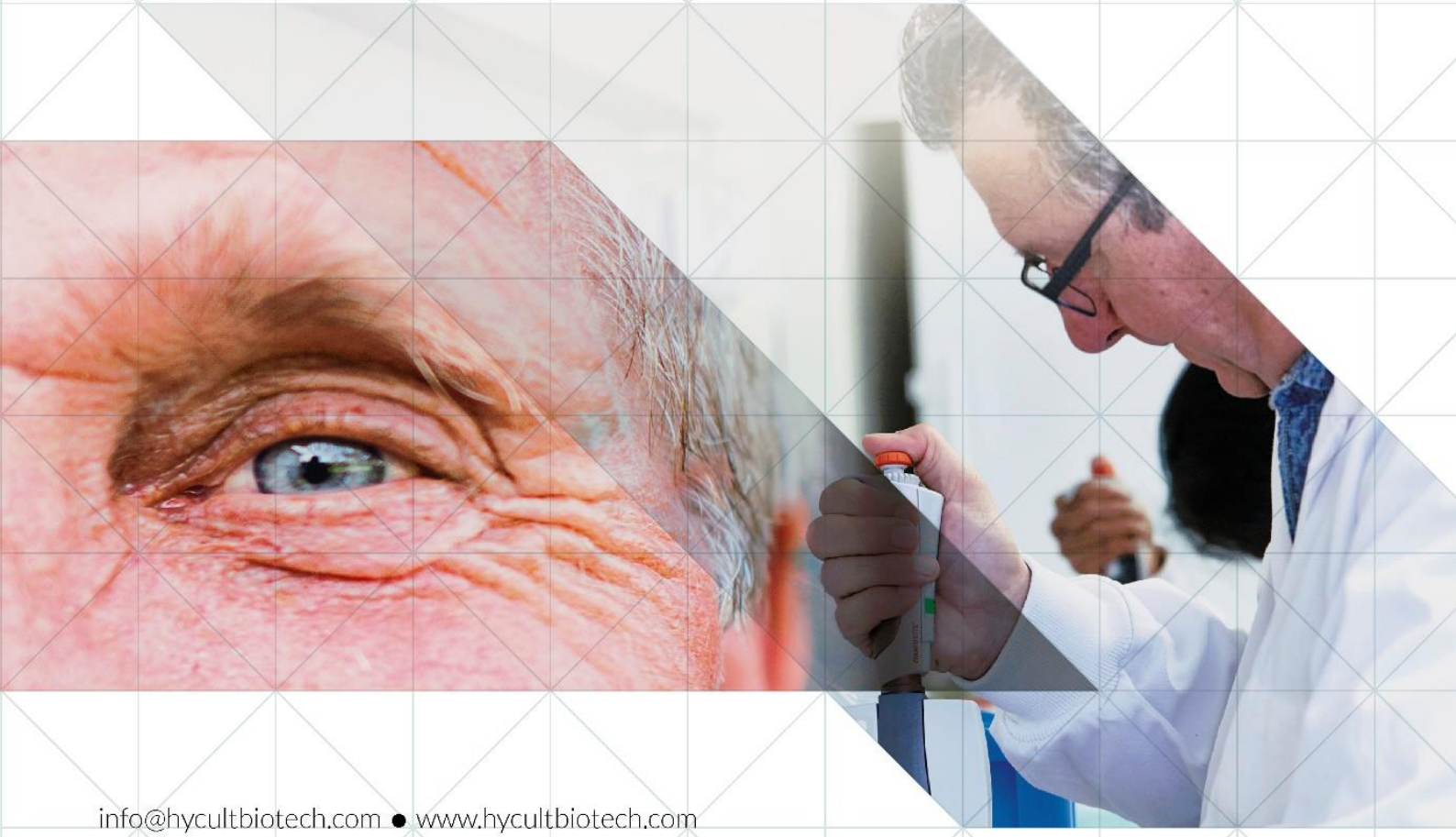
Complement Factor H, H402 and Y402 variant detection

HK353

Edition 10-18

ELISA KIT
PRODUCT INFORMATION & MANUAL

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



info@hycultbiotech.com ● www.hycultbiotech.com

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 complement factor H, H402 and Y402 variant detection ELISA kit is to be used for the *in vitro* detection of H402 and Y402 variants of complement factor H in serum and plasma 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

