INSTRUCTION FOR USE

SENSI*Spec* ELISA Buckwheat 96/48 Tests

(Cat. nr. HU0030144, HU0030145)

Enzyme Immunoassay for the Quantitative Determination of Buckwheat in Food

Sensitivity (Buckwheat)	0.06-0.35 ppm
Recovery	72-102%
Incubation Time	60 min

1. GENERAL INFORMATION

Buckwheat (Fagopyrum esculentum) belongs to the polygonaceae. With 11 % the fraction of proteins in buckwheat is high. Some of these proteins are known for being allergenic, such as the 13S globulin Fag e 1, Fag e 2 a 2S albumin or the Fag e 3 vicilin. Especially in the Asian pacific area buckwheat represents one of the most important food allergens. For buckwheat allergic persons hidden buckwheat allergens in food are a critical problem. Already very low amounts of buckwheat can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, buckwheat allergic persons must strictly avoid the consumption of buckwheat or buckwheat containing food. Cross-contamination, mostly in consequence of the production process is often noticed. The application of different grains in the same grain mill is a representative example. This explains why in many cases the existence of buckwheat residues in foods cannot be excluded. For this reason, sensitive detection systems for buckwheat residues in foodstuffs are required.

The **SENSI*Spec* ELISA Buckwheat** represents a highly sensitive detection system and is particularly capable of the quantification of buckwheat residues in grain flours, cookies, pasta and meat.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Buckwheat** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against buckwheat proteins is bound on the surface of a microtiter plate. Buckwheat containing samples or standards are given into the wells of the microtiter plate. After 20 minutes incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A peroxidase conjugated second antibody directed against buckwheat proteins is given into the wells and after 20 minutes of incubation the plate is washed again. A substrate solution is added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is inhibited by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of buckwheat is directly proportional to the colour intensity of the test sample.

3. PRECAUTIONS

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results:

1) Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).
2) All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
3) Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.
4) Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.
5) Use a separate disposable tip for each specimen to prevent cross-contamination.
6) All specimens and standards should be run at the same time, so that all conditions of testing are the same.
7) Do not mix components from different batches.
8) Do not use reagents after expiration date.
9) Check both precision and accuracy of the laboratory equipment used during the procedure (micropipettes, ELISA reader etc.).

4. HEALTH AND SAFETY INSTRUCTIONS

1) Do not smoke or eat or drink or pipet by mouth in the laboratory.
2) Wear disposable gloves whenever handling patient specimens.
3) Avoid contact of substrate and stop solution with skin and mucosa (possible irritation, burn or toxicity hazard). In case of contact, rinse the affected zone with plenty of water.

4) Handling and disposal of chemical products must be done according to good laboratory practices (GLP).

5. REAGENTS

The kit contains reagents for 96/48 determinations. They have to be stored at 2-8°C. Expiry data are found on the labels of the bottles and the outer package.

1) Microtiter plate consisting of 12/6 strips with 8 breakable wells each, coated with anti-buckwheat antibodies.
2) Buckwheat Standards (0; 2; 5; 15; 30 ppm of buckwheat): 5 vials with 2.0 mL each, dyed red, ready-to-use
3) Conjugate (anti-buckwheat-peroxidase): 15/7.5 mL, dyed red, ready-to-use.
4) Substrate Solution (TMB): 15 mL, ready-to-use.
5) Stop Solution (0.5 M H_2SO_4): 15 mL, ready-to-use.
6) Extraction and sample dilution buffer (Tris): 2/1 x 120 mL as 10x concentrate, dyed red. Dilute 1+9 with distilled water. Stored at 4°C the diluted buffer is stable for at least one week. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.
7) Washing Solution (PBS + Tween 20): 60 mL as 10x concentrate. Dilute 1+9 with distilled water. Stored at 4°C the diluted buffer is stable for at least 4 weeks. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.
8) Plastic bag to store unused microtiter strips.

6. ADDITIONAL INSTRUMENTATION AND REAGENTS (NOT PROVIDED)

6.1 Instrumentation

- 100 - 1000 µL micropipettes
- Volumetric flask
- Analytical balance
- Mortar, mixer
- Water bath
- Centrifuge
- ELISA reader (450 nm)

6.2 Reagents

- double distilled water
- Extraction additive for polyphenol containing samples (HU0030100)

7. SAMPLE PREPARATION

Due to high risk of cross-contamination all applied instruments like applicator, mortar, glass vials etc. have to be **cleaned thoroughly** before and after each sample. Buckwheat proteins could adhere to different surfaces. To identify possible cross-contamination caused by previous extractions it is strongly recommended to note the sequence of the extractions.

