinations from 50 years onwards. Colon carcinomas slowly develop over a period of 10 - 12 years from macroscopically visible adenomas which often exist without change for a long time. If these forms are recognised and removed at an early stage, the prospects of healing and complete recovery are very good. As a direct method of detection, colonoscopy is the reference. Carcinomas and some of the larger adenomas intermittently release blood/haemoglobin into the lumen of the bowel. The haemoglobin/haptoglobin complex (Hb/Hp complex), which plays an important role in the recovery of haemoglobin from lysated erythrocytes, enters the intestine by the same route. Both haemoglobin and the Hb/Hp complexes can be used for the determination of concealed (non-visible) blood in stools.

The non-invasive test method which is most often used for the detection of occult blood in stool today is based on the conversion of the colourless guaiac gum by the non-specific pseudo-peroxidase activity of the porphyrin ring (haem), the central functional component of the haemoglobin, into a blue dye. The test therefore does not rely on the species-specific protein chain of human haemoglobin and this can lead to false positive results in the presence of haemoglobin and myoglobin which have come from animal meat products or food components with peroxidase activity (e.g. fruit and vegetables such as radishes and horseradish). False negative results can be produced by secondary plant materials like antioxidants (e.g. in red wine) and by Vitamin C (ascorbic acid) which is added to many foods as a preservative. Without a diet which is sufficiently controlled before and while the specimens are taken, meaningful results cannot be guaranteed when using the guaiac gum test method.

This to some extent explains the low sensitivity (28 - 40 %) of this type of test method.

By using specific antibodies which only detect human haemoglobin or the human haemoglobin/haptoglobin complex, modern immunological tests have a distinct advantage over the biochemical detection of the guaiac tests. A diet before the test is not necessary and false positive results which arise from food components are virtually ruled out. The immunological test is also sensitive to human haemoglobin concentrations 100 times lower than those required for the biochemical methods.

The determination of the haemoglobin/haptoglobin complexes has an extra diagnostic advantage. Since the Hb/Hp complex is very resistant to decomposition by acids or proteolytic enzymes, it can still be detected in the stools after long periods in the intestine. Thus, blood admixtures from larger intestinal polyps and colon carcinomas located higher up in the intestine can also be recorded with high sensitivity.
Since carcinomas and polyps can bleed intermittently to different extents, it is advisable to examine several (2 to 3) stool specimens also when using immunological detection methods.
The more reliable and, under certain circumstances, earlier diagnosis achieved with immunological tests usually makes it possible to remove polyps, adenomas and carcinomas early during a following colonoscopy. This improves the healing prognoses and leads to lower subsequent costs for the patients.

3. Test principle

In the RIDASCREEN® Haemo-/Haptoglobin Complex, specific antibodies are used in a sandwich-type method. Polyclonal antibodies against epitopes from human haptoglobin are applied to the surface of the well in the microwell plate. A suspension of the stool specimen to be tested is pipetted into a well of the microwell plate and incubated. This is followed by a wash step and second incubation phase together with a monoclonal anti-haemoglobin antibody which is conjugated with horseradish peroxidase. In the presence of haemoglobin/haptoglobin complex, a sandwich complex forms which is made up of the immobilised antibodies, the Hb/Hp complex and the conjugated antibodies. Unattached enzyme-labelled antibodies are removed during a further washing phase. If the test is positive, after adding the substrate, the attached enzyme changes the colour of the previously colourless solution in the wells of the microwell plate to blue. On adding the stop reagent, the colour changes from

blue to yellow. The measured extinction is proportional to the concentration of haemoglobin/haptoglobin complex present in the specimen.

4. Reagents provided

One kit is sufficient for 96 determinations.

Tab. 1: Package content for RIDASCREEN® Haemo-/Haptoglobin Complex (G09031)

Plate	96 det.	Microwell plate, 12 microwell strips (which can be divided) in the strip holder; coated with polyclonal antibodies (from rabbit) against human haptoglobin
Diluent | 3	2 x 15 ml	Sample dilution buffer 3 (for final dilution), protein-buffered NaCl-solution; contains 0.1 % NaN_3; ready for use; red coloured
Wash 10x	100 ml	Wash buffer 10x (10-fold concentrate), phosphate-buffered NaCl-solution; contains 0.1 % Thimerosal
Calibrator	4 pieces	Calibrator (for standard calibration); 0.5 ml lyophilised
Control | +	4 pieces	Positive control; 0.5 ml lyophilised
Low control | +	4 pieces	Low positive control; 2 ml lyophilised
Conjugate	12 ml	Conjugate; Peroxidase conjugated, monoclonal antibody against human haemoglobin (from mouse) in stabilised protein solution; ready for use
SeroSC	12 ml	Substrate; Hydrogen peroxide / TMB; ready for use
Stop	12 ml	Stop reagent, 1 N sulphuric acid; ready for use

Details of hazardous substances according to labeling obligations. For more details see Material Safety Data Sheets (MSDS) at www.r-biopharm.com.

