Human LL-37

HK321

Edition 08-16

ELISA KIT
PRODUCT INFORMATION & MANUAL

Read carefully prior to starting procedures!
For use in laboratory research only
Not for clinical or diagnostic use



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Note that this user protocol is not lot-specific and is representative for the current specifications of this product. Please consult the vial label and the Certificate of Analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions.

For research use only. Not for use in or on humans or animals or for diagnostics. It is the responsibility of the user to comply with all local/state and federal rules in the use of this product. Hycult Biotech is not responsible for any patent infringements that might result from the use or derivation of this product.

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1. INTENDED USE

The human LL-37 ELISA kit is to be used for the *in vitro* quantitative determination of human LL-37 in cell culture supernatant, cervicovaginal secretions, plasma, saliva, serum and urine 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

Cathelicidins are a family of antimicrobial proteins predominantly found in the peroxidase-negative granules of neutrophils. The cathelicidins are synthesized as preproproteins. Within the neutrophils, they are stored in granules as inactive proforms after removal of the signal peptide. The active biologic domains of the cathelicidins generally reside in the C-terminus. The C-terminal antimicrobial peptides are activated when cleaved from the proforms of the cathelicidins by serine proteases from azurophil granules. Human cationic antimicrobial protein (hCAP)18 is the only human cathelicidin identified to date. The antibacterial C-terminus of hCAP-18, LL-37 (37 amino acids), has been shown to exert broad antimicrobial activity towards gram-negative as well as gram-positive bacteria and to have synergistic antibacterial effects with the defensins. For instance, deficiency in saliva LL-37 accords with occurrence of periodontal disease in patients with morbus Kostmann. Moreover, it functions as a chemotactic agent for neutrophils, monocytes and T cells. LL-37 is markedly resistant to proteolytic degradation and to a limited extent also cytotoxic towards mammalian cells.

LL-37 was demonstrated to be present in plasma in levels from 25 – 250 ng/ml and to be enhanced in infectious diseases.

3. KIT FEATURES

- Working time of 3½ hours.
- Minimum concentration which can be measured is 0.1 ng/ml.
- Measurable concentration range of 0.1 to 100 ng/ml.
- Working volume of 100 µl/well.

Cross-reactivity

Potential cross-reacting proteins detected in the human LL-37 ELISA:

Cross reactant	Reactivity
Bovine LL-37	negative
Dog LL-37	negative
Goat LL-37	negative
Horse LL-37	negative
Mouse LL-37	negative
Pig LL-37	negative
Rabbit LL-37	negative
Rat LL-37	negative
Sheep LL-37	negative

Table 1

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

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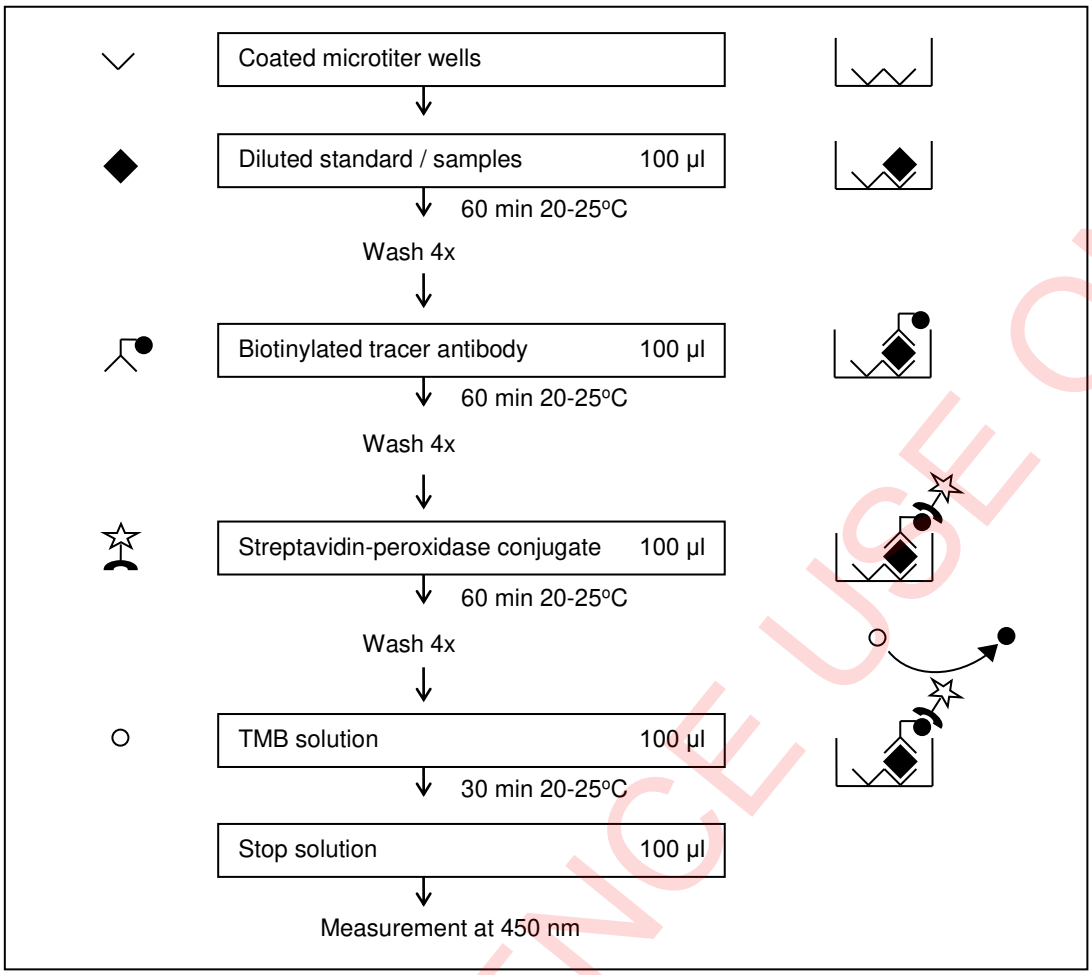
4. PROTOCOL OVERVIEW



