iVYLISA GIP Stool

For research use only

KT-5739R: Immunosorbent assay in a sandwich ELISA format that allows the detection of the presence of Gluten Immunogenic Peptides (GIP) in stool samples

INTRODUCTION

Celiac disease is a chronic disorder of autoimmune origin that is characterized by a permanent intolerance to the proteins of gluten.

It appears in genetically predisposed people, both adults and children. It has an prevalence in the population of above 1:100, most frequently in children. The disease affects the small intestine and provokes the atrophy of the intestinal villi and thereby interferes with the absorption of nutrients such as proteins, fats, carbohydrates, salts and vitamins. It doesn't only affect the intestine but is in fact a systemic disease which can cause lesions in the skin, joints and other organs.

The strict adherence to the Gluten Free Diet (GFD) is essential for reducing those symptoms of the disease, avoiding nutritional deficiencies and improving the quality of life. However, based on multiple different studies, dietary transgressions, whether voluntary or involuntary, are frequent (32.6-55.4%) in celiac patients, especially in adults.

At present, there is no direct method for the monitoring the adherence to the GFD. Gluten immunogenic peptides (GIP) are fragments of gluten proteins which are resistant to gastrointestinal digestion and trigger immune responses in celiac patients. The recuperation of measurable quantities of GIP in stool indicates that a patient has consumed gluten. The present of GIP in the stools constitutes a precise, direct marker for the control of the GFD in the long and short term.

INTENDED USE AND CHARACTERISTICS

Indication	Sanitary product
Function	Detection and quantification of GIP in stool samples
Target	Monitoring of the GFD (celiac disease)
Method Type	Manual
Analysis Type	Quantitative
Sample Type	Stool
Intended user	Celiac patients, patients with non-celiac wheat sensitivity, gluten-allergy or other disorders related to gluten.
Intended Use	For research use only
Assay Type	Immunosorbent assay in sandwich ELISA format

TECHNICAL SPECIFICATIONS

Sample quantity	1 gramo
Diagnostic sensitivity *	97,1% (IC95:90,2-100%)
Diagnostic specificity *	83,3% (IC95:63,3-100%)
Positive predictive value *	91,9% (IC95:81,8-100%)
Negative predictive value *	93,75% (IC95:78,8-100%)
Reproducibility	CV ≤ 22%
Repeatability	CV ≤ 17%
Lower detection limit **	0,3 µg GIP***/g of stools
Dynamic range	(0,3–1,25) µg GIP***/g stools
Concordance between extraction methods	Lin Coeficient: 96

** Associated with people without restrictions on gluten consumption in the diet. Intake not controlled.*
*** Defined as the value where 95% of the samples at that concentration have a positive result.*
**** Based on the 33-mer peptide of α-gliadin.*

INTERFERENCES

No interference detected to date.

KIT CONTENTS

References	Description	Quantity	Uds	References	Description	Quantity	Uds
RS-6528R*	Extraction Solution_iVYL	40 mL	1	RS-5865R	Negative control	1,2 mL	1
RS-5860R	Dilution Solution	125 mL	1	RS-6252R	Inner Control	1,2 mL	1
RS-5861R*	Wash Solution	100 mL	1	RS-5874R	Standard 1 [25 ng/mL GIP]	1,2 mL	1
RS-5863R	Substrate Solution	15 mL	1	RS-5875R	Standard 2 [12,5 ng/mL GIP]	1,2 mL	1
RS-5862R	Stop Solution	15 mL	1	RS-5876R	Standard 3 [6,25 ng/mL GIP]	1,2 mL	1
RS-5879R	Plate Coated with G12	1 ud	1	RS-5877R	Standard 4 [3,13 ng/mL GIP]	1,2 mL	1
AB-5866R	G12-HRP conjugated	15 mL	1	RS-5878R	Standard 5 [1,56 ng/mL GIP]	1,2 mL	1
RS-5864R	Positive Control	1,2 mL	1	RS-6526R	Extraction Tube_iVYL	40 uds	40

*NOTE: For the preparation of the reagents, consult the protocol.

