Hycult Biotech

# Human

# Alternative Complement Pathway

## HK3012

**Edition 10-25**

**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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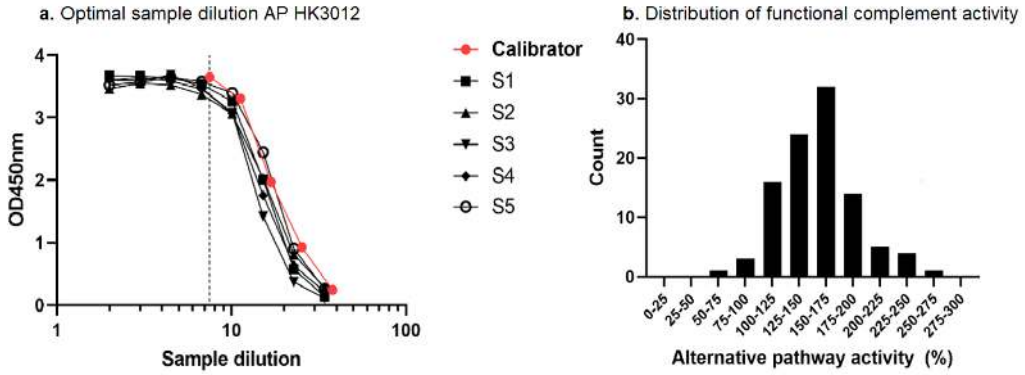
## 1. INTENDED USE

The Human Alternative Complement Pathway ELISA kit is to be used for the *in vitro* quantitative determination of Alternative Complement Pathway activity in serum samples.

The ELISA can also be used to determine the effect of inhibitory compounds for the alternative pathway.




Representative data showing characteristics of the human alternative pathway (AP) assay (HK3012):
**a)** A calibrator curve and 1.5-fold serial dilution of complement preserved serum samples. The assay works with an optimal 7.5x start dilution.
**b)** The distribution of the normal alternative pathway complement activity measured in 100 healthy representative blood donors.

Figure 1

This kit is intended for laboratory research use only (RUO) and is not for use in diagnostic or therapeutic procedures.

The analysis should be performed by trained laboratory professionals.

## 2. INTRODUCTION

The complement system plays important roles in both innate and adaptive immune response and can produce an inflammatory and protective reaction to challenges from pathogens before an adaptive response can occur. It consists of a complex family of proteins and receptors which are found in the circulation, in tissues and other body-fluids. There are three pathways of complement activation. The classical pathway (CP) is initiated by immune complexes; the lectin pathway (LP) by surface bound lectins; and the alternative pathway (AP) by all the surfaces that are not specifically protected against it. More specifically, the initiation of the AP depends on the spontaneous hydrolysis of C3 and this activated C3 binds fB and forms a C3 convertase ($C3(H_2O)Bb$). This convertase can be stabilized by properdin and the activation of the AP is regulated by proteins like fH and fI. Each pathway generates a C3 convertase, a serine protease that cleaves the central complement protein C3 and generates the major cleavage fragment C3b. At this point the three pathways converge and C3b initiates the formation of the second convertase. This so-called C5 convertase cleaves C5 into C5a and C5b. The latter initiates and amplifies the activity of the complement pathways and ultimately generates the cytolytic MAC.

The assessment of complement proteins as a whole, also expressed as complement activity, is used to identify if the complement system has been affected *in vivo* and in the therapeutic era of complement to monitor and screen for complement inhibitor function. Complement activity in sera can provide data in order to identify complement abnormalities in the clinic. Historically, one used haemolysis related assays to measure functional complement activity. Among others due to the opsonization, stability of the erythrocytes, complicated and time-consuming steps this assay is hard to standardize. ELISA based assays can overcome this, but can be hampered by high serum dilutions and restrictive quantification. The HK3012 AP complement activity assay has been developed to address these elements. Besides the

screening for deficiencies and abnormalities for complement proteins in individuals, innovations and the success of complement inhibiting drugs in the clinic and new ones in clinical trials drives a new requirement for accurate, more standardized and high throughput assessment of complement activity. The current aim of complement activity assessment is not only to screen the system in acute or chronic disease but also to identify and screen new candidates for complement inhibition. The costs of complement inhibitors are high and powerful screening for more potent inhibitors or therapy monitoring to introduce more patient specific treatment strategies authorize the introduction of improved measurements of complement activity.

