BioVendor Group

ENG

Instructions for Use:

HUMAN LACTOFERRIN ELISA

Catalogue number:

RHK329-01R

RHK329-02R

For research use only!

BioVendor R&D

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HISTORY OF CHANGES

Previous version	Current version
	ENG.001.A
New edition	

1. INTENDED USE

The human lactoferrin ELISA kit is to be used for the *in vitro* quantitative determination of human lactoferrin in plasma, urine, feces, breast milk and cell culture supernatant samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.
The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

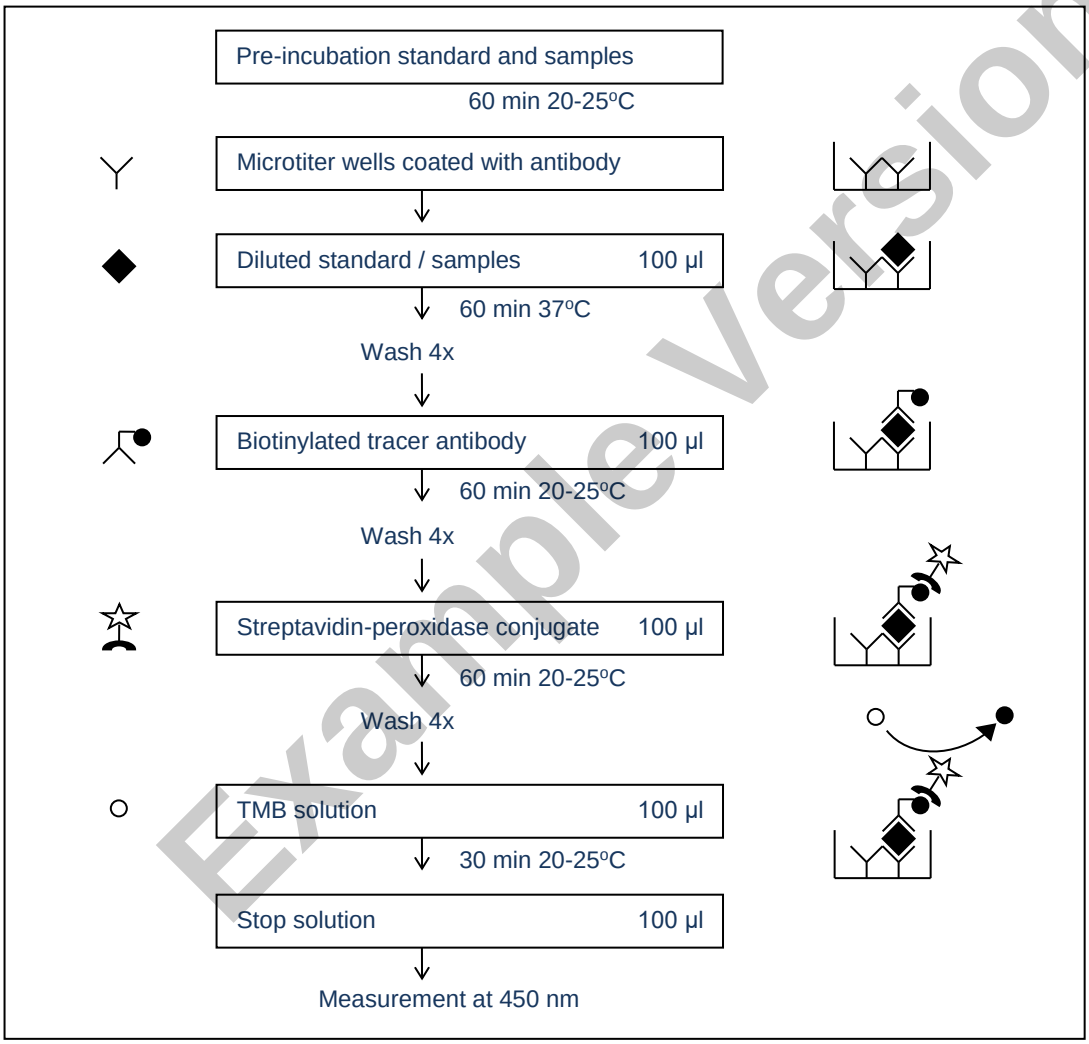
Human lactoferrin (LF) is an 80 kDa glycoprotein found concentrated in the secondary granules of the neutrophils. In addition, lactoferrin can be found in epithelia and most body fluids and secretions. Lactoferrin was first isolated from human milk and plays an important part in the immune system by helping to fight infections. It has the ability to bind iron and possesses five different enzyme activities: DNase, RNase, ATPase, phosphatase, and malto-oligosaccharide hydrolysis. Lactoferrin is a natural anti-bacterial, anti-fungal and anti-viral protein, it is an antioxidant and also possesses immunomodulatory properties. Furthermore, lactoferrin promotes the health of the gastro-intestinal system by improving the intestinal microbalance. Lactoferrin is secreted in plasma by neutrophils. Plasma of healthy individuals contains ~190-500 ng/ml LF. The lactoferrin plasma concentration represents a positive relation to the total pool of neutrophils and the rate of neutrophil turnover. Upon inflammation, lactoferrin is released from the secondary neutrophil granules into the extracellular medium. Therefore, the extracellular lactoferrin concentration can be used as an index for neutrophil activation.
The iron binding property of lactoferrin is considered to be an important antimicrobial function. Human lactoferrin binds via its highly positively charged amino-terminus to bacterial products. It kills various bacteria, most probably by inducing intracellular changes in these bacteria without affecting the membrane permeability. Cleavage by pepsin of lactoferrin leads to the release of lactoferricin H. This 47amino acid peptide has more antimicrobial activity than its precursor and it can inhibit the classical but not the alternative complement pathway.
Urine or breast milk of healthy persons contain ~30 ng/ml and ~500 µg/ml LF, respectively. During infection, the LF concentration can raise 10-100-fold. In feces of healthy persons, ~1 µg/g LF can be detected, whereas in feces derived from colon cancer or inflammatory bowel disease (IBD) patients, LF levels range from ~75-310 µg/g. Faecal lactoferrin is useful as a sensitive and specific marker in identifying intestinal inflammation such as Crohn's disease and IBD. Combination of several markers, such as calprotectin, defensin, elastase, MPO, I-FABP and MAdCAM, may be useful for classifying IBD, as well as for identifying tumor grade and to confirm remission/response to treatment. Therefore, the human lactoferrin ELISA is a sensitive, non-invasive tool for monitoring disease activity.