Non-provided, required materials:
Sample collection pot, 100 µL – 1000 µL pipette and disposable tips, 50mL Falcon® type tubes with screw cap, 1,5 mL plastic vials Eppendorf® (It is recommended to use low biding materials), powder-free nitrile gloves, laboratory coat, (96-100%) ethanol, distilled water.

Required equipment:
Plate reader with 450nm filters, vortex, thermostatic bath or thermoblock with temperature control (adjustable to 50°C). Falcon centrifuge (only in protocol B).

Recommended materials and equipment:
Multichannel pipette, automatic washer

SECURITY INFORMATION

Component	Dangerous Reagent	Hazard pictograms	Hazard Warnings	Precautions
RS-5862	Sulfuric acid (95-97) %		314	280, 305+351+338, 310, 303+361+353, 363

Safety Indications:

H314 Causes severe skin burns and eye damage

Precaution:

P280 Wear eye protection, face protection, protective clothing, protective gloves

P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P310 Immediately call a doctor or a POISON CENTER

P303+P361+P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower.

P363 Wash contaminated clothing before reuse.

Note:
The non-included ethanol (96-100%) is classified, according to Regulation (CE) 1272/2008 (CLP) as a category 2 reagent due to two risk classes:

2.6. Flammable liquids (H225 Highly flammable liquids and vapours)

3.3. Serious injuries or ocular irritation (H319 Causes serious eye irritation)

Follow the indications from the safety data sheet of the provider.

STORAGE AND TRANSPORT CONDITIONS

Storage conditions	4°C a 8°C / 39,2°F a 46,4°F
Transport conditions	4°C a 8°C
Stability in use *	3 months

* Once the reagents have been prepared and aliquoted as indicated in the procedure. NOTE: The washing solution prepared at 1X has a shelf life of 15 days, so this dilution must be prepared for each test.

STABILITY

The product is stable until the expiration date indicated on the label provided that the storage conditions indicated on the kit are maintained.

RECOMMENDATIONS, PRECAUTIONS AND LIMITATIONS

Any serious incident which occurs related to the use of the kit should be communicated to the manufacturer and the relevant authority.

- This kit has been designed for research use.
- Use the appropriate PPE (disposable protective gloves, laboratory coat) when working with kit reagents and samples.
- The kit should be used at standard room temperature conditions.
- Close the bottle and vial that contain the components of the kit immediately after use and do not interchange lids.
- Do not freeze any of the reagents supplied in the kit.
- Do no combine nor exchange reagents between kits.
- Any remaining unused RS-5879 Plates Coated with G12 (Multiwell Strips) should be returned to the foil bag containing the desiccant, tightly closed and stored at (4-8)°C.
- The RS-5863 Substrate Solution component and AB-5866 G12-HRP conjugate must be stored at the required temperature (4-8) °C protected from light as well as at the required temperature.
- The 1X diluted wash solution will be stable for up to 15 days when stored at (4-8) °C.
- Dispose of the unused reagents and waste material in accordance with the relevant regulation.
- It is important to use the indicate amount of sample. The usage of more or less than recommended could result in erroneous results.
- Homogenise the sample well. An insufficient homogenisation could cause variability in the replicates of a sample.
- In the case of excessively liquid or hard stools, the use of a sample of the subsequent deposit (where possible) is recommended.
- Pay attention to the mixing process. A weak or shortened mixing process could lead to false negatives.
- Make sure the washing steps are done correctly. Improper washing or drying of the plate can cause poor precision and/or increased background.
- Make sure to add the stop solution in the same order as the substrate.
- Do not reuse pipette tips. Use new tips for each reagent.
- Do not return unused amounts of reagents to the original bottles.
- Do not touch the bottom of the wells with the pipette tip.
- Avoid the generation of foam when mixing reagents and throughout the procedure.
- The controls and the curve must be analyzed in each assay.
- Once the procedure has started, all steps must be completed without interruption.
- The usage of this kit should comply with the safety documentation provided
- The avoidance between clinical samples can be avoided solely by the application of good laboratory practise and the careful compliance with the protocol provided in the instructions for use. All reagents should be monitored for impurities and contaminations. Any reagent with suspected issues should be disposed of.
- Control should be taken during the management of clinical material. Incorrect manipulation of samples could result in a contamination and/or deterioration of components of the kit. BIOMEDAL S.L. will not be responsible for the incorrect usage of the kit.
- Alcohol based disinfectants can damage the labels on the bottles and vials.

