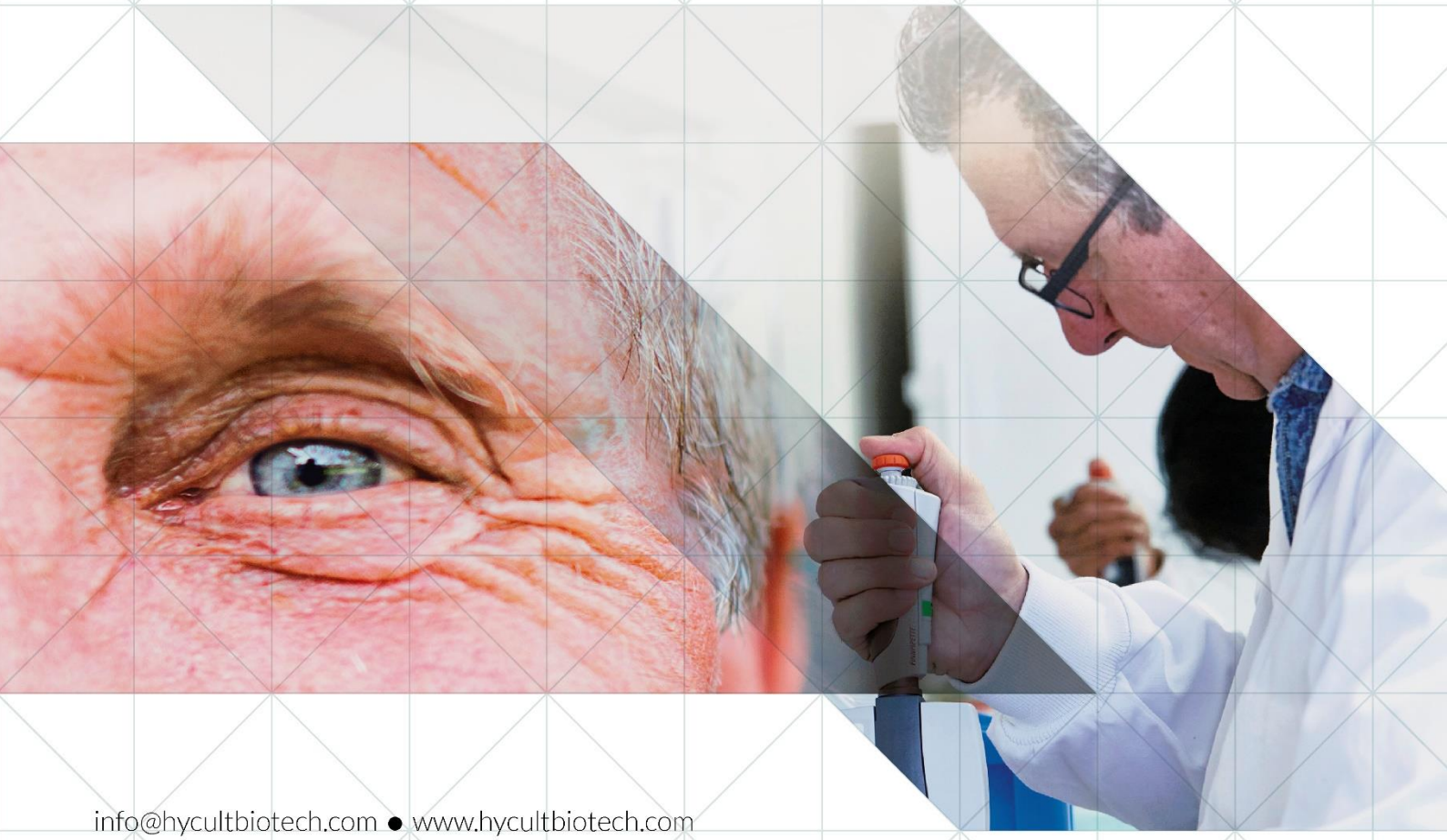
Human C3NeF detection assay

HK3051

Edition 06-26

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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Please note that the user protocol provided is not specific to any particular lot and represents the general specifications for this product. We advise consulting the vial label and the Certificate of Analysis for information regarding specific lots. Additionally, be informed that shipping conditions for this product may differ from its recommended storage conditions.

