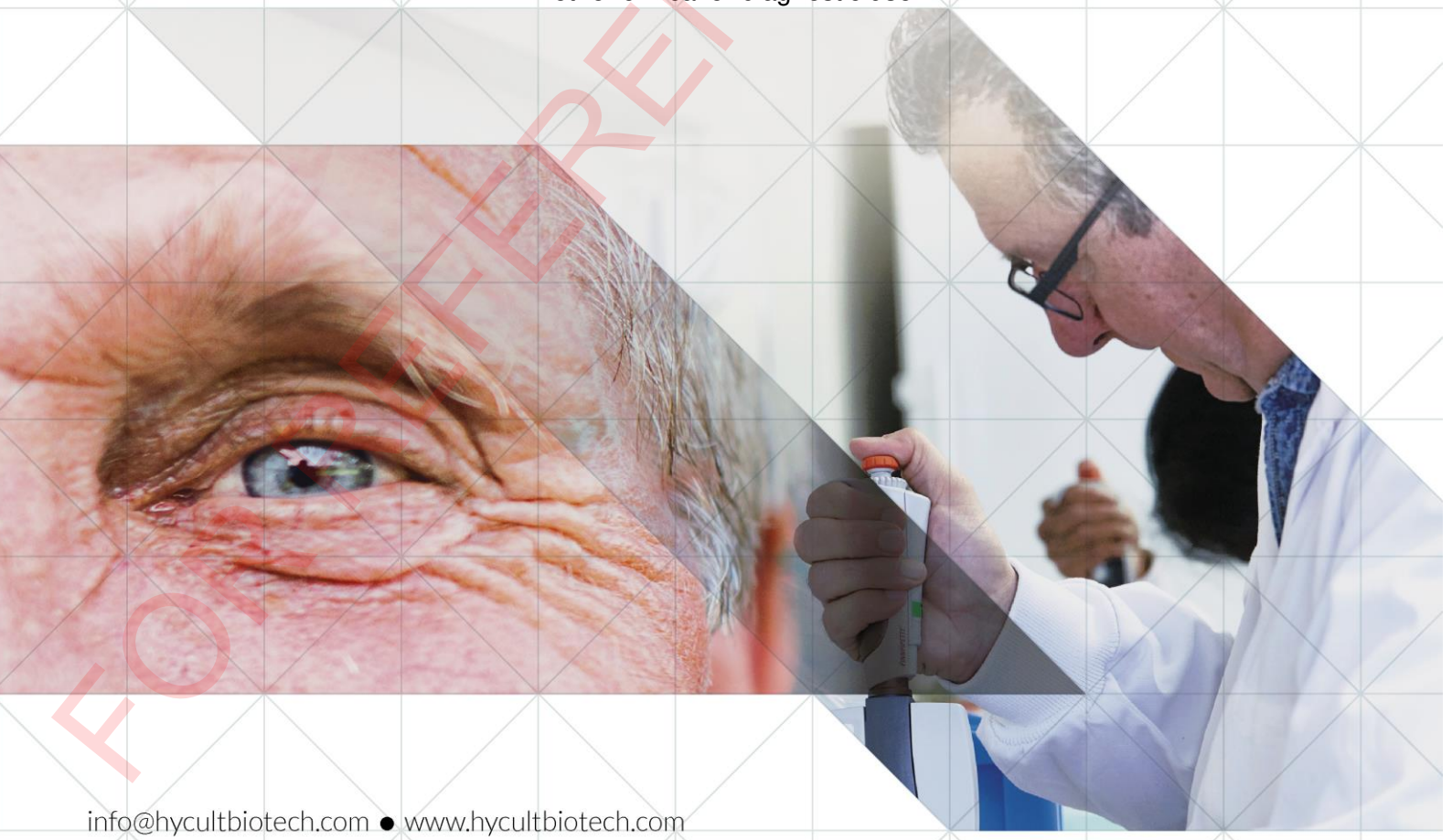
Pig Alternative Complement Pathway Assay