Procedural Limitations:

- Read all of the instructions thoroughly before employing the kit.
- Adequate systems for collection, transportation, storage and processing of samples are required for the optimal usage of the product.
- Do not use the kit after the expiry date.
- The kit is not validated for automation. In the event of using automated equipment, it is the user's responsibility to ensure that the equipment has been properly validated.

Clinical Limitations

- This kit is not valid for the analysis of neonatal samples.

COLLECTION AND STORAGE OF SAMPLES

Sample collection recommendations:

- If gluten ingestion is suspected, please consider the following information:

Maximum detection time from ingestion	1-6 days
Optimal detection time from ingestion*	1-2 days
Minimum amount of detectable gluten	50mg

**To maximize the probability of detection of the kit, sampling is recommended 1-2 days after gluten intake.*

At least 1-2 g or mL (for liquid samples) of stool should be collected. These samples must be collected in a clean, dry container without any added chemical additives or preservatives. The sample should be prevented from coming into contact with urine or toilet water.

For a follow-up of the gluten-free diet, it is recommended to collect two stools samples a week (for example: Monday and Thursday).

Storage of stool samples:

Storage at room temperature	Maximum time 72 hours
Storage at 4°C	Maximum time 72 hours
Storage at -20°C	Maximum time 24 months
Freeze/Thaw Cycles	Maximum six cycles

PROTOCOL

PREPARATION OF REAGENTS:

- Preparation of wash buffer 1X
  The Wash Solution is supplied as a 10-fold concentrate (RS-5861R_Wash Solution), so a 1:10 dilution in distilled water should be made prior to use (e.g., if you are using only 16 wells, you may prepare 30 mL by adding 3 mL of 10X Wash Solution to 27 mL of distilled water).
  Please note that the Wash Solution, once diluted, is only stable for up to 2 weeks when stored at (4-8) °C so it is recommended to prepare only the amount to be used.
- Preparation of extraction solution
  Add 60 mL of 100% ethanol or 62.5 mL of 96% ethanol to the RS-6528R_Extraction Solution/Solución Extracción_iVYL bottle. Once the ethanol has been added, the solution is ready for use. Remember to check the appropriate box on the product label as a reminder that you have added the ethanol.

PREPARATION OF SAMPLES:

There are two methods of extraction of GIP in feces. In case that the samples are frozen, thaw the samples completely before performing the extraction. Select one of the two protocols described below:

- Protocol A:

1. Aliquot the previously prepared extraction solution (RS-6528R) into the extraction tubes included in the kit (RS-6526R). Add 0.7 mL of extraction solution to each extraction tube.
2. Adequately homogenise the stool sample using the spoon of the extraction tube (RS-6526R_Extraction Tube/Tubo de Extracción_iVYL) or a clean pipette tip.

3. Take the stool sample with the spoon attached to the tube RS-6526R_Extraction Tube/Tubo de Extracción_iVYL. The correct amount of sample is the one that fills the spoon.
4. Put the spoon with the sample back into the tube and move it to detach the sample from the spoon. Close the tube tightly and mix vigorously using a vortex or a similar device to dissolve the sample. Mixing should go on until no sample remains on the spoon or on the tube walls.
5. Incubate the sample at 50 °C for 60 minutes in a temperature-controlled thermoblock or water bath and mix the sample periodically (every 15 minutes) by inverting or vortexing the tube for 10 seconds.
6. Unscrew the small white cap of the tube with the sample and using the tube dropper transfer the supernatant to an Eppendorf®-like 1.5 mL tube for further analysis.