3. KIT FEATURES

- Working time of 4½ hours.
- Minimum concentration which can be measured is 0.4 ng/ml.
- Measurable concentration range of 0.4 to 100 ng/ml.
- Working volume of 100 µl/well.

Cross-reactivity
Cross-reactivity for other species or proteins/peptides has not been tested.

4. TEST PRINCIPLE



- The human lactoferrin ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time 4½ hours.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples and standards are incubated in microtiter wells coated with antibodies recognizing human lactoferrin.
- Biotinylated tracer antibody will bind to captured human lactoferrin.
- Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.
- Streptavidin-peroxidase conjugate will react with the substrate, tetramethylbenzidine (TMB).

- The enzyme reaction is stopped by the addition of oxalic acid.
- The absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human lactoferrin standards (log).
- The human lactoferrin concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

5. TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.
- If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
- Since exact conditions may vary from assay to assay, a standard curve must be established for every run. Samples should be referred to the standard curve prepared on the same plate.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, sample, tracer, streptavidin-peroxidase and buffers should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of supplied covers during incubation steps is necessary.
- The waste disposal should be performed according to your laboratory regulations.

6. REAGENT SUPPLIED

Kit component	Quantity RHK329-01R	Quantity RHK329-02R	Color code
Wash buffer 40x	1 vial (30 ml)	1 vial (30 ml)	Colorless
Dilution buffer 10x	1 vial (20 ml)	1 vial (20 ml)	Green
Sample dilution buffer 10x	1 vial (15 ml)	1 vial (15 ml)	Blue
Standard	2 vials, lyophilized	4 vials, lyophilized	White
Tracer, biotinylated	1 vial, 1 ml lyophilized	2 vials, 1 ml lyophilized	White
Streptavidin-peroxidase 100x	1 tube, 0.25 ml in solution	1 tube, 0.25 ml in solution	Brown
TMB substrate	1 vial (11 ml)	1 vial (22 ml)	Brown
Stop solution	1 vial (20 ml)	1 vial (20 ml)	Red
12 Microtiter strips, pre-coated	1 plate	2 plates	