- The human LL-37 ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay with a working time of 3½ 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 coated micro titer wells recognizing human LL-37.
- Biotinylated tracer antibody will bind to captured human LL-37.
- 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 LL-37 standards (log).
- The human LL-37 concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

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5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat. #	Quantity HK321-01	Quantity HK321-02	Color code
Wash/dilution buffer A 20x	WBDB53	1 vial (60 ml)	1 vial (60 ml)	Colorless
Wash/dilution buffer B 40x	WBDB31	1 vial (30 ml)	1 vial (30 ml)	Colorless
Standard		2 vials, lyophilized	4 vials, lyophilized	White
Tracer, biotinylated		1 vial, 1 ml lyophilized	2 vials, 1 ml lyophilized	White
Streptavidin-peroxidase 100x	CON03	1 vial, 0.25 ml in solution	1 vial, 0.25 ml in solution	Brown
TMB substrate	TMB050/TMB100	1 vial (11 ml)	1 vial (22 ml)	Brown
Stop solution	STOP110	1 vial (22 ml)	1 vial (22 ml)	Red
12 Microtiter strips, pre-coated		1 plate	2 plates	
Certificate of Analysis		1	1	
Manual		1	1	
Data collection sheet		2	2	

Table 2

- 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.
- After reconstitution the standard is ready to use and 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 is not stable when stored 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.

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.

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6. 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 micro wells 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.

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7. SAMPLE PREPARATION

Collection and handling

Serum and plasma

Please be aware that human LL-37 is released from neutrophils into serum in the process of blood coagulation. This will lead to false positive results. Most reliable results are obtained if EDTA plasma is used. 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: 1500xg 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: 1500xg at 4°C for 15 min.

Cervicovaginal secretion samples

Place swabs in a tube containing 5 ml sterile phosphate buffered saline and immediately put on ice. Centrifuge samples at 800 g for 10 minutes at 4°C and separate supernatants from the cell pellet. Store samples at −80°C.

Urine

Collect urine using normal aseptic techniques. Centrifuge the urine to remove debris (1500xg at 4 °C for 15 min). Transfer urine to a fresh polypropylene tube.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of human LL-37. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of human LL-37 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

Serum and plasma samples

Human LL-37 can be measured accurately if plasma samples are diluted at least 20x with supplied wash/dilution buffer in polypropylene tubes.

Note that most reliable results are obtained with EDTA plasma.

Cervicovaginal secretions samples

Human LL-37 can be measured accurately if cervicovaginal secretions samples are diluted at least 10x with supplied wash/dilution buffer in polypropylene tubes.

Saliva samples

Human LL-37 can be measured accurately if saliva samples are diluted at least 5x with supplied wash/dilution buffer in polypropylene tubes.

Urine samples

Human LL-37 can be measured accurately if urine samples are diluted at least 10x with supplied wash/dilution buffer in polypropylene tubes.