- Protocol B:

1. Adequately homogenise the stool sample using a clean pipette tip or spoon.
2. Use 50 mL Falcon®-type tubes (not supplied) to weigh the sample and tare the balance after each sample with a clean tube. Weigh 0.1-0.2 g of each sample into a sample collection tube using a clean plastic stirrer or a pipette tip. Write down each weight, you will need this value for future calculations. A sample of that weight is about the size of a pea. You can leave the tip or stirrer inside the tube with the sample.

3. Add the required amount of Extraction Solution to each sample. This amount is given by the formula:
   Extraction Solution Volume (mL) = 5 x sample weight (g)
   For example, the volume required for a 0.18 g sample is:
   Volume = 5 x 0,18 g = 0,9 mL of Extraction Solution

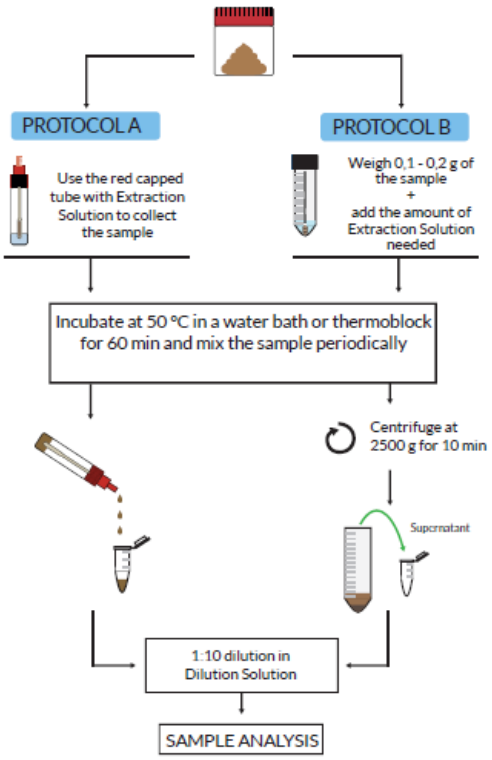
As an alternative to tare, you can first weigh the empty tube (a) and then the tube with the sample (b). The difference of these weights will give you the weight of the sample:

(b)-(a) = sample weight

If you decide to leave the pipette tip or scoop/spatula inside the sample tube, be sure to include its weight in the calculations or tare with the scoop/spatula inside.

4. Close the tube completely and shake vigorously to dissolve the sample using a vortex or similar mechanism. You should continue mixing until there are no large pieces of sample adhering to either the stirrer or scoop or the walls of the tube.
5. Incubate the sample for 60 minutes at 50°C in a thermostatic bath or thermoblock, shaking the tube periodically (every 15 minutes) by inversion or using a vortex for 10 seconds.
6. Centrifuge the suspension at 2,500 g for 10 minutes and transfer the supernatant to a 1.5 mL Eppendorf®-type vial for further analysis.

DIAGRAMATIC PROTOCOL



PROTOCOL:

1. Allow all reagents to reach room temperature (20-28 °C) before starting the assay, with the exception of the conjugated antibody (AB-5866R_G12-HRP conjugated/Conjugado G12-HRP) which should be kept at 4-8 °C until use.
   Immediately after finishing the assay, store the reagents to the indicated temperature (4-8) °C
2. Prepare the appropriate dilution of the sample in microcentrifuge tubes (like Eppendorf® tubes) using Dilution Solution (RS-5860R_Dilution Solution/Solución Dilución). The sample dilution must be at least 1:10. A final volume of 300 µL is sufficient for the analysis of each sample. To obtain this volume, add 30 µL of the extracted sample to 270 µL of Dilution Solution.

   ⚠ In those cases where the sample has an amount of GIP greater than the quantification limit and it is desired to know the amount of GIP, the analysis must be repeated, performing a higher dilution factor. Once the sample has been analyzed with the new dilution factor applied, it must be taken into account for the calculation of the amount of GIP in faeces following the following formula:

   ⚠ $$\mu g \text{ of GIP/g sample} = \frac{ng\ de\ GIP/mL\ x\ 5\ mL/g\ of\ stool\ x\ d}{1000}$$ d is the new dilution applied. For instance 1:200

   The diluted samples should be analyzed as soon as possible.

