Quantitative complement alternative pathway activity assay

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Introduction

The measurement of 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. The historically used hemolysis assays are **hard to standardize** due to opsonization, stability of the erythrocytes and complicated and time-consuming steps. ELISA based assays can overcome this, nonetheless, can be hampered by **high serum dilutions** and **restrictive quantification**. The alternative pathway (AP) complement activity assay (HK3012) has been developed to address these elements and aimed to improve functional complement measurement by **accurate**, **more standardized and high throughput** assessment of complement activity.

The AP complement activity assay (HK3012) measures complement function *in vitro* in serum. The wells of the assay 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 (figure 1).

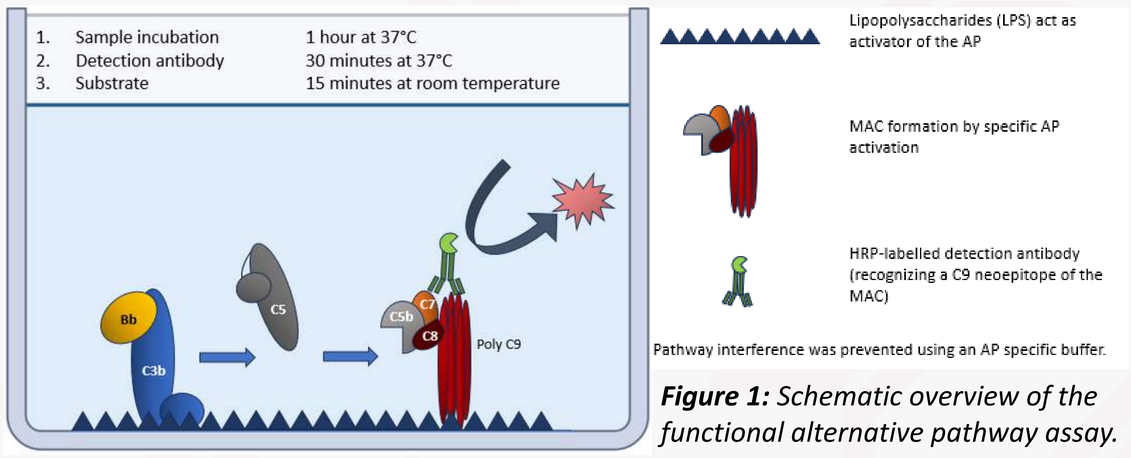

Figure 1: *Schematic overview of the functional alternative pathway assay.*

Quantification of Complement Activation

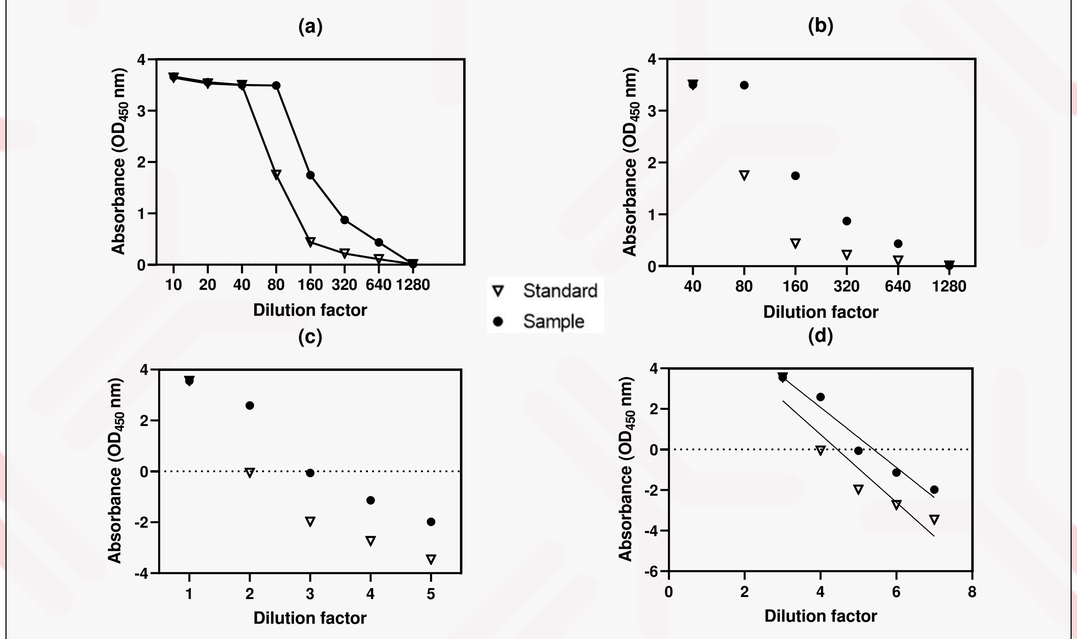

Figure 2: *(a-d) Regression analysis on logistically transformed optical density (OD) values. Pathway activity expressed as percentage of activity of calibrator. (a) OD values of a calibrator serum and a donor serum. (b) Max OD is established and dilutions with similar OD or higher are omitted. (c) Transformation OD values between 0 and 1, hereafter the values are logistically transformed using y'=ln[y/(1-y)]. (d) A linear regression is applied to the data points. [1]*