HIT432

Edition 07-17

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 pig alternative complement pathway ELISA is a qualitative/ semiquantitative ELISA to be used for the *in vitro* determination of activation of the alternative pathway of the complement system in serum 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

The HIT432 measures the alternative pathway activity mediated via LPS. Complement deficiencies or other defects in the complement system can easily be screened by running an assay for each pathway in parallel or separately. The ELISA contains a positive control which can be used as process control to ensure that the alternative complement cascade has run completely. This positive control cannot be used to determine the level of activation between batches. We advise to include in your study your own reference sample for 100% complement activity and negative control (eg non-preserved activated serum) to determine the level of activation of your samples. Beware that complement activity levels are pig strain dependent and might be affected by the way the samples are collected and processed.

The innate immune system is important in the first defense against foreign pathogens. A major component of this response is the complement system. The complement system consist of a complex family of proteins and receptors which are found in the circulation, in tissues and other body-fluids. Nowadays, also several links to the adaptive immunity are described. The system consist of three defined pathways which are activated by a pathway specific panel of molecules. Complement activation proceeds in a sequential fashion through the proteolytic cleavage of a series of proteins leading to the generation of activated products that mediate various biological activities through their interaction with specific cellular receptors and other serum proteins The three pathways, designated classical, lectin and alternative pathway, converge at a central component into a final common pathway. That is activation of C3 leading to formation of C3a and C3b. This cleavage activates the terminal complement pathway leading to eventually the formation of the terminal C5b-9 complement complex (TCC). The classical pathway is initiated by binding of C1q to antibody complexes, whereas the alternative and lectin pathway are activated in an antibody-independent fashion through the interaction of complement components with respectively specific carbohydrate groups and lipopolysaccharides (LPS) on the surface of foreign pathogens. The alternative pathway also acts as an amplification loop of the other pathways. Under certain conditions, the complement system can be unfavorable to the host leading to e.g. autoimmune diseases and infections. Deficiency of C3 is e.g. associated with SLE. Alterations in the alternative pathway, like properdin or ficolin deficiency, increase the susceptibility to infection. Mannose binding lectin (MBL), a major component of the lectin pathway, is associated with bacterial, fungal and viral infection. A common way to measure the activity of the classical or alternative pathway is the haemolysis of erythrocytes. Furthermore some assays have been described to measure the activity of the MBL pathway. Not all assay are easy to perform. The pig pathway ELISAs are easy to use and specific per pathway by making use of a combination of specialized coatings and buffers. Complement deficiencies or other defects in the complement system can easily be screened by running an assay for each pathway in parallel or separately.

