

Sensitivity (Lysozyme)	2/10 ppb
Recovery	85 - 99%
Incubation Time	60 min

Test Instruction

Lysozyme

96/48 Tests

Enzyme Immunoassay
for the Quantitative
Determination of
Lysozyme in Food

Cat.-No.: LYS-E01/E04

Version: March 19th, 2020

This document represents a combined test instruction for the products LYS-E01 (96 well) and LYS-E04 (48 well).

General Information

Hen's egg (*Gallus gallus*) is very rich of proteins and represents an important food source for humans. While proteins of egg yolk only have minor allergenicity, many proteins of egg white are known to be allergenic. In addition to ovalbumin, ovotransferrin, ovomucoid and livetin, lysozyme represents an important allergen. Primarily lysozyme is used as a preservative in wine and cheese industry. For allergic persons the consumption of lysozyme represents a critical problem. Already very low amounts of the allergen can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, lysozyme allergic persons must strictly avoid the consumption of lysozyme containing food. Non-declared addition of lysozyme in food is hazardous for allergic people. Cross-contamination, mostly in consequence of the production process, is also problematic. Since July 1, 2012 the European Union requests allergen labeling for wine if milk or egg proteins are used during the production and are still present at a detection level of 0.25 mg/L or greater. Thus for the detection of lysozyme residues, sensitive assay systems are required.

The **Immunolab Lysozyme ELISA** represents a highly sensitive detection system for lysozyme and is particularly capable of the quantification of lysozyme residues in wine and cheese.

Principle of the Test

The **Immunolab Lysozyme** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against lysozyme is bound on the surface of a microtiter plate. Lysozyme 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 lysozyme 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 lysozyme is directly proportional to the colour intensity of the test sample.

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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 (micropipets, ELISA reader etc.).

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).

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-lysozyme antibodies.
2. Lysozyme standards (0; 25; 50; 100; 250 ppb of lysozyme): 5 vials with 2.0 mL each, dyed red, ready-to-use
3. Conjugate (anti-lysozyme-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 H₂SO₄): 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.
9. Instruction Manual.

Additional Instrumentation and Reagents (not provided)

Instrumentation

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

Reagents

- double distilled water
- NaCl (if applicable)

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. 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 wine samples:

1. 1 ml of the wine sample is diluted in 19 mL of **pre-diluted** extraction buffer.
2. 100 µL of the wine 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 cheese and all other kinds of 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 10 mL of **pre-diluted** extraction buffer (in which 10 g / 100 mL NaCl is diluted prior to use). 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. Dilute 100 µL of supernatant in 900 µL **pre-diluted** extraction buffer (without NaCl).
5. 100 µL of this 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.

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-lysozyme-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 H₂SO₄) 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.

Calculation of results

The ready-to-use standards are prepared for a direct determination of wine sample concentrations. The dilution of wine samples in the extraction process as described in the above stated sample preparation procedure for wine samples is already considered. In case of using the extraction process for cheese or other samples than wine, the determined concentration **has to be multiplied by 5** in order to get the sample concentration. Additional dilution due to high sample concentration has also to be accounted 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 stan-

dard against its concentration in ppb 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.

- Using the mean optical density value for each sample, determine the corresponding concentration of lysozyme in ppb from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

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 250 ppb standard. These values are only an example and should not be used instead of the standard curve which has to be measured in each new test.

Lysozyme (ppb)	% binding of 250 ppb
250	100
100	63
50	30
25	16
0	2

Performance

Sensitivity

The limit of detection (LOD) of the **Immunolab Lysozyme test** is 2 ppb applying sample preparation method for wine and 10 ppb applying sample preparation method for other matrices.

The limit of quantification (LOQ) of the **Immunolab Lysozyme test** is 25 ppb applying sample preparation method for wine and 125 ppb applying sample preparation method for other matrices.

Due to the variety of sample matrices and their influence on the blank, results less than the LOQ should be treated as negative.

Cross-reactivity

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

Adzuki	Cow's milk	Pecan
Almond	Cumin	Pepper
Apricot	Duck	Pine seed
Barley	Fenugreek	Pistachio
Bean, white	Gliadin	Poppy seed
Beef	Goat's milk	Pork
Bovine gelatin	Guar gum	Potato
Brazil nut	Hazelnut	Prawn, cooked
Buckwheat	Kidney bean	Prawn, raw
Caraway	Kiwi	Pumpkin seed
Carob gum	Lamb	Rice
Carrot	Lentil	Rye
Cayenne	Linseed	Saccharose
Celery	Lupin	Sesame
Cherry	Macadamia	Shrimps
Chestnut	Mustard	Soy
Chia	Nutmeg	Soy lecithin
Chickpea	Oats	Split peas
Chili	Onion	Sunflower seeds
Cocoa	Paprika	Tomato
Coconut	Pea	Turkey
Cod	Peach	Walnut
Corn	Peanut	Wheat

The following cross-reactions were determined:

Cashew	< 0.000005%
Conalbumin	< 0.0001%
Chicken	< 0.000006%
Egg white protein, total	2.2%
Ovalbumin	< 0.0001%
Ovomucoid	< 0.0001%

Precision

Intra-assay Precision	2 - 6%
Inter-assay Precision	2 - 3%

Linearity

The serial dilution of spiked samples (white wine, red wine & cheese) resulted in a dilution linearity of 87% - 98%.

Recovery

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

Wine	99%
Cheese	85%