The HK3012 AP complement activity assay measures complement function *in vitro* in serum. The wells of the assays are coated with lipopolysaccharide (LPS) and when the cascade has elapsed the *in vitro* formed MAC complex is detected with a C9 neo-epitope antibody. The assay can be performed with an initial serum dilution of 7.5x and pathway interference has been prevented using a specific buffer. Using the power of a 1.5-fold dilution combined with regression analysis of logistically transformed values, complement activity and inhibitory capacity can accurately be measured.

## 3. KIT FEATURES

- Working time of 1 ¾ hours.
- Working volume of 100 µl/well.

### Cross-reactivity

Potential cross-reacting complement activity detected using the Human Alternative Complement Pathway ELISA:

| Cross reactant species | Reactivity |
|---|---|
| Pig | Yes |
| Monkey (non-human primate) | Yes |

Table 1

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

## 4. PROTOCOL OVERVIEW

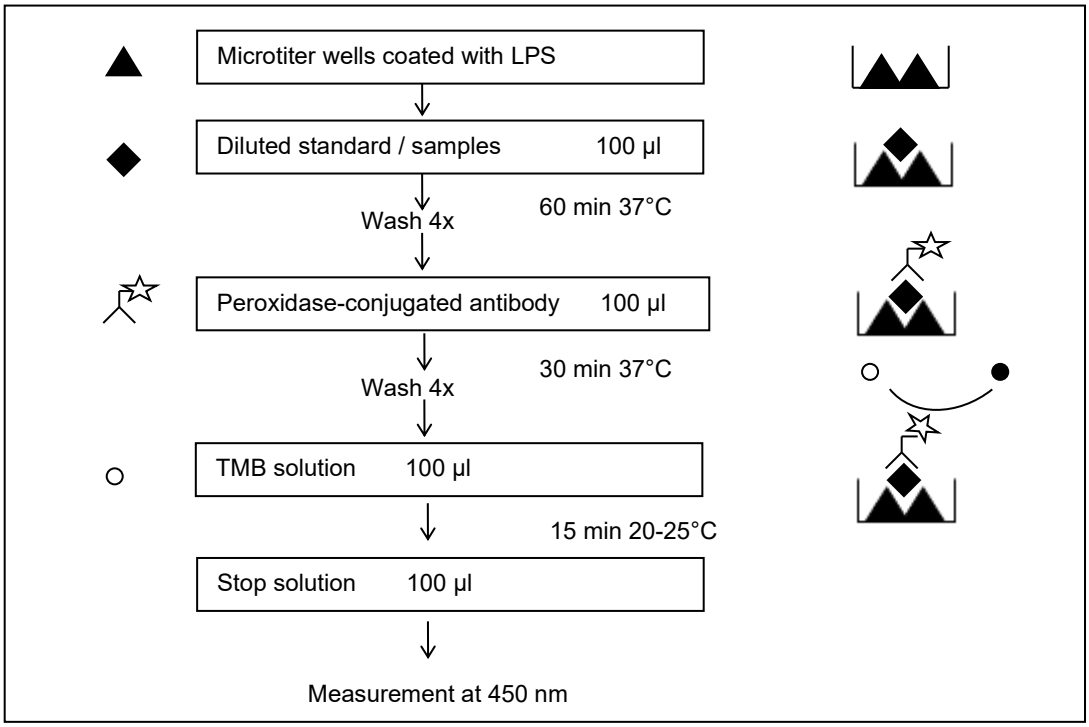



Figure 2

- The Human Alternative Complement Pathway ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent activity assay based on the sandwich principle with a working time of 1 hour and 45 minutes.
- 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.
- Peroxidase-conjugated antibody will bind a neo-epitope of to the formed TCC complex.
- 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.
- The Human Alternative Complement Pathway activity of samples, which are run concurrently with the standards, can be evaluated on OD-data using regression analysis on logistically transformed values. Complement activity can be expressed as percentage of the activity of a positive standard serum. (See 11. Technical hints)