The following sample preparation should be applied for solid samples:

1) To maximize homogeneity and representativeness of the sample drawing, a minimum of 5 g sample should be pulverized finely in a mortar, impact mill, etc.
2) 1 g of the homogenized mixture is suspended in 20 mL of **pre-diluted** extraction and sample dilution buffer. Afterwards the suspension is incubated for 15 min in a preheated water bath at 60°C. To ensure good homogeneity, the samples should be shaken every two minutes.
3) The samples are centrifuged for 10 minutes at ≥2000 g. If it is not possible to separate the supernatant from the precipitate completely, the suspension should be filtrated if necessary.
4) 100 µL of particle-free solution are applied per well. If the results of a sample are out of the measuring range, further dilution with the **pre-diluted** extraction and sample dilution buffer is necessary. The additional dilution has to be considered when calculating the concentration.

The following sample preparation should be applied for liquid food samples:

1 mL of liquid sample is diluted in 19 mL of **pre-diluted** extraction and sample dilution buffer. Afterwards the suspension is incubated for 15 min in a pre-heated water bath at 60°C. To ensure good homogeneity, the samples should be shaken every two minutes. The process is continued at point 3 of solid sample extraction process.

Attention:

- *Do not shake the final extract to prevent from re-suspension.*
- *If after centrifugation a third layer at the top appears due to a high fatty matrix, only the middle aqueous phase should be applied to the wells.*

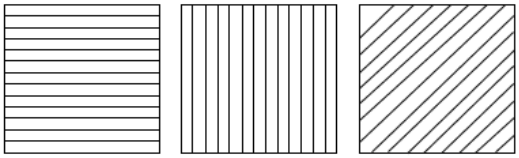
The following sample preparation should be applied for rinse water samples:

1) Adjust the pH of the sample to 8.2 (+/- 0.5)
2) 1 mL of liquid sample is diluted in 4 mL of **pre-diluted** extraction and sample dilution buffer. The process is continued at point 4 of solid food sample extraction process.

The following sample preparation should be applied for swab samples on dry surfaces:

1) Mark out 5x5 cm area or use swab directly on (e.g. uneven) area.
2) Moisten the swab in 1 mL **pre-diluted** extraction and sample dilution buffer previously applied in a test tube.

3) Swab marked area by using crosshatch (1. horizontally, 2. vertically, 3. diagonally) technique while rotating the tip.



4) Place swab into the test tube.
5) Shake the test tube for 1 minute to release the sample from the swab. The process is continued at point 4 of solid food sample extraction process.

For wet surfaces exactly the same procedure is applied without prior need to moisten the swab.
Samples prepared by applying the procedures above are at least stable for 8 h at room temperature.

8. PROCEDURE

The washing solution is supplied as 10x concentrate and has to be **diluted** 1+9 with double distilled water before use.

In any case the **ready-to-use** standards provided should be determined twofold. When samples in great quantities are determined, the standards should be pipetted once before the samples and once after the samples. For final interpretation the arithmetic mean is used for calculation.

In consideration of GLP and quality control requirements a duplicate measurement of samples is recommended.

The procedure is according to the following scheme:

1) Prepare samples as described above.
2) Pipet 100 µL **ready-to-use** standards or prepared samples in duplicate into the appropriate wells of the microtiter plate.
3) Incubate for 20 minutes at room temperature.
4) Wash the plate three times as follows: Discard the contents of the wells (dump or aspirate). Pipet 300 µL of diluted washing solution into each well. After the third repetition empty the wells again and remove residual liquid by striking the plate against a paper towel. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbencies.
5) Pipet 100 µL of conjugate (anti-buckwheat-peroxidase) into each well.
6) Incubate for 20 minutes at room temperature.
7) Wash the plate as outlined in 4.
8) Pipet 100 µL of substrate solution into each well.
9) Allow the reaction to develop in the dark (e.g. cupboard or drawer; the chromogen is light-sensitive) for 20 minutes at room temperature.
10) Stop enzyme reaction by adding 100 µL of stop solution (0.5 M H_2SO_4) into each well. The blue colour will turn yellow upon addition.
11) After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The colour is stable for 30 minutes.

9. CALCULATION OF RESULTS

The following evaluation procedure should be applied for all **food samples** prepared by the procedure as stated in *Sample Preparation*:
The ready-to-use standards are prepared for a direct determination of food sample concentrations. The dilution (1:20) of samples in the extraction process as described in the above stated sample preparation procedure is already considered. Additional dilution due to high sample concentration has to be account-ted for.