Table 1

- Upon receipt, store individual components at 2 - 8°C. Do not freeze.
- Do not use components beyond the expiration date printed on the kit label.
- The standard and tracer in lyophilized form and the streptavidin-peroxidase in concentrated solution are stable until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The exact amount of the standard is indicated on the label of the vial and the Certificate of Analysis.
- The standard is single use. After reconstitution the standard cannot be stored.
- Once reconstituted the tracer is stable for 1 month if stored at 2 - 8°C.
- The streptavidin-peroxidase can only be stored in concentrated solution and cannot be stored once diluted.
- Upon receipt, foil pouch around the plate should be vacuum-sealed and unpunctured. Any irregularities to aforementioned conditions may influence plate performance in the assay.
- Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality guaranteed for 1 month if stored at 2 - 8°C.

7. MATERIALS REQUIRED BUT NOT PROVIDED

- Calibrated micropipettes and disposable tips.
- Distilled or de-ionized water.
- Plate washer: automatic or manual.
- Polypropylene tubes.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.
- Adhesive covers can be ordered separately. Please contact your local distributor.
- Centrifuge for 1 ml tubes.

8. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not under any circumstances add sodium azide as preservative to any of the components.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by manufacturer.
- The assay has been optimized for the indicated standard range. Do not change the standard range.
- Open vials carefully: vials are under vacuum.
- It is advised to spin down streptavidin-peroxidase tubes before use.
- Do not ingest any of the kit components.
- Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
- The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
- The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advice.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.
- Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.
- The standard is of human origin. It was tested for various viruses and found negative. Since no test method can offer complete assurance that infectious agents are absent, this reagent should be handled as any potentially infectious human serum or blood specimen. Handle all materials in contact with this reagent according to guide-lines for prevention of transmission of blood-borne infections.

9. PREPARATION OF REAGENTS

Allow all the reagents to acclimatize to room temperature (20 – 25 °C) prior to use. Return to proper storage conditions immediately after use.

Wash buffer

Prepare wash buffer by mixing 30 ml of 40x wash buffer with 1,170 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 40x wash buffer with 39 parts of distilled or de-ionized water.

Dilution buffer

Prepare dilution buffer by mixing 20 ml of the 10x dilution buffer with 180 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the

desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer with 9 parts of distilled or de-ionized water. Concentrated dilution buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37 °C. Do not shake the solution.

Sample dilution buffer

Acclimatize the 10x sample dilution buffer to room temperature (20 – 25 °C) prior to use. Make sure to homogenize the 10x sample dilution buffer carefully, do not shake the solution. Prepare sample dilution buffer by mixing 15 ml of the 10x sample dilution buffer with 135 ml of prepared dilution buffer, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of sample dilution buffer by diluting 1 part of the 10x sample dilution buffer with 9 parts of dilution buffer.

Standard solution

The standard is reconstituted by pipetting the amount of sample dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure 1. Prepare each human Lactoferrin standard in polypropylene tubes by serial dilution of the reconstituted standard with sample dilution buffer as shown in Figure 1*. After reconstitution the standard cannot be stored for repeated use.