## 5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

| Kit component | Cat.# | Quantity HK3012 | Color code |
|---|---|---|---|
| Wash buffer 20x | WB31 | 1 vial (30 ml) | Colorless |
| Dilution buffer 1x | DB67 | 1 vial (60 ml) | Green |
| Standard | | 2 vials, lyophilized | White |
| Negative Control | | 2 vials, lyophilized | Blue |
| Peroxidase-conjugated antibody | | 1 vials, 1 ml lyophilized | Blue |
| TMB substrate | TMB050 | 1 vial (12 ml) | Brown |
| Stop solution | STOP110 | 1 vial (22 ml) | Red |
| 12 Microtiter strips, pre-coated | | 1 plate | |
| Certificate of Analysis | | 1 | |
| Manual | | 1 | |
| Data collection sheet | | 1 | |

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 peroxidase-conjugated detection antibody in lyophilized form is stable until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The standard is single use. After reconstitution the standard cannot be stored.
- 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 one 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.
- Centrifuge for 1 ml tubes.

## 6. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Handle standard and sample solutions carefully, prevent unnecessary pipetting and mechanical forces in order to prevent complement activation.
- Under any circumstances, do not 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 the manufacturer.
- The assay has been optimized for the indicated range. Do not change the range.
- Open vials carefully: vials are under vacuum.
- 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 has been 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.
- Concentrated dilution buffer may contain crystals. In case the crystals do not disappear at room temperature within one hour, concentrated dilution buffer can be warmed up to 37°C.
- Note: The basal level of complement cascade proteins is person dependent.
- Note: The figures are not batch-specific, but a representation of the assay procedure

## 7. SAMPLE PREPARATION

### Collection and handling

### Serum samples

Collect blood using normal aseptic techniques. Blood samples should be kept on ice. Allow blood to clot for one hour at room temperature (18-23 °C). Separate serum from blood after clotting by centrifugation (1500xg at 4°C for 15 min). Transfer the serum to a fresh polypropylene tube and avoid excessive heat. Careful handling is advised in order to prevent complement activation (prevent unnecessary pipetting and mechanical forces). Avoid multiple freeze-thaw cycles which may cause loss of complement activity and give erroneous results.

### Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect complement activating 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

Human Alternative Complement Pathway can be measured accurately if serum samples are diluted with supplied dilution buffer in polypropylene tubes. The complement cascade can only pass on if none of the involved proteins is rate limiting. It is advised to determine the activity of each sample by a serial dilution (e.g.: 7.5x, 11.3x, 16.9x, 25.3x), for recommended dilution see Table 2. The recommended ELISA plate layout can be found in Figure 3.

| | Dilution | Pre-dilution | Amount of sample or pre-dilution required | Amount of dilution buffer required |
|---|---|---|---|---|
| **1.** | 7.5x | Not necessary | 50 µl (sample) | 325 µl |
| **2.** | 11.3x | Recommended: 7.5x (see nr.1) | 250 µl (pre-dilution) | 125 µl |
| **3.** | 16.9x | Recommended: 11.3x (see nr.2) | 250 µl (pre-dilution) | 125 µl |
| **4.** | 25.3x | Recommended: 16.9x (see nr.3) | 250 µl (pre-dilution) | 125 µl |