3. KIT FEATURES

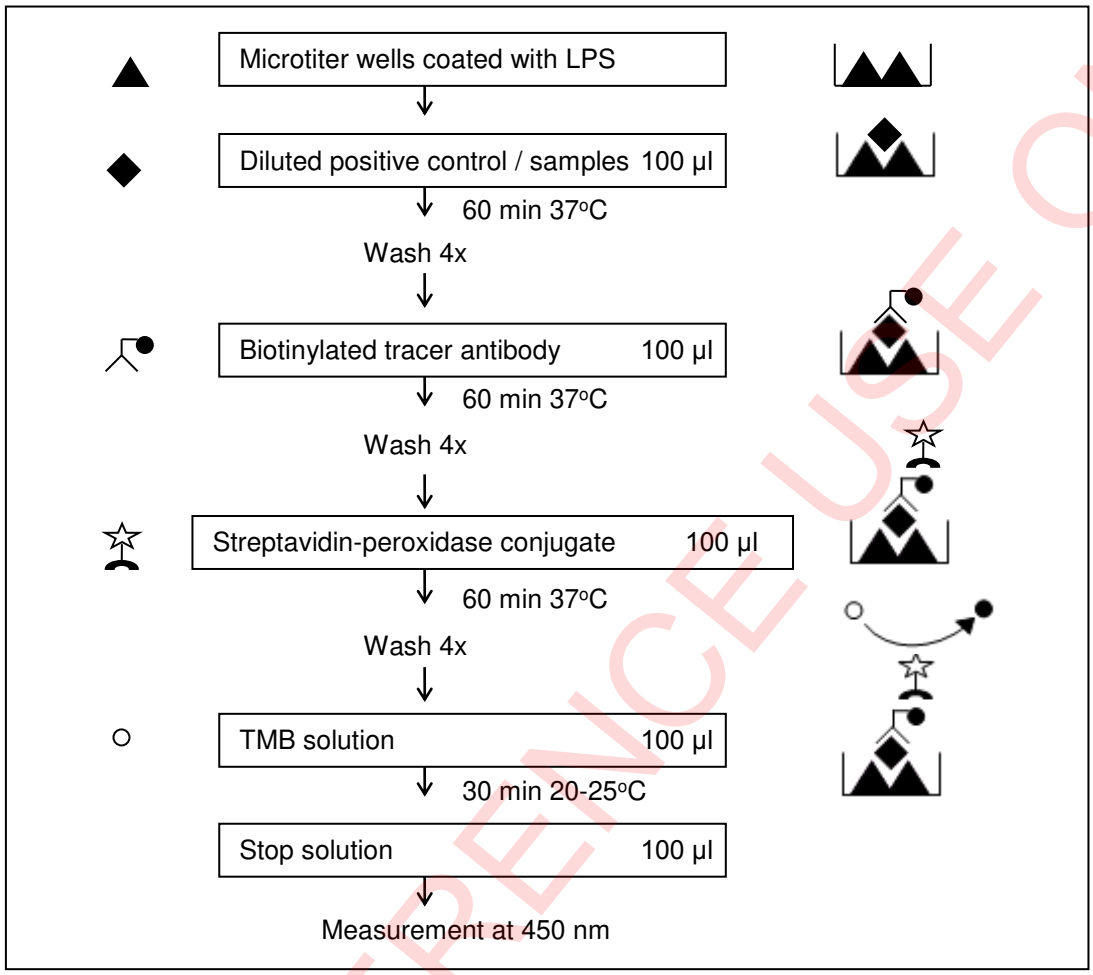
- Working time of 3½ hours.
- Positive control.
- Working volume of 100 µl/well.

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Cross-reactivity

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

4. PROTOCOL OVERVIEW



- The Pig Alternative Complement Pathway ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on a sandwich principle 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 positive control are incubated in microtiter wells coated with LPS.
- Biotinylated tracer antibody will bind to the bound samples and control.
- Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.
- Streptavidin-peroxidase 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.

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5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat.#	Quantity	Color code
Wash buffer 20x	WB21	1 vial (60 ml)	Colorless
Sample Dilution buffer 1x	SDB65	1 vial (30 ml)	Green
Dilution buffer 20x	DB51	1 vial (6 ml)	Green
Positive control		2 vials, lyophilized	White
Tracer, biotinylated		1 vial, 1 ml lyophilized	White
Streptavidin peroxidase 100x	CON03	1 tube, 0.25 ml in solution	Brown
TMB substrate	TMB050	1 vial (11 ml)	Brown
Stop solution	STOP110	1 vial (22 ml)	Red
12 Microtiter strips, pre-coated	HIT4AP	1 plate	
Certificate of Analysis		1	
Manual		1	
Data collection sheet		2	

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 positive control 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 reconstitution volume of the positive control is indicated on the Certificate of Analysis and on the label of the vial.
- The positive control is single use. After reconstitution the positive control 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 add under any circumstances 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 range. Do not change the 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 advise 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 advise.
- 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.
- Use polypropylene tubes for preparation of positive control and samples. Do not use polystyrene tubes or sample plates.