3. Put the required number of multiwell strips in the holder. Immediately return unused strips to the foil bag with the desiccant and seal tightly to minimize exposure to moisture and light. Store at required temperature ((4-8) °C).
4. Plate loading: add 100 µL of each sample (including calibration standards and positive, negative and inner controls) to each well. It is recommended to set up the plate as shown in next image:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 1 [25 ng/mL GIP]	Standard 5 [1,56 ng/mL GIP]	M1									
B	Standard 1 [25 ng/mL GIP]	Standard 5 [1,56 ng/mL GIP]	M1									
C	Standard 2 [12,5 ng/mL GIP]	Inner Control	M2									
D	Standard 2 [12,5 ng/mL GIP]	Inner Control	M2									
E	Standard 3 [6,25 ng/mL GIP]	Positive Control	...									M39
F	Standard 3 [6,25 ng/mL GIP]	Positive Control										M39

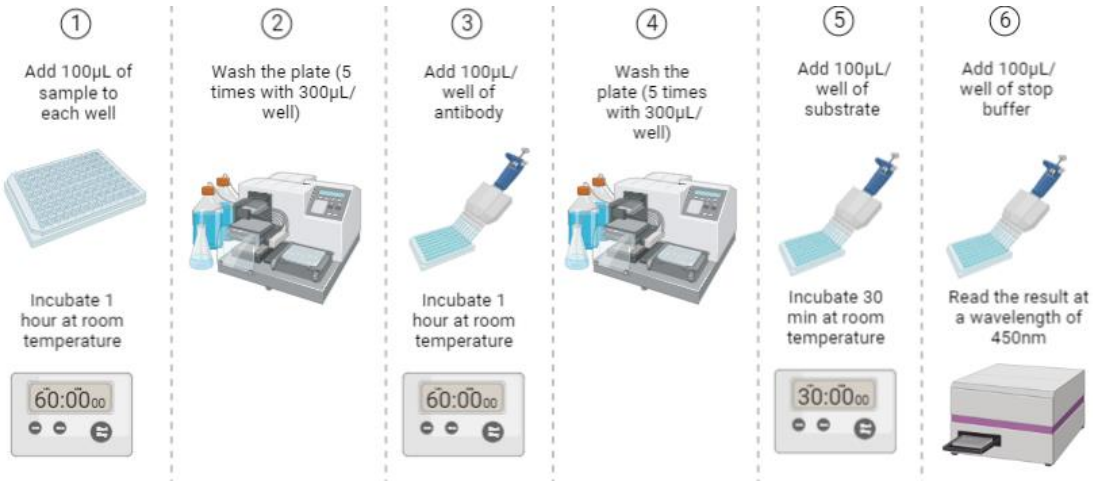
G	Standard 4 [3,13 ng/mL GIP]	Negative Control										M40
H	Standard 4 [3,13 ng/mL GIP]	Negative Control										M40

4. Cover the plate with a foil or alike and incubate at room temperature for 60 minutes.
5. Wash plate 5 times with automatic washer using 300µL per well of previously prepared 1X Wash Solution. After the last wash, invert the plate and tap gently on absorbent paper to remove residual liquid.
   If you do not have an automatic washer, you can do it manually, removing the contents of the wells by inversion.
6. Add 100 µL of conjugates antibody (AB-5866R_G12-HRP conjugated/Conjugado G12-HRP) to each well. Cover the plate with a foil paper to protect from light and incubate at room temperature for 60 minutes

   To avoid any potential microbial and/or chemical contamination, it is recommended to extract the amount necessary for the assay. Do not return unused antibody to its original container.