Table 2

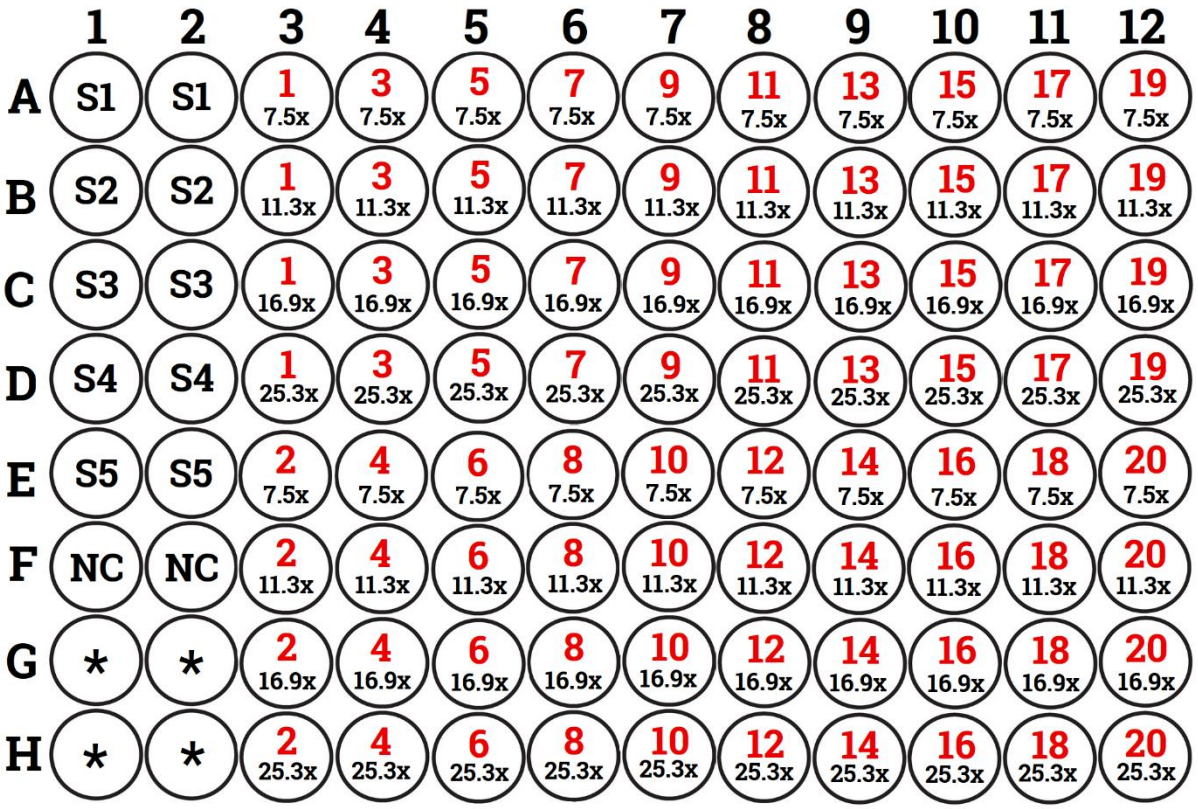



Figure 3

*The wells G1, G2, H1, and H2 are recommended for control samples or other refences.

## 8. REAGENT PREPARATION

The controls, standards, and dilution buffer should be kept on ice. Allow all remaining reagents to equilibrate 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 20x wash buffer with 570 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.

### Standard solution

The standard is reconstituted by pipetting the amount of dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure *4*. Prepare each Human Alternative Complement Pathway standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Figure *4*. After reconstitution the standard cannot be stored for repeated use.

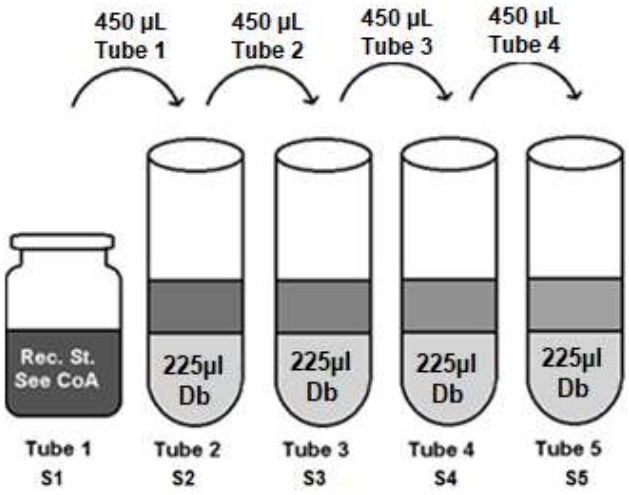



Figure 4

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

**Negative control**

The negative control is reconstituted by pipetting 563 µl dilution buffer. Transfer 100 µl of the negative control to the assigned wells.

**Peroxidase-conjugated antibody**

The peroxidase-conjugated antibody is reconstituted by pipetting 1 ml distilled or de-ionized water. Please note that, although the conjugate may appear cloudy after reconstitution, this has no effect on the test. Dilute the reconstituted 1 ml detection antibody with 11 ml dilution buffer, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of peroxidase-conjugated antibody by diluting 1 part of the reconstituted vial with 11 parts of dilution buffer.

## 9. ELISA PROTOCOL

The controls, standards, and dilution buffer should be kept on ice. Allow all remaining reagents to equilibrate to room temperature (20 – 25°C) prior to use.

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. The coated microtiter plate should be kept on ice.
3. Dilute controls and samples on ice with cold dilution buffer.
4. Transfer 100 µl in duplicate of the standard curve solutions, negative control and samples into appropriate wells in the coated microtiter plate, which is kept on ice. Do not touch the side or bottom of the wells.
5. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
6. Incubate for 1 hour at 37°C.
7. 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 7b.
d. Repeat the washing procedure 7b/7c three times.
e. Empty the plate and gently tap on thick layer of tissues.