Complement is a major defense system of innate immunity and aims to destroy microbes. There are three pathways of complement activation. The classical pathway is initiated by immune complexes, the lectin pathway by surface bound mannan binding lectin, and the alternative pathway by all the surfaces that are not protected against it. One of the central complement regulators of the alternative pathway is complement factor H (CFH). CFH is the major soluble inhibitor of the alternative pathway of the complement system and plays a key role in controling complement activation *in vivo*. CFH is a 150-kDa plasma protein that is mainly produced in the liver and is present in plasma at a concentration of approximately 500 µg/ml. The molecule is made up entirely of a string of 20 folded globular domains known as short consensus repeats (SCRs).

Alternative pathway activation results from a failure to appropriately regulate the constant low level of the abundant complement C3 to C3(H_2O) due to spontaneous turnover. Complement non-activating cells and other host surfaces are protected from alternative pathway complement attack through binding of CFH. CFH inhibits the alternative pathway by binding C3b and reducing the formation and activation of the alternative pathway C3-convertase C3bBb. It also accelerates the decay of this convertase and works as a cofactor for the serine proteinase factor I in the degradation of C3b into inactive C3b (iC3b).

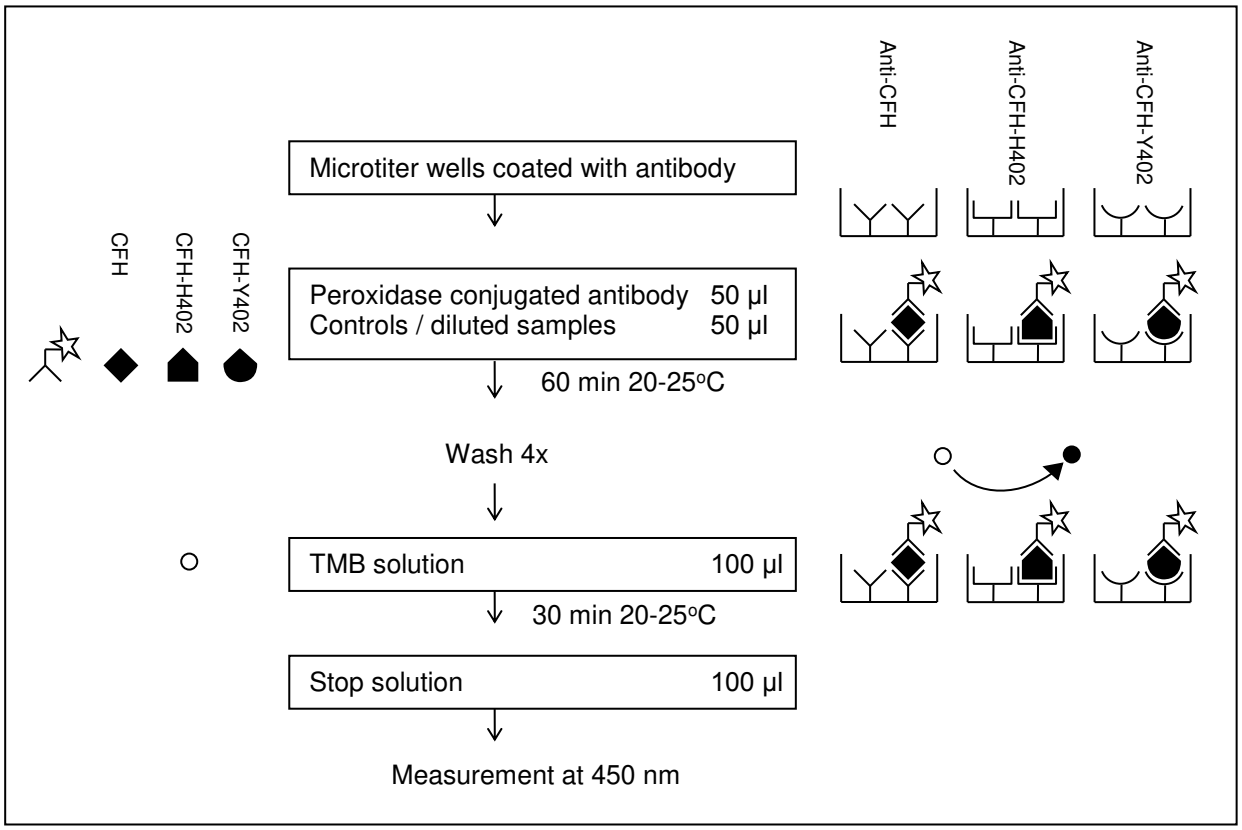
CFH is associated with several diseases like atypical hemolytic syndrome (aHUS), membranoproliferative glomerulonephritis type II (MNGN) and age-related macular degeneration (AMD). In the western world, AMD is the leading cause of natural irreversible blindness in the elderly, affecting 50 million individuals worldwide. A common polymorphism in the *CFH* gene on chromosome 1, has been determined to be strongly associated with a person's risk for developing AMD. The *CFH* gene polymorphism is characterized by a T-to-C single nucleotide substitution resulting in a tyrosine-histidine change at amino acid position 402 . The sequence change is in a region of CFH that binds heparin and C-reactive protein. Individuals homozygous for H402 (genotype CC; CFH-HH402) have up to 12-fold increased risk for developing AMD, while heterozygotes (genotype CT; CFH-HY402) have a 2.5-fold greater risk for developing AMD than individuals homozygous for Y402 (genotype TT; CFH-YY402). The CFH-HH402 and CFH-HY402 variants, likely explain about 43% of AMD in older adults.