7. Wash the plate following step 6 of this procedure.
8. Add 100µL of Substrate (RS-5863R) to all wells. Cover plate to protect from light and incubate at room temperature for 30 minutes.
9. Add 100µL of the stop solution (RS-5862R) to each well in the same order as the substrate was added.
10. Read the absorbance of each well at a wavelength of 450nm within 10 minutes of adding the stop solution.

DIAGRAMATIC PROTOCOL



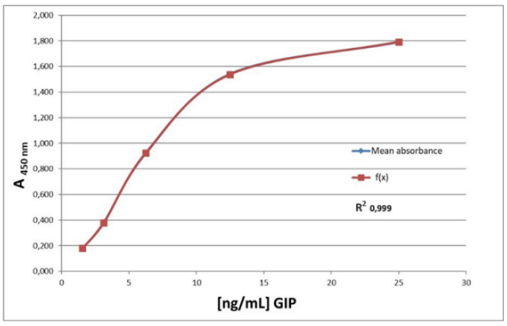
RESULTS CALCULATION

It is recommended to use the spreadsheet provided by Biomedal S.L. (download from: **http://ivydal.biomedal.com/client/**).

In the case of not using the supplied spreadsheet, follow these steps:

1. Determine the mean absorbances for duplicates of both the samples and each point on the standard curve and your controls.
2. Calculate the coefficients of variation between said duplicates.
3. Construct a standard curve plotting absorbance value (y-axis) against GIP concentration for each of the standards supplied in the kit (x-axis).
   Calculate the equation that defines the standard curve using a 4-parameter logistic regression (4PL).
   The equation obtained must follow this type: y = log [GIP] (ng/mL) = (a-d)/(1+(x/c)b)+d)
4. Calculate the GIP content in the samples using the following steps:
   a. Enter in the obtained equation the value of y (absorbance value, λ=450 nm). Then raise this value to 10 to obtain the value in ng/mL of GIP in the diluted samples.
   b. To transform the units of ng/mL of GIP to ng of GIP/g of feces, you must follow the following formula: ng GIP/g sample = ng GIP/mL x 5 mL/g of stools[a] x 10[b]

Where, a is the dilution corresponding to the extraction step and b the corresponding dilution in the dilution step.



	X-axis			Y-axis	
	ng/mL	A450nm 1	A450nm 2	Media	C.V.%
Standard 1	25	1,802	1,777	1,790	0,99
Standard 2	12,5	1,540	1,544	1,542	0,18
Standard 3	6,25	0,893	0,949	0,921	4,30
Standard 4	3,13	0,382	0,385	0,384	0,55
Standard 5	1,56	0,178	0,176	0,177	0,80

RESULT INTERPRETATION

A result above the detection limit of the technique will indicate a positive result. To quantify the amount of GIP in the sample, it must be within the dynamic range of the technique (1.56 to 25 ng/mL).

A sample is considered negative if its result is below the detection limit of the technique.

For the test to be considered valid, the following requirements must be met:

- ✓ The coefficient of variation between the absorbances should not exceed 30% (CV<30%)
- ✓ The absorbance of the internal control must be 9ng/mL±30%
- ✓ The positive control must have an optical density greater than standard 1
- ✓ The negative control must have an optical density lower than standard5

TROUBLESHOOTING

If you notice unusual product performance or other deviations from expected results, please re-read these instructions carefully, especially this chapter. If this does not help immediately, contact the technical department of Biomedal, S.L. or with your local distributor.

Problem	Possible causes and suggestions
Nonspecific color development	Incomplete washing of the plate: Wash the plate at least 5 times with 300 µl/well of wash buffer. More than 5 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
	Cross-contamination of ELISA wells: Take care while pipetting and mixing sample to minimize risk.
	Enzyme Substrate Solution or antibody solution is contaminated: Do not return unused amounts of reagents to the original bottles.
Low optical density	Pipetting error: Ensure pipets are calibrated and used according to manufacturer's instructions.
	Incubation temperature too low: Incubation of the ELISA should be performed at room temperature (20-25) °C.
	Incorrect plate reader filter used: Plate should be read at 450 nm.
	Reagents are too cold: All reagents, except for the G12 antibody-conjugated, must be brought to room temperature prior to commencing the assay. This takes approximately 30 minutes.
High background	Incomplete washing of the plate: Wash the plate at least 5 times with 300 µl/well of wash buffer. More than 5 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.
	Incubation temperature too high: Incubation of the ELISA should be performed at room temperature (20-25) °C.
Nonlinear standard curve	Incomplete washing of the plate: Wash the plate at least 5 times with 300 µl/well of wash buffer. More than 5 washing cycles may be required depending on the washer being used. A soak time of at least 5 seconds between cycles should be used.