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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 LL-37 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 LL-37.

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 Wash/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.	1000x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	990 µl
7.	2000x	Recommended: 20x (see nr.2)	10 µl (pre-dilution)	990 µl
8.	5000x	Recommended: 50x (see nr.3)	10 µl (pre-dilution)	990 µl

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8. REAGENT PREPARATION

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

Wash / dilution buffer

Dilute 20x concentrated wash/dilution buffer A by mixing 60 ml with 540 ml distilled or de-ionized water. Dilute 40x concentrated wash/dilution buffer B by mixing 30 ml with 570 ml distilled or de-ionized water. Finally combine both solutions equally and mix well. The wash/dilution buffer is sufficient for 2 x 96 tests.

In case less tests are required, prepare the required volume by dilution of 1 part 20x concentrated wash/dilution buffer A with 9 parts of distilled or de-ionized water and 1 part 40x concentrated wash/dilution buffer B with 19 parts of distilled or de-ionized water. Finally combine both solutions equally and mix well.

Concentrated buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated buffer can be warmed up to 37°C. Do not shake the solution.

Standard solution

The standard is reconstituted by pipetting the amount of wash/dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure 1. Prepare each human LL-37 standard in polypropylene tubes by serial dilution of the reconstituted standard with wash/dilution buffer as shown in Figure 1*. After reconstitution the standard cannot be stored for repeated use.

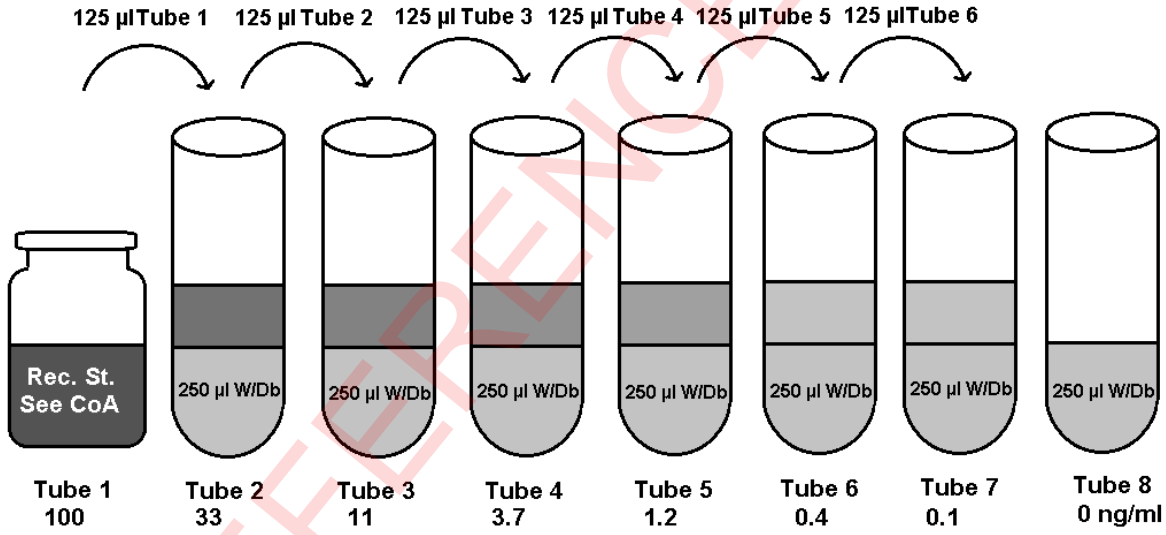

Figure 1

*) CoA: Certificate of Analysis, Rec. St: Reconstituted Standard, W/Db: Wash/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 wash/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 wash/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 wash/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 wash/dilution buffer.

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9. ELISA PROTOCOL

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. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.
3. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
4. Incubate the strips or plate for 1 hour at room temperature.
5. Wash the plates 4 times with wash/dilution 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/dilution buffer to each well, wait 20 seconds, empty the plate as described in 5b.
   d. Repeat the washing procedure 5b/5c three times.
   e. Empty the plate and gently tap on thick layer of tissues.
6. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
7. Cover the tray and incubate the tray for 1 hour at room temperature.
8. Repeat the wash procedure described in step 5.
9. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
10. Cover the tray and incubate the tray for 1 hour at room temperature.
11. Repeat the wash procedure described in step 5.
12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
13. Cover the tray and 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 micro well strips to direct sunlight. Covering the plate with aluminum foil is recommended.
14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
15. 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.