This product is intended solely for research purposes and is not approved for human or animal use, or for diagnostic procedures. Users must adhere to all applicable local, state, and federal regulations when utilizing this product. Hycult Biotech disclaims any liability for patent infringements that may arise from the use or adaptation of this product.

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1. INTENDED USE

The C3NeF detection assay is to be used for the *in vitro* qualitative determination of C3 nephritic factor (C3NeF) autoantibodies in heat-inactivated serum or plasma. 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 C3NeF detection assay enables in vitro qualitative detection of C3NeF autoantibodies in heat-inactivated serum or plasma. C3NeF is an IgG autoantibody that binds to the Bb portion of the C3 convertase C3bBb(P), thereby stabilizing the complex and prolonging its half-life, resulting in sustained activation of the complement alternative pathway (AP). Sustained AP activation can contribute to disease and indeed the presence of C3NeF has been reported in several complement-mediated disorders, most prominently in C3 glomerulopathy, including dense deposit disease and C3 glomerulonephritis, but also in atypical hemolytic uremic syndrome and related conditions characterized by dysregulated alternative pathway activation.

The assay consists of a C3b coated plate to which functional Factor B (FB) and Factor D (FD) should be added for the formation of the C3bBb convertase to which the potentially present C3NeFs can bind. Bound C3NeFs are detected using a HRP-labelled anti-human IgG antibody, which catalyzes conversion of the TMB substrate into a measurable color signal.

3. KIT FEATURES

- Working time of 1½ hours.
- Working volume of 100 µl/well.
- Distinguishes between C3NeF positive and negative samples based on a cut-off OD.

4. PROTOCOL OVERVIEW

- The C3NeF detection ELISA is a qualitative enzyme-linked immunosorbent assay with a working time of ~1½ hours. See Figure 1 for a schematic overview of the assay workflow.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples are incubated in microtiter wells coated with C3b in the presence of Factor B and Factor D, allowing in situ formation of the C3bBb convertase and subsequent binding of C3 nephritic factors, if present. In parallel, samples are also incubated in C3b-coated wells in the absence of Factor B and Factor D, which serve as background controls for non-specific binding to C3b or the plate surface, ensuring that the measured signal specifically reflects antibody binding to the assembled C3bBb convertase.
- HRP-labeled detection antibody will bind to the captured C3NeFs.
- HRP 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.
- OD values for each sample are calculated by subtracting the OD in the C3b wells from the OD in the C3bBb wells. $Sample\ OD = OD_{C3bBb\ wells} - OD_{C3b\ wells}$
- Samples are classified as C3NeF positive when the sample OD is above the $OD_{cut\text{-}off}$ and regarded negative when the sample OD is below the $OD_{cut\text{-}off}$.
- C3NeF positivity is determined using the predefined cut-off*.

*No positive control is provided with the assay. Therefore, laboratories should use internally collected C3NeF-positive and C3NeF-negative samples to validate the assay and monitor assay performance prior to routine sample testing. During assay validation, also an in-house cut-off value should be determined. The cut-off is established using at least 10–20 independent C3NeF-negative samples and is calculated as the mean optical density of the negative samples plus three times the standard deviation ($OD_{cut\text{-}off} = mean\ OD_{neg} + 3 \times SD\ OD_{neg}$). During routine testing, both in-house C3NeF-positive and C3NeF-negative samples should be included in each run as internal controls, alongside the provided negative (technical) control, to verify assay performance and cut-off robustness.

Comment regarding the evaluation of properdin-dependent C3NeFs

Some C3 nephritic factors require properdin for stable binding to the C3 convertase (properdin-dependent C3NeFs), whereas others bind and stabilize C3bBb independently of properdin. Properdin dependence can be assessed by adding properdin (0.5 – 3.1 µg/mL; not provided) to the Factor B and Factor D mixture to allow formation of the C3bBbP complex and performing the assay according to the standard procedure. By incubating the same samples in parallel in C3bBb- and C3bBbP-coated wells, C3NeF binding can be directly compared under properdin-independent and properdin-dependent conditions. Please note that when measuring C3bBbP binding C3NeFs, also a cut-off OD should be set based on C3bBbP wells in C3NeF negative samples.