and duplicate variability	Poor mixing: Mix reagents thoroughly by inversion or gentle vortexing prior to their addition to the plate.
	Pipetting error: Ensure pipets are calibrated and used according to manufacturer's instructions.
	Interruption of the steps of the procedure: All reagents must be prepared before starting the procedure to avoid stops during the procedure.

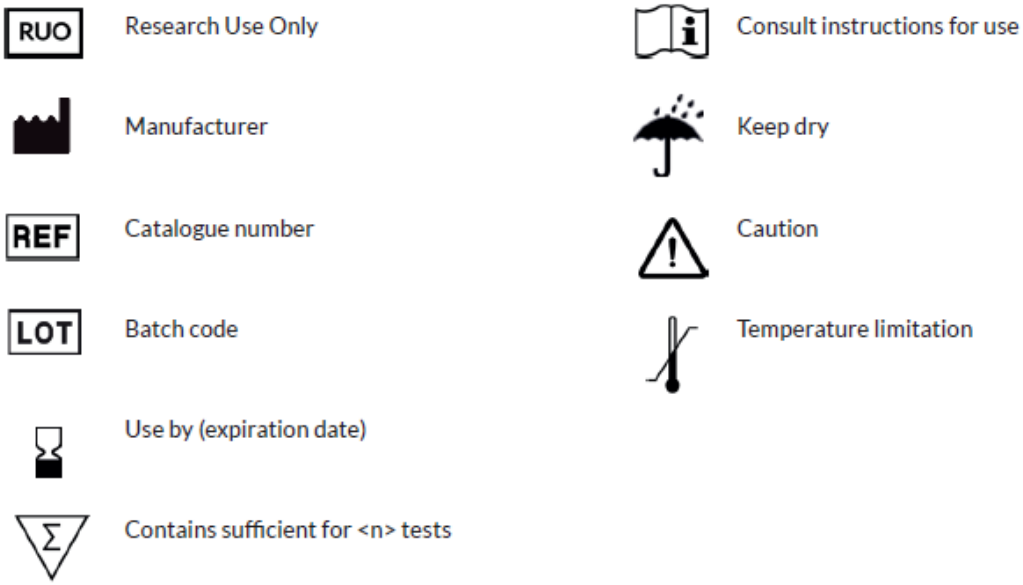
INTERNAL QUALITY CONTROL

Each batch is subject to internal quality control before release. Quality control is performed using both positive and negative stool samples and spiked negative stool samples.

The batch acceptance criteria are as follows:

- ✓ The coefficient of variation of the absorbances between the replicas of the standards and the samples must not exceed 30% (CV<30%).
- ✓ The R2 of the curve must be ≥0,99.
- ✓ There must be discrimination between two consecutive points on the curve (p-value< 0,05).
- ✓ The absorbance of the internal control must be between the absorbances of standard 2 and 3.
- ✓ The limit of blank (LoB) must be below the obtained limit of quantification (LLoQ).
- ✓ Positive stool samples must be above the limit of quantitation (LLoQ).
- ✓ At least 2 of the 3 fortified samples must be above the limit of quantification (LLoQ).

SYMBOLS



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- Coto L, Sousa C, Cebolla A. "Individual variability in patterns and dynamics of fecal gluten immunogenic peptides excretion after low gluten intake". Eur J Nutr. 2022 Jun;61(4):2033-2049.

MODIFICATIONS IN THE LAST VERSION

For more information, please visit our website or contract us: info@biomedal.com