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10. INTERPRETATION OF RESULTS

- 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.

11. 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-contamination, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- The waste disposal should be performed according to your laboratory regulations.

Technical support

Do not hesitate to contact our technical support team at support@hycultbiotech.com for inquiries and technical support regarding the human LL-37 ELISA.

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12. QUALITY CONTROL

The Certificate of Analysis included in this kit 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 Hycult Biotech immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advised to work according to good laboratory practice.

13. PERFORMANCE CHARACTERISTICS

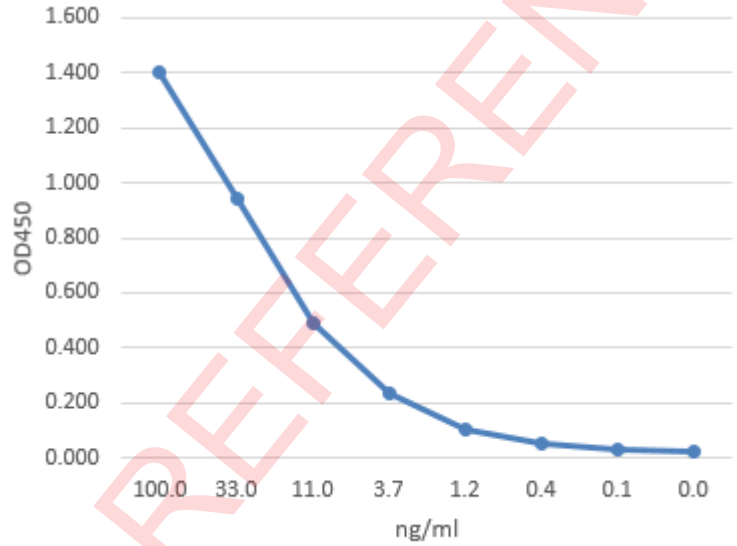
Inter and intra variation

The inter variation of the HK321 ELISA kit had an average CV of 6.1%. The lowest CV value was 1.1% and the highest 6.5%.

The intra variation had an average CV of 5.3%. The lowest CV value was 3.9% and the highest 6.0%.

Measurement of hCAP18

The HK321 LL-37 ELISA does not discriminate between LL-37 (the processed form of full length hCAP18 protein) and full length, precleaved hCAP18 protein. In general it is considered that in circulation the processed form LL-37 occurs, after being processed within granules from the CAP18 protein into processed form. The graph below shows the result of an experiment to test the reactivity of hCAP18 in the HK321 ELISA kit.



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 support@hycultbiotech.com.

Suggestions summarized below in Table 3 can be used as a guideline in the case of unexpected assay results.

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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 micro titer reader
	•	•			Air bubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 3

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15. REFERENCES

1. Martineau, A et al; IFN-γ- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37, J Immunol 2007, *178*: 7190
2. Coffelt, S et al, Ovarian cancers overexpress the antimicrobial protein hCAP-18 and its derivative LL-37 increases ovarian cancer cell proliferation and invasion, Int J Cancer, 2008, *122*: 1030
3. Schwaab, M et al; Human antimicrobial proteins in ear wax, Eur J Clin Microbiol Infect Dis 2011
4. Goleva, E et al; Steroid requirements and immune associations with vitamin D are stronger in children than adults with asthma, J Allergy Clin Immunol 2012
5. Levinson, P et al; HIV-Neutralizing Activity of Cationic Polypeptides in Cervicovaginal Secretions of Women in HIVSerodiscordant Relationships, Plos One 2012, *7*:e31996
6. Davidopoulou, S et al; Salivary concentration of the antimicrobial peptide LL-37 in patients with oral lichen planus. J Oral Micro 2014, *6*:26156
7. Nielsen, K et al; Role of Urinary Cathelicidin LL-37 and Human α-Defensin 1 in Uncomplicated Escherichia coli Urinary Tract Infections. Inf and Imm 2014, *82*:1572
8. Van der Does, A et al; Vitamin D3 and phenylbutyrate promote development of a human dendritic cell subset displaying enhanced antimicrobial properties. JLB 2014, *6*:883
9. Larcombe, L et al; Vitamin D, serum 25(OH)D, LL-37 and polymorphisms in a Canadian First Nation population with endemic tuberculosis. Int J Circum Health 2015, *74*:28952
10. Stukes, T et al; Circulating Cathelicidin Concentrations in a Cohort of Healthy Children: Influence of Age, Body Composition, Gender and Vitamin D Status. PLos One 2016, *11*: e0152711

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