5. Storage instructions

All reagents as well as the lyophilised Calibrator, Positive control and Low positive control must be stored at 2 - 8 °C and can be used up to the expiry date printed on the label of the reagent concerned. After re-solving the Calibrator, Positive control and Low positive control have to be used immediately in the test. Remaining liquids have to be discarded after the test is finished. The diluted Wash buffer 10x can be used for a maximum of 4 weeks when stored at 2-8 °C. Microbial contamination must be prevented. After the expiry date, the quality guarantee is no longer valid. The aluminium bag containing the microwell strips must be opened in such a way that the clip seal is not torn off. Any microwell strips which are not required must immediately be returned to in the aluminium bag and stored at 2 - 8 °C. The colourless 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. Additional necessary reagents – and necessary equipment

6.1. Reagents

- Distilled or deionised water

6.2. Accessories

- RIDA®TUBE Haemoglobin (GZ3012)
- Vortex mixer (optional, see 9.4)
- Micropipette for 20 - 100 µl and 1 ml volume
- Measuring cylinder (1000 ml)
- Stop clock
- Microplate washer or multichannel pipettes (300 µl)
- Microplate reader (450 nm; reference wavelength ≥ 620 nm)
- Filter paper (laboratory towels)
- Waste container containing 0.5 % hypochlorite solution

7. Precautions for users

For *in vitro* diagnostic use only.

This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories have to be followed. The instruction manual for the test procedure has to be followed. The Calibrator, Positive control and Low positive control contain human blood which has been tested for HIV and Hepatitis with

negative results. In spite of this, they and the stool specimens must be treated as potentially infectious and handled in accordance with the national safety regulations.

Do not pipet samples or reagents by mouth. Avoid contact with bruised skin or mucosal membranes. During handling reagents or samples, wear appropriate safety clothing (appropriate gloves, lab coat, safety goggles) and wash your hands after finishing the test procedure. Do not smoke, eat or drink in areas where samples or reagents are being used.

The Wash buffer 10x contains 0.1 % Thimerosal as a preservative. This substance must not be allowed to come into contact with skin or mucous membranes.

The extraction and Sample dilution buffer contain 0.1 % NaN_3 as a preservative. This substance must not be allowed to come into contact with the skin or mucous membrane.

Hydrogen peroxide can cause burns. Handle with care.

The Stop reagent contains 1 N sulphuric acid. Avoid contact with the skin and clothing. If the skin is contaminated with the reagent, rinse it 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 1 hour. CAUTION: To prevent the formation of poisonous gases, any liquid waste containing Stop reagent must be neutralised before adding to hypochlorite solution.

All reagents and materials used have to be disposed properly after use. Please refer to the relevant national regulations for disposal.

8. Specimen collection and storage

The stool samples are collected with RIDA®TUBE Haemoglobin. The sample is stable for 5 days at room temperature (up to 30 °C) in the extraction buffer.

9. Test procedure

9.1. General information

All reagents and the Microwell plate Plate must be brought to room temperature (20 - 25 °C) before use. The microwell strips must not be removed from the aluminium bag until they have reached room temperature. The reagents must be thoroughly mixed immediately before use. After use, the microwell strips (in sealed bags) and the reagents must be stored at 2 - 8 °C. Once used, the microwell strips must not be

used again. The reagents and microwell strips must not be used if the packaging is damaged or the vials are leaking. In order to prevent cross contamination, the samples 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 microwell plate or placing film on it to prevent evaporation losses.

9.2. Preparing the wash buffer

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

9.3. Preparing the calibrator and positive control (optional low positive control)

The Calibrator Calibrator, Positive control Control | + and Low positive control Low control | + are lyophilised and must be freshly re-suspended in Sample dilution buffer 3 Diluent | 3 each time immediately before the start of the test. Therefore Calibrator Calibrator and Positive control Control | + are re-solved in 500 µl and the Low positive control Low control | + is re-solved in 2 ml of Sample dilution buffer 3 Diluent | 3. The lyophilisate must be completely dissolved before use without any residues. A vortex mixer can be used for this purpose. Remaining liquids must be discarded after the test is finished.