3. KIT FEATURES

- Working time of 1½ hours.
- Working volume of 50 µl/well for samples or controls.
- Specific detection of Y402 and H402 CFH variants.

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

4. PROTOCOL OVERVIEW



- The complement factor H, H402 and Y402 variant detection ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 1½ hours.
- The efficient format of 1 plate with 4 disposable 8-well strips per type (3 types; anti-CFH; colorless, anti-CFH-H402; red and anti-CFH-Y402; blue) allows free choice of batch size for the assay.
- Samples and controls are incubated together with peroxidase-conjugated second antibody (conjugate) in microtiter wells coated with antibodies recognizing CFH (colorless), CFH-H402 (red) and CFH-Y402 (blue).
- During incubation CFH, CFH-H402 or CFH-Y402 are captured by the solid bound antibody. The conjugated antibodies will bind to the captured CFH variants.
- The peroxidase conjugated antibody 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 polymorphism (Y402H) of complement factor H can be determined from the controls.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat.#	Quantity	Color code
Wash buffer 20x	WB21	1 vial (60 ml)	Colorless
Dilution buffer 10x	DB81	1 vial (15 ml)	Green
CFH-HH402 control		2 vial, 0.25 ml lyophilized	Red
CFH-YY402 control		2 vial, 0.25 ml lyophilized	Blue
CFH-HY402 control		2 vial, 0.25 ml lyophilized	White
Conjugate, HRP labeled		1 vial, 1 ml lyophilized	Blue
TMB	TMB050	1 vial (11 ml)	Brown
Stop solution	STOP110	1 vial (22 ml)	Red
Coated microwell strips CFH		4 x 8 wells	Colorless
Coated microwell strips CFH-H402		4 x 8 wells	Red
Coated microwell strips CFH-Y402		4 x 8 wells	Blue
Manual		1	
Data collection sheet		2	
Certificate of Analysis		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 controls and conjugate are stable in lyophilized form until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- Once reconstituted, the controls are stable for 4 hours. The controls cannot be stored for repeated use.
- Once reconstituted, the conjugate is stable for 1 month if stored at 2 - 8°C.
- 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.

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 (since it influences HRP activity).
- 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 controls. Do not change the controls.
- 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 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 controls and samples. Do not use polystyrene tubes or sample plates.
- The controls are of human origin. They were tested for various viruses and found negative. Since no test method can offer complete assurance that infectious agents are absent, these reagents 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.

7. SAMPLE PREPARATION

Collection, handling and storage

Serum or plasma

Collect blood using normal aseptic techniques. If serum is used, separate serum from blood after clotting at room temperature within 1 hour by centrifugation (1500 x g at 4°C for 15 min). Transfer the serum to a fresh polypropylene tube.

If plasma is used, separate plasma from blood within 20 minutes after blood sampling by centrifugation (1500 x g at 4°C for 15 min). Transfer the plasma to a fresh polypropylene tube.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of 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 or plasma samples

For accurate detection of HH402, YY402 and HY402 variants of complement factor H in serum or plasma, dilute samples at least 500 times with supplied dilution buffer in polypropylene tubes.