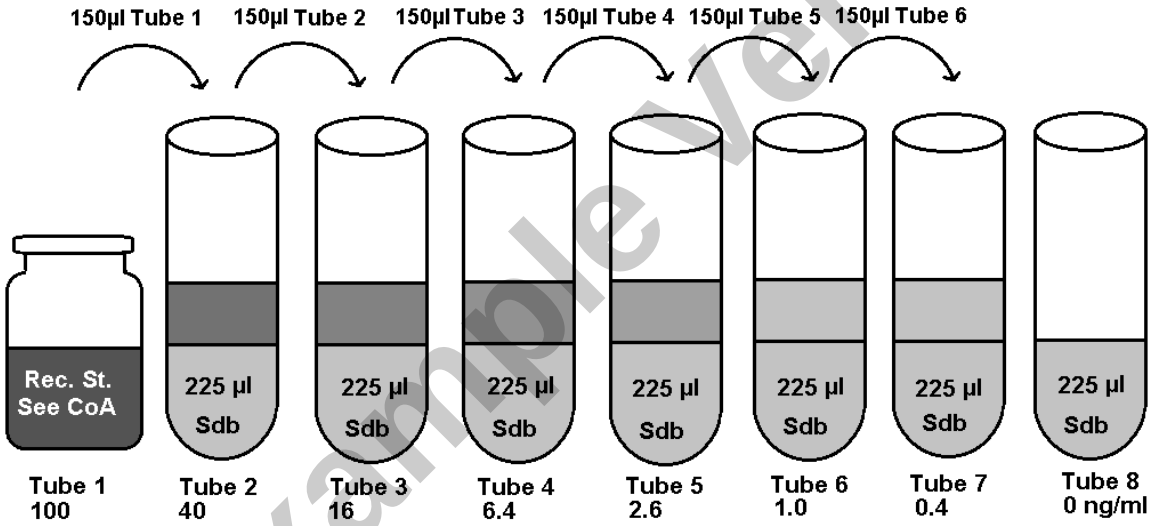


Figure 1

*) CoA: Certificate of Analysis, St: Standard, Sdb: Sample Dilution buffer

Tracer solution

The tracer is reconstituted by pipetting 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml tracer with 11 ml dilution buffer, which is sufficient for 1 x 96 tests. Where less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 11 parts of dilution buffer.

Streptavidin-peroxidase solution

It is advised to spin down streptavidin-peroxidase tubes before use. Prepare the streptavidin-peroxidase solution by mixing 0.25 ml of the 100x streptavidin-peroxidase solution with 24.75 ml dilution buffer, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of streptavidin-peroxidase solution by diluting 1 part of the 100x streptavidin-peroxidase solution with 99 parts of dilution buffer.

10. PREPARATION OF SAMPLES

Collection and handling

Plasma

Please be aware that human lactoferrin is released from neutrophils into serum in the process of blood coagulation. This will lead to false positive results. It is therefore advised to use 'careful plasma', which can be obtained as follows.
Keep freshly collected blood on ice. Within 20 minutes after blood sampling, separate plasma by centrifugation: 1,500g at 4 °C for 15 min. Remove plasma and transfer to fresh polypropylene tube. Be careful to not disturb white cells in the buffy coat. Recentrifuge the transferred plasma in order to avoid every contamination with white blood cells: 1,500g at 4 °C for 15 min. Most reliable results are obtained if EDTA or heparin plasma is used.

Feces

Lactoferrin can be measured in feces if samples are extracted using the following extraction buffer: 0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM CaCl2, 0.1 M citric acid monohydrate, 5 g/l BSA and 0.25 mM thimerosal (pH 8.0). Add 5 ml extraction buffer to 100 mg sample (giving a dilution factor of 51, assuming the density of feces to be 1 g/ml). Vortex samples and filter the samples to remove coarse particles (> 0.6 mm). Shake the filtrate for 20 minutes and centrifuge samples: 10,000 g at 4 °C for 20 minutes. Use supernatant for analysis.

Storage

Store samples below -20 °C, preferably at -70 °C in polypropylene tubes. Storage at -20 °C can affect recovery of human lactoferrin. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of human lactoferrin activity and give erroneous results. Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.
Before performing the assay, samples should be brought to room temperature (18 – 25 °C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Lactoferrin is highly absorbing to Ig and other proteins. Therefore, samples can only be measured accurately if diluted with supplied sample dilution buffer.

Plasma samples

Human lactoferrin can be measured accurately if plasma samples are diluted at least 4x with supplied sample dilution buffer in polypropylene tubes. Incubate 1 hour at room temperature before pipetting into the plate.
Note that most reliable results are obtained with heparin plasma.

Urine samples

Human lactoferrin can be measured accurately if urine samples are diluted at least 20x with supplied sample dilution buffer in polypropylene tubes. Incubate 1 hour at room temperature before pipetting into the plate.