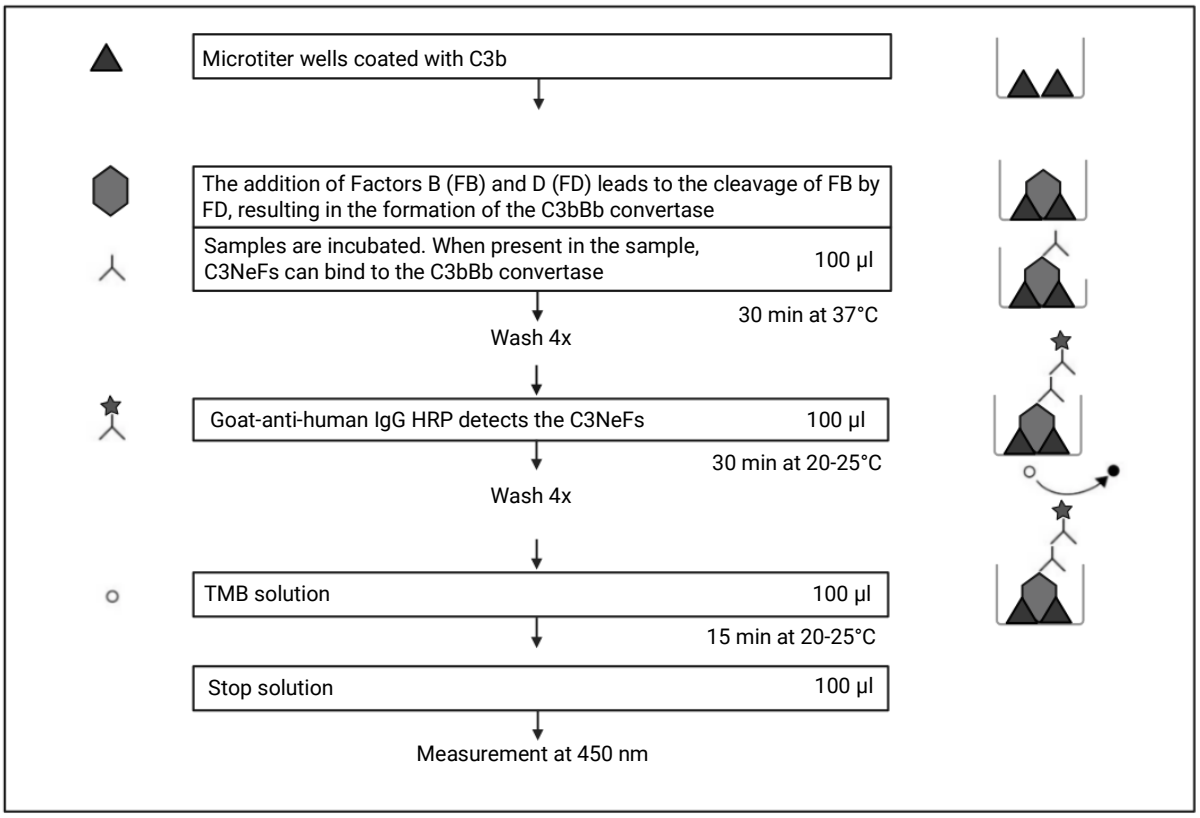

Figure 1. Schematic overview assay workflow

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat.#	Quantity HK3051-01	Quantity HK3051-02	Color code
Wash buffer 20x	WB21	1 vial (60 ml)	1 vial (60 ml)	Colorless
Dilution buffer 10x	DB109	1 vial (15 ml)	1 vial (15 ml)	Green
Factor B	HK3051-FB	1 vial, lyophilized	2 vials, lyophilized	White
Factor D	HK3051-FD	1 vial, lyophilized	2 vials, lyophilized	Red
Negative (technical) control	HK3051-NC	1 vial, lyophilized	2 vials, lyophilized	Blue
Detection antibody, HRP-labeled	HK3051-T	1 tube, 250 µl in solution	1 tube, 250 µl in solution	Brown
TMB substrate	TMB	1 vial, (22 ml)	1 vial, (22 ml)	Brown
Stop solution	STOP	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		1	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.
- Lyophilized components are stable until the expiration date indicated on the kit label if stored at 2 - 8°C. After reconstitution, these components are stable for one month if stored below -20ºC.
- The HRP-labeled detection antibody 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 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.
- 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 the manufacturer.
- Open vials carefully: vials are under vacuum.
- It is advised to spin down the HRP-labeled detection antibody 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 let wells stand in wash buffer for extended periods as this 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 or contaminated samples may give erroneous results.
- Use polypropylene tubes for preparation assay reagents and samples. Do not use polystyrene tubes or sample plates.
- The negative control serum and purified proteins (FB and FD) are 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. Handle all materials in contact with this reagent according to guide-lines for prevention of transmission of blood-borne infections.