1) Calculate the average optical density (OD 450 nm) for each set of reference standards or samples.
2) Construct a standard curve by plotting the mean optical density obtained for each reference standard against its concentration in ppm on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis. Alternatively, the evaluation can be carried out by software. In this case the 4-parameter method should be preferred.
3) Using the mean optical density value for each sample, determine the corresponding concentration of buckwheat in ppm from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

The following evaluation procedure should be applied for **rinse water samples** prepared according to the procedure stated *Sample Preparation*:

1) Apply the evaluation procedure food samples as stated above.
2) Divide the result by 4 in order to compensate the different dilution factor of the extraction procedure to receive the sample concentration in mg/L.

The following evaluation procedure should be applied for **swab samples** prepared according to the procedure stated in *Sample Preparation*:

1) Apply the evaluation procedure food samples as stated above.
2) Multiply the result (ppm) by 2 in order to compensate the different dilution factor of the extraction procedure to receive the sample concentration in ng/cm^2.

10. TYPICAL STANDARD VALUES

The following table contains an example for a typical standard curve. The binding is calculated as percent of the absorption of the 30 ppm standard. These values are only an example and should not be used instead of the standard curve which has to be measured in every new test.

Buckwheat (ppm)	% binding of 30 ppm
30	100
15	70
5	32
2	15
0	2

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Buckwheat test** is 0.06 ppm related to the standard curve.

Validation experiments with common matrices resulted in the following mean LODs [ppm].

Barley flour	0.10
Rye flour	0.20
Wheat flour	0.12
Rice flour	0.09
Corn flour	0.10
Soy flour	0.09
Cookies	0.19
Pasta	0.24
Meat	0.18

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Buckwheat test** is 2 ppm buckwheat.

As matrices can have variable influence on the LOD in specific cases, and the range of matrices that was tested is of course limited, the end user if needed may evaluate its own LOD values depending on the matrices to be analyzed.
Alternatively, any results below LOQ should be just reported quantitatively as "< LOQ".

11.2 Precision

Intra-Assay Precision	5.3%
Inter-Assay Precision	7.5%
Inter-Extraction Precision	8.7%

11.3 Linearity

The serial dilution of spiked samples (grain flours, cookie, pasta and meat) resulted in a mean dilution linearity of 90-109%.

11.4 Cross-reactivity

For the following foods no cross-reactivity could be detected:

Adzuki bean	Duck	Pecan
Almond	Egg white, dried	Pepper, black
Apricot	Egg, dried	Pine seed
Barley	Fennel	Pistachio
Bean, white	Fenugreek	Poppy
Beef, cooked	Garden cress	Pork
Beef, raw	Garlic, fresh	Potato
Bovine gelatin	Garlic, granulated	Prawn, cooked
Brazil nut	Ginger, ground	Prawn, raw
Cabbage, white	Ginger, fresh	Pumpkin seed
Caraway seeds	Gliadin	Radish
Cardamom	Goat's milk	Rapeseed
Carob gum	Guar gum	Rice
Carrot	Gum arabic	Rye
Cashew	Horseradish	Saccharose
Celery	Kidney bean	Sesame
Cherry	Kiwi	Shrimps
Chestnut	Lamb	Soy flour
Chicken	Leek	Soy lecithin
Chickpea	Lentil	Split pea
Cinnamon	Lupin	Sunflower seed
Clove	Mustard, yellow	Thyme
Cocoa	Nutmeg	Tofu
Coconut	Oats	Tomato
Cod	Onion	Turkey
Corn	Pea	Turmeric
Cow' milk	Peach	Walnut
Cumin	Peanut	Wheat
Dill		

For the following commodities of the table above the results were between 0.5*LOQ and LOQ of the kit. So, it cannot be completely excluded that these matrices may provide values above the LOQ in specific cases:

Oats	Walnut

The following cross reactions were determined:

Cayenne	0.0012%
Chia seeds	0.0003%
Chili	0.0017%
Flaxseed	0.0004%
Hazelnut	0.0003%
Macadamia	0.0007%
Paprika	0.0010%

11.5 Recovery

Mean recovery was determined by spiking samples with different amounts of buckwheat:

Barley flour	99%
Rye flour	85%
Wheat flour	99%
Rice flour	96%
Corn flour	99%
Soy flour	84%
Cookie	95%
Pasta	101%
Meat	78%

12. LIABILITY

Gold Standard Diagnostics Budapest shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.

Gold Standard Diagnostics Budapest shall not be liable for the unsafe use of the kit out of the current European safety regulations