8. Add 100 µl of diluted peroxidase-conjugated antibody to each well using the same pipetting order as applied in step 4. Do not touch the side or bottom of the wells.
9. Cover the tray and incubate for half an hour at 37°C.
10. Repeat the wash procedure described in step 7.
11. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 4. Do not touch the side or bottom of the wells.
12. Cover the tray and incubate the tray for 15 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.
13. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 11. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
14. 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.

## 10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, and determine activity of samples.
- For accurate quantification of complement activity is advised to apply regression analysis on logistically transformed optical density (OD) values to determine the complement activity as percentage of the activity of the positive reference. This method can be found in following reference: Palarasah Y et al. Novel assays to assess the functional capacity of the classical, the alternative and the lectin pathways of the complement system. Clinical & Experimental Immunology 2011; 164: 388 (http://dx.doi.org/10.1111/j.1365-2249.2011.04322.x)
- ELISA calculation sheets can also be found on Hycult Biotech website: https://www.hycultbiotech.com/elisa-calculation-sheet/. These sheets represent a mathematical approach to the method as described in the Palarasah paper. Please be aware that the calculation sheet makes use of non-validated software.
- If individual calculated values differ by more than 15%, the result is considered suspect and the sample should be retested.
- Ensure that no *in vitro* complement activation has been initiated in your test samples
- The mean absorbance of the negative control should be less than 0.2.
- The inhibiting or modifying effect of a compound/biological or suspected aberrant complement levels in samples should always be verified in a second or independent test.
- Samples that give an absorbance above the absorbance for the highest standard concentration or below the lowest standard concentration are out of range of the assay. These samples should be neglected or retested at a different dilution.
- For any results not covered in this section, please visit: https://www.hycultbiotech.com/faq/#pathwayassays.

## 11. TECHNICAL HINTS

- Be aware of challenges with measuring complement activity in serum samples and that pre-analytical sample handling is performed in an appropriate manner to avoid erroneous results. See also:
  - Website: https://www.hycultbiotech.com/how-to-analyze-complement/
  - Reference: Brandwijk R et al. Pitfalls in complement analysis: A systematic literature review of assessing complement activation. Front Immunol. 2022; 13: 1007102 (https://doi.org/10.3389%2Ffimmu.2022.1007102).
- 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, detection antibody 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.
- 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 Alternative Complement Pathway ELISA.

Hycult Biotech, Frontstraat 2A, 5405 PB Uden, the Netherlands

T: +31 (0)413 251 335, F: +31 (0)413 248 353

## 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 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

### Intra-assay variation

The data was collected from 3 samples over 9 experiments. The average activity and the coefficient of variation (CV) is shown in Table 3. The specification at HB for the CV for intra-assay variation is < 15%.

Intra-assay variation

| Sample | 1 | 2 | 3 |
|---|---|---|---|
| **Average Activity (%)** | 119 | 94 | 79 |
| **CV** | 7.3 | 8.9 | 9.6 |

Table 3

### Inter-assay variation

The data was collected from 1 experiment performed by 2 operators from 3 samples in triplicate. The averages of the three samples are displayed per operator with intra assay CV in Table 4 and Table 5. The inter-assay variation is shown in Table 6. The specification at HB for the CV for inter-assay variation is < 20%.

Operator 1

| Sample | 1 | 2 | 3 |
|---|---|---|---|
| **Average Activity (%)** | 119 | 96 | 77 |
| **CV** | 4.7 | 4.4 | 4.3 |

Table 4

Operator 2

| Sample | 1 | 2 | 3 |
|---|---|---|---|
| **Average Activity (%)** | 130 | 105 | 89 |
| **CV** | 10.3 | 3.9 | 10.3 |

Table 5

Inter-Assay variation

| Sample | 1 | 2 | 3 |
|---|---|---|---|
| **Average Activity (%)** | 123 | 101 | 83 |
| **CV** | 8.1 | 6.1 | 11.2 |

Table 6

## 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 the generated data can be sent to support@hycultbiotech.com.

Suggestions summarized below in Table *7* 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 had reached 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 |
| | | • | | | Imprecise sealing of the plate after use |
| • | | | | | Wrong storage conditions |
| • | | | | | Lamp in microplate reader is not functioning optimally |

Table 7