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

Each RIDA®TUBE Haemoglobin comes pre-filled with 2.5 ml of ready-to-use extraction buffer and accommodates a 10 mg stool sample. When processing liquid stool samples, 10 µl of the stool sample can be measured 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 in each package of RIDA®TUBE Haemoglobin and can be downloaded from www.r-biopharm.de.

Stool extracts must not be stored longer than 5 days at 2 to 30 °C.

a. Manual sample dilution

50 µl of the stool suspension obtained using the RIDA®TUBE Haemoglobin (Art. No. GZ3012) – as described in Section 8 of the instructions for product GZ3012 – is further diluted directly in the micro titer plate in 50 µl of RIDASCREEN® Sample dilution buffer 3 Diluent | 3.

b. Automated sample dilution

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 coated wells is placed in the microwell holder of the RIDASCREEN® Haemoglobin Microwell plate Plate. When using the automated ELISA system, the stool suspension extracted with a RIDA®TUBE Haemoglobin is automatically diluted 1:2 (final dilution: 1:500) on the Microwell plate Plate. For this purpose 50 µl of the stool suspension is pipetted directly from the RIDA®TUBE Haemoglobin (Art. No. GZ3012) into the RIDASCREEN® Haemoglobin microwell plate and diluted with 50 µl of Sample dilution buffer 3 Diluent | 3 in the Microwell 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 placing a sufficient number of wells in the holder, add 100 µl of diluted Calibrator Calibrator (in duplicate), of Sample dilution buffer 3 Diluent | 3 (= Negative control), of Positive control Control | + and of the stool suspension being analysed to the relevant wells. If used, also add 100 µl of the Low positive control Low control | + to the relevant wells. Then incubate the plate at room temperature (20 - 25 °C) for 1 hour.

9.6. First washing

Careful washing is important in order to achieve the correct results and should therefore be carried out strictly in accordance with the instructions. The incubated substance in the wells must be emptied into a waste container containing hypochlorite for disinfection. After this, tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells.

Then wash the plate 5 times using 300 µl diluted wash buffer (see 9.2) each time. Make sure that the wells are emptied completely by tapping them after each wash on a part of the absorbent paper which is still dry and unused.

When using a microplate washer, make sure that the machine is correctly adjusted to the type of microwell 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 make sure that all of the liquid is sucked away during each washing phase. After washing for the last time, tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells.

9.7. Second incubation

Pipette 100 µl Conjugate Conjugate into each well. Then incubate the plate at room temperature (20 - 25 °C) for 1 hour.

9.8. Second washing

The incubated substance in the wells must be emptied into a waste container containing hypochlorite solution for disinfection. Tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells. Then wash the plate 5 times using 300 µl diluted wash buffer each time. Tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells.

9.9. Third incubation

Add 100 µl Substrate SeroSC to each well. Then incubate the plate at room temperature (20 - 25 °C) in the dark for 15 minutes. After this, stop the reaction by adding 50 µl Stop reagent Stop to each well.

After careful mixing (by lightly tapping the side of the plate) measure the extinction at 450 nm (reference wavelength ≥ 620 nm) in a plate reader.

10. Quality control – indications of reagent expiry

For quality control purposes, the Calibrator Calibrator (in duplicate), the positive control Control | + and the Sample dilution buffer 3 Diluent | 3 as a negative control must be used every time the test is performed to ensure that the reagents are stable and the test has been carried out correctly.

The test has been carried out correctly if the extinction (OD) of the negative control at 450 nm/620 nm is smaller than 0.05 and the determined extinction (OD) of the calibrator is within the lot-specific range given on the data sheet enclosed. For further validation of test results it is recommended to use the positive control in routine. In that case the positive control should be within the lot-specific range given on the data sheet.
If running RIDASCREEN® Haemo-/Haptoglobin Complex on ELISA processors, the measured OD value for the Calibrator Calibrator can differ from the value given on the lot-specific data sheet depending on the used instrument. Therefore the positive control is decisive for the validity of results and **must** always be processed when running the test on ELISA processors. The use of the Low positive control Low control | + is not mandatory.

If the values differ from those required; if the substrate is turbid or has turned blue before adding it the wells, this may indicate that the reagents have expired. If the stipulated values are not met, the following points must be checked before repeating the test:

- Expiry date of the reagents used
- Functionality of the equipment being 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 repeating the test, 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

The concentration of the haemoglobin/haptoglobin complex in µg/g stool is determined with RIDASCREEN® Haemo-/Haptoglobin Complex according to the 4-parameter-logistic-log-model (4PL).