Feces samples

Human lactoferrin can be measured accurately if feces samples are diluted at least 100 to 1,000x with supplied sample dilution buffer in polypropylene tubes. Incubate 1 hour at room temperature before pipetting into the plate.

Breast milk

Human lactoferrin can be measured accurately if breast milk samples are diluted at least 10,000x with supplied sample dilution buffer in polypropylene tubes. Incubate 1 hour at room temperature before pipetting into the plate.

Remark regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline. The recovery of human lactoferrin from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human lactoferrin.
Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

Guideline for dilution of samples

Please see the table below for recommended sample dilutions. Volumes are based on a total volume of at least 230 µl of diluted sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend to use at least 10 µl of sample.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of Sample Dilution buffer required
1.	10x	Not necessary	25 µl (sample)	225 µl
2.	20x	Not necessary	15 µl (sample)	285 µl
3.	50x	Not necessary	10 µl (sample)	490 µl
4.	100x	Not necessary	10 µl (sample)	990 µl
5.	500x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	490 µl
6.	1,000x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	990 µl
7.	2,000x	Recommended: 20x (see nr.2)	10 µl (pre-dilution)	990 µl
8.	5,000x	Recommended: 50x (see nr.3)	10 µl (pre-dilution)	990 µl

Table 2

11. ASSAY PROCEDURE

Bring all reagents to room temperature (20 – 25 °C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 – 8 °C.
2. Dilute samples and standard series in sample dilution buffer and incubate 1 hour at room temperature.
3. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.
4. Cover the tray. Tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
5. Incubate the strips or plate for 1 hour at 37 °C.
6. Wash the plates 4 times with wash buffer using a plate washer or as follows*:
   a. Carefully remove the cover, avoid splashing.
   b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
   c. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate as described in 6b.
   d. Repeat the washing procedure 6b/6c three times.
   e. Empty the plate and gently tap on thick layer of tissues.
7. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 3. Do not touch the side or bottom of the wells.
8. Cover the tray and incubate the tray for 1 hour at room temperature.
9. Repeat the wash procedure described in step 6.
10. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 3. Do not touch the side or bottom of the wells.
11. Cover the tray, incubate the tray for 1 hour at room temperature.
12. Repeat the wash procedure described in step 6.
13. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 3. Do not touch the side or bottom of the wells.
14. Cover the tray, incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminium foil is recommended.
15. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 13. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
16. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

*) In case plate washer is used, please note: use of a plate washer can result in higher background and decrease in sensitivity. We advise validation of the plate washer with the manual procedure.

Make sure the plate washer is used as specified for the manual method.

12. CALCULATIONS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.3.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

13. QUALITY CONTROL

The Certificate of Analysis is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the Certificate of Analysis are to be used as a guideline only. The results obtained by your laboratory may differ.
This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the BioVendor immunoassay, the possibility of interference cannot be excluded.
For optimal performance of this kit, it is advised to work according to good laboratory practice.

14. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before expiry date of the product. A written complaint containing lot number of the product and experimental data should sent to info@biovendor.com
Suggestions summarized below in Table 3 can be used as a guideline in the case of unexpected assay results.

Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
•	•		•	•	Kit materials or reagents are contaminated or expired
•					Incorrect reagents used
•		•	•		Lyophilized reagents are not properly reconstituted
•	•	•	•	•	Incorrect dilutions or pipetting errors
•		•			Improper plastics used for preparation of standard and/or samples
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•					Assay performed before reagents were brought to room temperature
•	•	•	•	•	Procedure not followed correctly
				•	Omission of a reagent or a step
		•			Poor mixing of samples
	•		•		Low purity of water
	•	•			Strips were kept dry for too long during/after washing
	•	•	•		Inefficient washing
	•	•			Cross-contamination from other samples or positive control
		•	•		TMB solution is not clear or colorless
•	•				Wrong filter in the microtiter reader
	•	•			Air bubbles

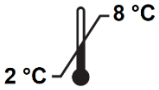
Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