Remark regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

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 buffer

Prepare wash buffer by mixing 60 ml of 20x concentrated wash buffer with 1140 ml of distilled or de-ionized water, which is enough 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 concentrated wash buffer with 19 parts of distilled or de-ionized water. Concentrated wash buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated wash buffer can be warmed up to 37°C.

Dilution buffer

Prepare dilution buffer by mixing 15 ml of the 10x dilution buffer with 135 ml of distilled or de-ionized water, which is enough 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 concentrated 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.

Control solution

Reconstitute the CFH-HH402, CFH-YY402 and CFH-HY402 controls by injection of 0.25 ml dilution buffer into each vial. The controls are ready to use after reconstitution.
After reconstitution, the controls must be used within 4 hours. The controls cannot be stored for repeated use.

Conjugate solution

Reconstitute the conjugate by pipetting of 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml conjugate with 5 ml dilution buffer, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of conjugate by diluting 1 part of the reconstituted conjugate with 5 parts of dilution buffer.

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 of the CFH (colorless), CFH-Y402 (blue) and CFH-H402 (red) 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. Add 50 µl of diluted conjugate to each well. Do not touch the side or bottom of the wells.
3. Transfer 50 µl of controls, samples and dilution buffer in duplicate into CFH (colorless), HH402 (red) and YY402 (blue) 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 the strips or plate for 1 hour at room temperature.
6. Wash the plate 4 times with wash buffer as follows*:
   a. Carefully remove the plate 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 TMB substrate to each well. Do not touch the side or bottom of the wells.
8. Cover the tray and incubate the tray for 30 minutes at room temperature. Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminum foil is recommended.
9. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 7. Gently tap the tray to mix the solution and to eliminate air bubbles trapped in the wells.
10. 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 controls 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 dilution buffer and controls should be as indicated in Table 2.

control	anti-CFH (colorless)	anti CFH-H402 (red)	Anti CFH-Y402 (blue)
CFH-HH402	> 0.5	> 0.5	< 0.5
CFH-YY402	> 0.5	< 0.5	> 0.5
CFH-HY402	> 0.5	> 0.5	> 0.5
Dilution buffer	< 0.2	< 0.2	< 0.2

Table 2

- Determine the polymorphism (Y402H) of complement factor H of the samples using the OD450 values.

anti-CFH (colorless)	anti CFH-H402 (red)	anti CFH-Y402 (blue)	genotype
+	+	–	CFH-HH402 (homozygous for CFH-H402)
+	–	+	CFH-YY402 (homozygous for CFH-Y402)
+	+	+	CFH-HY402 (heterozygous)

Table 3

+ = OD_{450} > 0.5
– = OD_{450} < 0.5

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 the controls only following the instructions.
- Improper or insufficient washing 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, the controls must be established for every run. If the controls are out of range, the result of the test samples are not reliable. The test should be repeated.
- 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 sample, conjugate 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 control, 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 complement factor H, H402 and Y402 variant detection 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 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

Human EDTA-plasmas of 317 persons (119 controls and 198 AMD patients) were tested to validate the specificity and sensitivity of the assay. 315 samples revealed a perfect match between genotype and ELISA outcome, resulting in a specificity of 99.8% (Table 4). Table 4 shows an overview of amino acid composition at position 402 of complement factor H as determined using the ELISA format and DNA sequence.

Sample type	Method	YH402	YY402	HH402	total
Control	ELISA	50 (42%)	57 (47.9%)	12 (10.1%)	119
	DNA sequence	51	56	12	
AMD	ELISA	85 (42.9%)	34 (17.2%)	79 (39.9%)	198
	DNA sequence	86	33	79	

Table 4

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 shall be sent to support@hycultbiotech.com.

Suggestions summarized below in Table 5 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 control 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 controls/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 during incubations
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 5

15. REFERENCES

1. Hakobyan, S et al; Measurement of factor H variants in plasma using variant-specific monoclonal antibodies: application to accessing risk of age-related macular degeneration. Invest Ophthalmol Vis Sci 2008, *49*: 1983
2. Szarvas, N et al; First-line therapy in atypical hemolytic uremic syndrome: consideration on infants with a poor prognosis. Ital J Pediatrics 2014, *40*:101