The evaluation software RIDA®SOFT Win.net is needed to determine the results. RIDA®SOFT Win.net or an update can be obtained from R-Biopharm AG or from your local R-Biopharm distributor.

The parameters (A - D) of the standard curve required for the 4PL calculation and the setpoint value of calibrator, positive control and low positive control are listed on the lot-specific datasheet supplied with the kit and must be compared to the values in the

evaluation software before each measurement using the corresponding information on this datasheet.

R-Biopharm AG has determined the standard curve (including the parameters A - D) as well as the set point value and allowed range of calibrator, positive control and low positive control under optimum conditions for each kit lot in the final quality control.

The Calibrator Calibrator is tested in routine in order to compensate test fluctuations and check the quality of each test run. In case of manual processing the positive control is only for lab internal validation. If running the test on ELISA processors the positive control is decisive for the validity of test results.

A correction factor F is calculated internally by RIDA®SOFT Win.net from the average value of the double determination of calibrator control and its setpoint value and is reconciled with the extinctions for the stool specimens. A safe and reliable evaluation of the test results can be carried out within the limits of the standard curve.

11.2. Test result

For a cut-off value of > 2 µg/g of the haemoglobin/haptoglobin complex in human stool samples, the result must be considered positive. If the results lie below the cut-off value, the specimens must be considered negative.

We recommend that a separate standard value range is established by each laboratory.

In case of concentrations of > 25 µg/g we recommend a 1:5 dilution of the extract in Diluent 3.

12. Limitations of the method

The RIDASCREEN® Haemo-/Haptoglobin Complex detects epitopes of the human haemoglobin/haptoglobin complex in stool specimens. Blood in stool can be caused by other factors apart from bowel cancer. The test cannot be used to derive a relationship between the determined extinction and the appearance of serious clinical symptoms. The results obtained must always be interpreted in combination with the clinical picture.

A positive result is a clear indication of a source of bleeding in the intestine but can also be present because of non-specific patient factors (such as menstrual bleeding in female patients or haemorrhoids).

A negative result does not rule out the possibility of bleeding pathological changes in the intestinal wall. This can be caused by intermittent segregation of blood and the

associated lack of antigens in the specimen or a non-homogeneous distribution of the haemoglobin/haptoglobin complex in the stool specimen. If there are anamnetic reasons for suspecting a serious intestinal disease, we recommend testing several specimens (2 to 3) from sequential defecations.

13. Performance characteristics

13.1. Test quality

The RIDASCREEN® Haemo-/Haptoglobin Complex was tested for sensitivity and specificity on 80 stool specimens during a study in an external laboratory and was compared with the Immunochemiluminometric assay (ILMA) which is routinely used there. The investigation showed the following correlation:

Sensitivity:	100.0 %
Specificity:	91.3 %

13.2. Detection limit

The detection limit of RIDASCREEN® Haemo-/Haptoglobin Complex was calculated from the addition of the B_0 value (n=22) and twice of its standard deviation.This resulted in a detection limit for the haemoglobin/haptoglobin complex of 0.38 µg/g stool.

13.3. Cross-reactivity

Different blood specimens from animals used for food were tested with the RIDASCREEN® Haemo-/Haptoglobin Complex. No cross-reactivities were found (see Tab. 5).

Tab. 5: Cross-reactivity of RIDASCREEN® Haemo-/Haptoglobin Complex with non-human blood specimens

Specimens	Dilution/ Concentration	OD
Negative control (Diluent | 3)		0.004
Positive control (human blood)	1/50000	1.581
Goat blood	1:10	0.006
Goat blood	1:100	0.003
Horse blood	1:10	0.004
Horse blood	1:100	0.003
Sheep blood	1:10	0.005
Sheep blood	1:100	0.004
Hen blood	1:10	0.004
Hen blood	1:100	0.004

13.4. Precision

The intra and inter assay reproducibility of RIDASCREEN® Haemo-/Haptoglobin Complex was determined by multiple determinations (n=72 or n=24) of two different blood concentrations at different days under optimum conditions. The results are shown in Tables 6 and 7.

Tab. 6: Intra-assay reproducibility

Intra-assay	RIDASCREEN® Haemo-/Haptoglobin Complex	
	Concentration 1	Concentration 2
Mean (OD)	0.285	1.186
SD	0.021	0.059
CV %	**7.3**	**5.0**

Tab. 7: Inter-assay reproducibility

Inter-assay	RIDASCREEN® Haemo-/Haptoglobin Complex	
	Concentration 1	Concentration 2
Mean (OD)	0.296	1.214
SD	0.013	0.050
CV %	**4.4**	**4.1**

References

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