7. SAMPLE PREPARATION

Collection and handling

Serum or plasma

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 one hour by centrifugation (1500xg 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 (1500xg at 4°C for 15 min). Transfer the plasma to a fresh polypropylene tube.

Most reliable results are obtained when all samples are prepared from the same matrix.

Samples should be heat-inactivated prior to use in the assay. Samples should be incubated at 56ºC for 30 minutes. Samples should be stored immediately on ice (short-term) after the heat-inactivation or samples should be stored immediately on ice and aliquoted and frozen for long-term storage.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect sample stability. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles.

Before performing the assay, samples should be thawed completely and mixed gently. Prepare all samples and assay reagents on ice prior to starting the assay procedure. Avoid foaming. Perform the assay itself on the temperature as indicated in the protocol overview (See chapter 4).

Dilution procedures

Serum or plasma samples

C3NeF positive and negative samples can be distinguished accurately if serum or plasma samples are diluted at least 25x with supplied dilution buffer in polypropylene tubes. Note that the most reliable results are obtained when all samples, including negative and positive controls, are derived from the same matrix and tested at the same dilution.

Assay is optimized for use with serum and EDTA-plasma samples, which are the recommended sample matrices. Use of other sample types has not been validated and may affect assay performance.

Comment regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline. It is advised to run pilot experiments for each study/sample cohort to determine the dilution factor that results in the broadest dynamic range between C3NeF positive versus negative samples (usually between 25x – 200x dilution). The same dilution should be used for all samples including the HK3051-NC (technical negative control). The cut-off value must be determined in the same dilution as is used for the test samples.

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

Guideline for dilution of samples

Please see table 2 for recommended sample dilutions. Volumes are based on a total volume of at least 125 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 5 µl of sample.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of dilution buffer required
1.	10x	Not necessary	15 µl (sample)	135 µl
2.	25x	Not necessary	5 µl (sample)	120 µl
3.	50x	Recommended: 10x (see nr.1)	25 µl (pre-dilution)	100 µl
4.	100x	Recommended: 10x (see nr.1)	15 µl (pre-dilution)	135 µl
5.	200x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	190 µl
6.	400x	Recommended: 25x (see nr.2)	10 µl (pre-dilution)	150 µl
7.	800x	Recommended: 25x (see nr.2)	5 µl (pre-dilution)	155 µl

Table 2

8. REAGENT PREPARATION

Allow the plate, HRP-labeled detection antibody, TMB substrate and stop solution to equilibrate to room temperature (20 – 25°C) prior to use. The dilution buffer, lyophilized proteins and lyophilized negative control remain cold. These reagents and sample dilutions should be prepared 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 2 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 15 ml of 10x dilution buffer with 135 ml of distilled or de-ionized water, which is sufficient for 2x 96 tests. In case less volume is required, prepare the desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer with 9 parts of distilled or de-ionized water.

Keep buffer on ice to keep it cold for preparation of assay reagents (like the lyophilized proteins) and sample dilutions.

The dilution buffer can be equilibrated to room temperature when used for diluting the HRP-labeled detection antibody.

Lyophilized proteins

The lyophilized proteins (Factor B and Factor D) should be reconstituted with 250 µl of dilution buffer. After reconstitution, the solution in the vial should be mixed gently by pipetting. Prior to use in the assay the reconstituted proteins should be 6x further diluted, thus adding 1250 µl of dilution buffer to the reconstituted 250 µl protein. For each protein 25 µl per well of protein solution is required per test well. In case less volume is needed, prepare desired amount by taking 1 part of the 250 µl reconstituted protein and 5 parts of dilution buffer. The reconstituted vials and/or protein solutions should be kept on ice until incubation in the assay plate. After reconstitution the undiluted protein vials can be stored below -20ºC for at least one month. Avoid multiple freeze-thaw cycles.

Prepare a pre-mix of diluted Factor B and Factor D in a 1:1 ratio to minimize pipetting steps. Make sure to keep this mix on ice until use. The mixture cannot be stored.