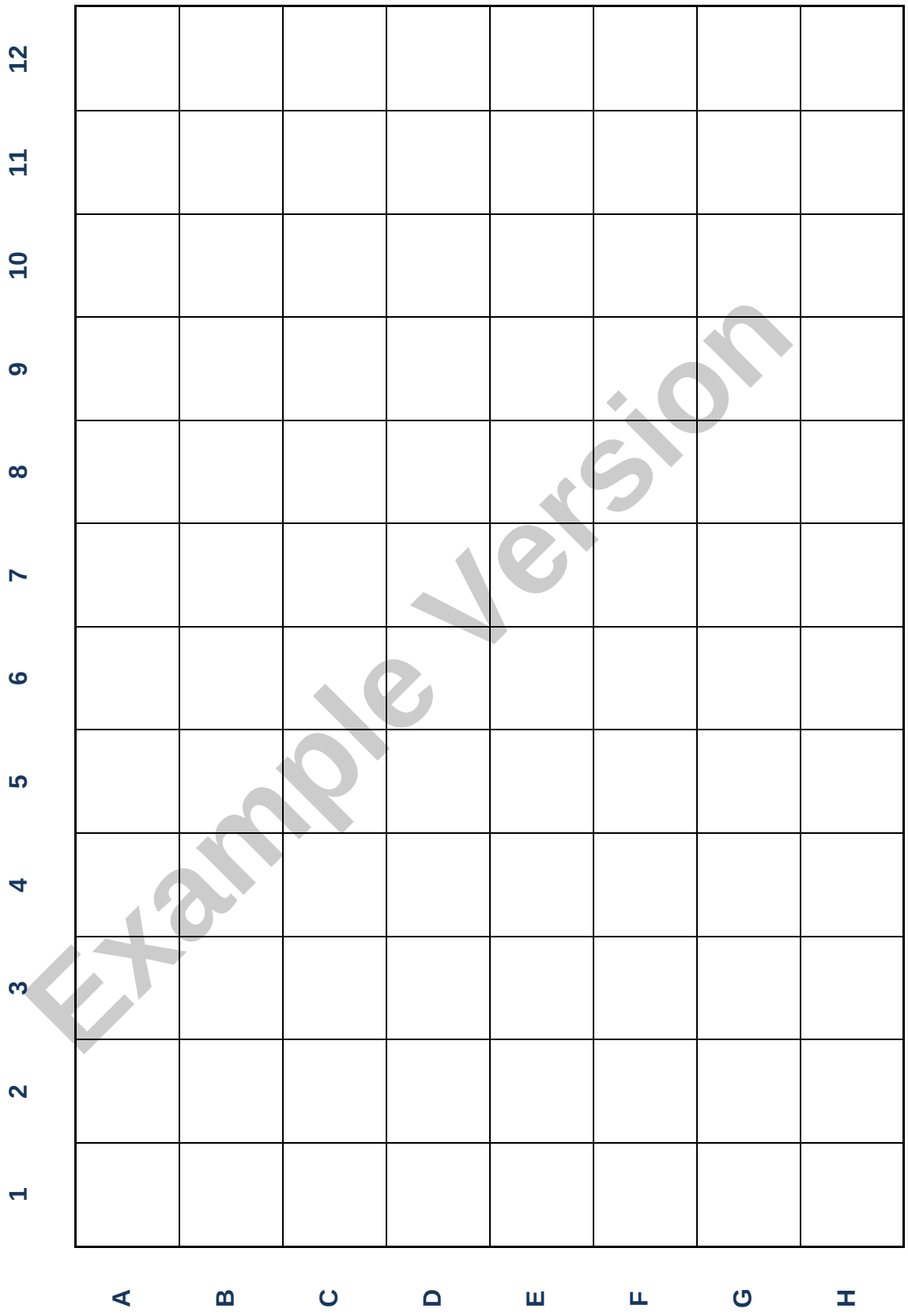
Table 3

15. REFERENCES

1. Lutykhina, I et al; Recombinant pseudoadenovirus nanostructure with the human lactoferrin gene: Production and study of lactoferrin expression and properties in vivo. Mol Gen Microbiology and Virology 2009, *24*:32
2. Sarsu, S et al; The llace of calprotectin, lactoferrin, and high-mobility group box 1 protein on diagnosis of acute appendicitis with children. Indian J Surg January 2016

16. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Caution
	Use by date
	Temperature limit
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks



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