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7. SAMPLE PREPARATION

Collection and handling

Serum

Collect blood using normal aseptic techniques. Blood samples should be kept on ice. If serum is used, separate serum from blood after clotting at room temperature within 1 hour by centrifugation (1500xg at 4°C for 15 min). Transfer the serum 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 inhibitory capacity. Before performing the assay, thaw and store samples on ice. Avoid multiple freeze-thaw cycles.

Keep diluted samples on ice until pipetting in the wells.

Dilution procedures

Serum samples

Pig Alternative Complement Pathway can be measured accurately if serum samples are diluted with supplied sample dilution buffer in polypropylene tubes. The complement cascade can only pass on if none of the involved proteins is rate limiting. The basal level of complement cascade proteins is species and strain dependent. To be able to identify if complement level in a sample is aberrant, samples should be diluted in such way that in a normal sample the complement protein with the lowest concentration is not limiting. It is advised to determine this specific dilution per study or sample collection. This can be tested by performing a serial dilution (eg. 0x, 5x, 10x, 15x, 20x) of your reference sample (100% complement activity expected). The optimal dilution is one dilution before the stationary phase with maximal OD-value ends.

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8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Keep the controls, samples and sample dilution buffer on ice. Return to proper storage conditions immediately after use.

Wash buffer

Prepare wash buffer by mixing 60 ml of 20x wash buffer with 1140 ml of distilled or de-ionized water, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 20x wash buffer with 19 parts of distilled or de-ionized water.

Dilution buffer

Prepare dilution buffer by mixing 6 ml of the 20x dilution buffer with 114 ml of distilled or de-ionized water, which is sufficient for 1 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 19 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.

Positive control

The positive control is reconstituted by pipetting the amount of cold sample dilution buffer mentioned on the Certificate of Analysis in the positive control vial. Keep the positive control on ice until usage. After reconstitution the positive control is ready to use. Transfer 100 µl of the reconstituted positive control to the assigned wells.

Negative control

The sample dilution buffer can be used as negative control. Transfer 100 µl of the negative control to the assigned wells.

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 just 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.

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9. ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use. Keep the controls, samples and sample dilution buffer on ice.

1. Determine the number of test wells required and fill out the data collection sheet. Return the unused strips of the coated microtiter plate to the storage bag with desiccant, seal and store at 2 - 8°C.
2. Dilute controls and samples on ice with cold sample dilution buffer.
3. Transfer 100 µl in duplicate of positive and negative control and samples into appropriate wells in the coated microtiter plate. Do not touch the side or bottom of the wells.
4. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
5. Incubate for 1 hour at 37°C.
6. Wash the microtiter plate 4 times with wash buffer using a plate washer or as follows*:
   a. Carefully remove 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 for 1 hour at 37°C.
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 and incubate for 1 hour at 37°C.
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 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 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.

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10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate positive 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.
- It is advised to run your own reference samples (ref-sample = considered maximum complement level, and RNC = deficient or activated sample) if you wish to measure in a semiquantitative way the level of complement activation of your samples.
- With your own reference sample, the complement activity can be calculated as follows: (sample - RNC)/(ref-sample - RNC) x 100%.
- The negative (NC) and positive (PC) control can be used to verify if activation of the complement cascade has occurred in the well during the assay procedure. Expected OD levels are mentioned on Certificate of Analysis.
- In case no appropriate reference sample or RNC are available, one can use the OD values of provided PC and NC. Beware that 100% complement activity within your sample type could be at a different level than the provided PC and cannot be compared. Furthermore, results cannot be compared from experiment to experiment or from batch to batch.

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.
- 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, controls must be established for every run. Samples should be referred to the controls 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.
- 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 Pig Alternative Complement Pathway ELISA 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. 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 shall be sent to support@hycultbiotech.com.

Suggestions summarized below in Table 2 can be used as guideline in 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 adapted 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
	•	•			Airbubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 2

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