Lyophilized negative control

The lyophilized negative control is prepared from 25 µl undiluted heat-inactivated C3NeF negative human serum. The negative control should be used in the same dilution as all other samples in the test. Consult table below for what reconstitution volume should be used based on the sample dilution.

	Sample dilution	Reconstitution volume of lyophilized negative control
1.	10x	250 µl
2.	25x	625 µl
3.	50x	1250 µl
4.	100x	2500 µl
5.	200x	Reconstitute according to 100x dilution (see nr.4) and dilute 2x further
6.	400x	Reconstitute according to 100x dilution (see nr.4) and dilute 4x further
7.	800x	Reconstitute according to 100x dilution (see nr.4) and dilute 8x further

Table 3

HRP-labeled detection antibody

The HK3051-T tube contains 250 µl of the HRP-labeled detection antibody. Dilute the detection antibody 100x for use in the assay. For 2x 96 tests 240 µl should be diluted with 23.76 ml dilution buffer. In case less volume is required, prepare the desired volume of detection antibody by diluting 1 part of the 100x concentrate with 99 parts of dilution buffer.

9. ELISA PROTOCOL

1. Determine the number of test wells required and place the corresponding microwell strips into the supplied frame. Include an equal number of background control wells in addition to the test wells. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.
2. For all following steps: Note that the best results are obtained when the steps are precisely followed and reagents are used immediately. Do not leave the ELISA plate in wash buffer in between assay steps, but proceed immediately.
3. Transfer 50 µl of dilution buffer to the background control wells.
4. Transfer 50 µl of the Factor B – Factor D mixture to the test wells (or alternatively 25 µl of Factor B and 25 µl of Factor D, see Chapter 8 'Lyophilized proteins').
5. Transfer 50 µl of heat-inactivated sample to each well and mix gently.
6. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
7. Incubate the plate for 30 minutes at 37°C.
8. Wash the plates 4 times with wash buffer using a plate washer or as follows*:
   a. Carefully remove the cover, avoid splashing.
   b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
   c. Add 200 µl of wash buffer to each well and empty the plate as described in 5b.
   d. Repeat the washing procedure 8b/8c three times.
      NOTE! Make sure to immediately proceed and not let the plate stand in wash buffer in between washing steps.
   e. Empty the plate and gently tap on thick layer of tissues.
9. Add 100 µl of the diluted peroxidase-labeled detection antibody to each well using the same pipetting order as applied in the previous step. Do not touch the side or bottom of the wells.
10. Cover the plate and incubate for 30 minutes at room temperature (20 – 25ºC). Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminum foil is recommended.
11. Repeat the wash procedure described in step 8a-e.
12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in previous steps. Do not touch the side or bottom of the wells.
13. Cover the plate and incubate for 15 minutes at room temperature. Avoid exposing the microwell 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 the previous step. 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.

10. INTERPRETATION OF RESULTS

- Calculate the signal obtained for each sample by taking the OD_{450nm} of the test well (C3bBb) and subtract the OD_{450nm} of the control well (C3b). Note: In all next steps when 'signal' is used, it refers to the signal after background correction and not the raw ODs.
- The signal of the provided negative (technical) control (HK3051-NC) should be <0.3.
- The average signal of the negative control samples + 3x the st.dev. of these samples is the *cut-off signal*. Preferably, the cut-off signal is established in advance using 10–20 C3NeF-negative samples. All tested samples that are above this cut-off are classified as C3NeF positive and all samples below the cut-off are classified as C3NeF negative.
- It is recommended to include multiple known positive and negative samples in each run to monitor assay performance. If the assay shows an insufficient dynamic range, refer to the troubleshooting suggestions for optimization.

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 several control samples (positive and negative) 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 positive and negative control sample should be included in each plate to compare results of different plates.
- 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 samples, 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 sample 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 C3bBb binding assay ELISA.

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12. QUALITY CONTROL

The Certificate of Analysis applies only to this specific lot and must not be used for any other lot. 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

Comparison between serum and plasma EDTA

The assay was tested for usability in serum and plasma EDTA samples. Both sample types were heat-inactivated for 30 minutes at 56ºC prior to use. Results show that distinction between positive and negative samples is suitable in both sample types. However, it is always recommended to determine the cut-off value for each sample type independently and distinguish positive and negative samples only based on the cut-off value for the matching sample type.

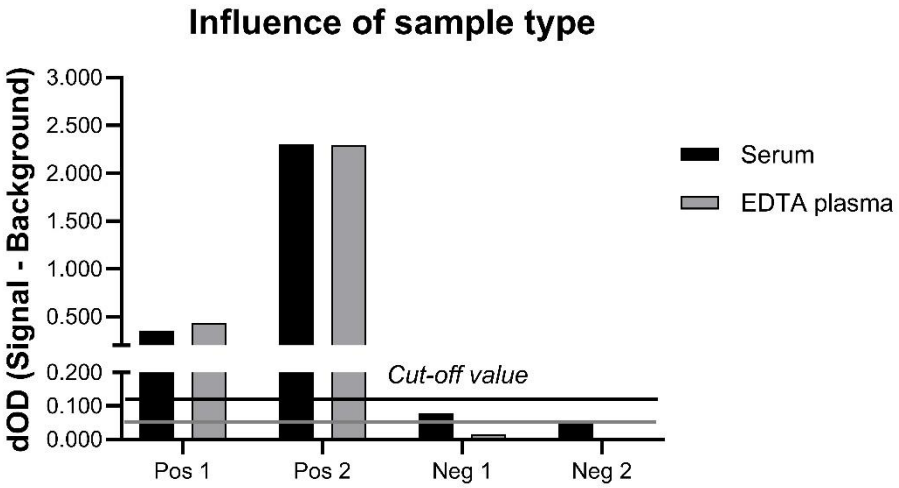

Figure 2: Comparison between serum and EDTA plasma. Cut-off value for serum samples was determined at 0.126 and for EDTA plasma samples at 0.049. All positive samples were above the set cut-off values and all negative samples were below. Both sample types can be used in this assay, but always compare samples to a cut-off value set with negative samples from the same sample type.

Inter-assay variation

The inter-assay variation was tested by performing the same assay multiple times by multiple operators (n=4).

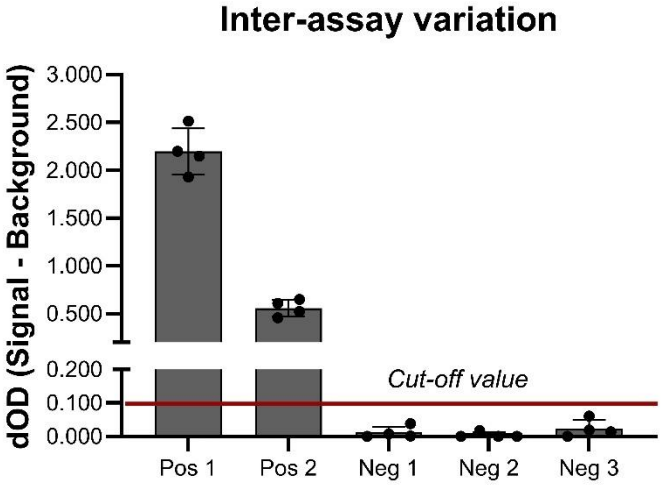

Figure 3: Inter-assay variation tested by comparing the results over multiple runs (n=4).

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

Suggestions summarized below in Table 4 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 sample dilutions
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•	•	•	•	•	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
•		•		•	Strips were kept in wash buffer 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 4

15. REFERENCES

1. Michels MAHM, Volokhina EB, van de Kar NCAJ, van den Heuvel LPWJ. Challenges in diagnostic testing of nephritic factors. Front Immunol. 2022 Nov 14;13:1036136. doi: 10.3389/fimmu.2022.1036136. PMID: 36451820; PMCID: PMC9702996.
2. Paixão-Cavalcante D, López-Trascasa M, Skattum L, Giclas PC, Goodship TH, de Córdoba SR, Truedsson L, Morgan BP, Harris CL. Sensitive and specific assays for C3 nephritic factors clarify mechanisms underlying complement dysregulation. Kidney Int. 2012 Nov;82(10):1084-92. doi: 10.1038/ki.2012.250. Epub 2012 Aug 1. PMID: 22854646; PMCID: PMC3608896.
3. Seino J, vd Wall Bake WL, van Es LA, Daha MR. A novel ELISA assay for the detection of C3 nephritic factor. J Immunol Methods. 1993 Feb 26;159(1-2):221-7. doi: 10.1016/0022-1759(93)90160-9. PMID: 8445254.