Results

Optimal sample dilution & quantification

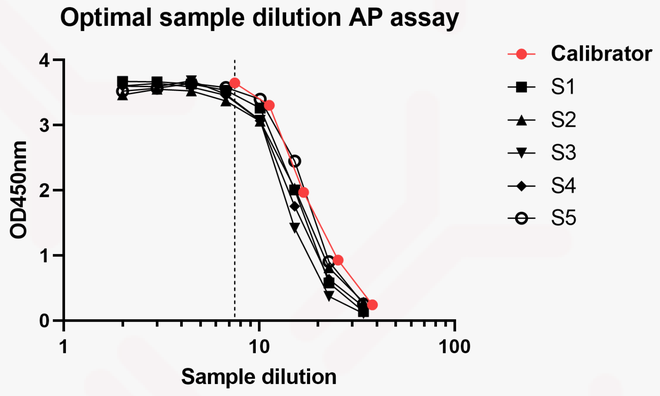

Figure 4: *A calibrator curve and 1.5-fold serial dilution of complement preserved serum samples. The assay works with an optimal 7.5x start dilution.*

The optimal serum dilution was determined by testing different serum donors for their key-point. At the key-point dilution (7.5x, figure 4) none of the complement components are rate limiting and the alternative pathway activity is 100%. The dilutions after the key-point can be used to quantitatively measure complement activity of samples compared to a calibrator consisting of pooled preserved serum. In case of aberrant activity levels, other dilutions can be tested, as long as the key-point dilution has been verified.

Conclusion

A normal distribution of alternative pathway activity was found using a cohort of 100 healthy serum donors (figure 8). Most donors have a pathway activity of 100-200% compared to the calibrator.

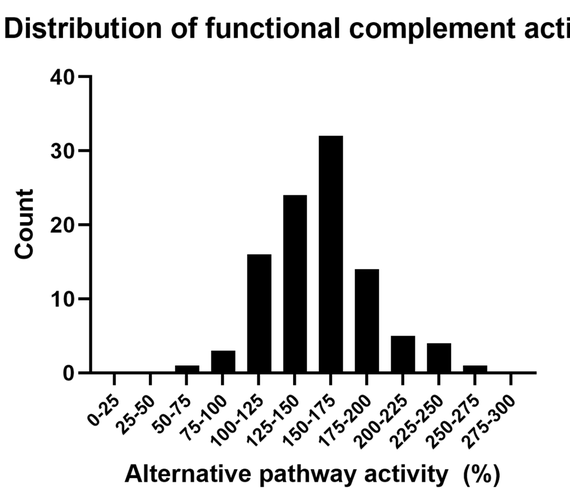

Figure 8: *Normal distribution of functional alternative pathway complement activity of 100 healthy serum donors*

Hycult Biotech has developed an easy-to-use and standardized AP complement pathway assay (HK3012), which enables reproducible and quantitative complement measurement for screening for complement therapeutics. The assay is able to measure complement activity in low serum dilutions (7.5x to 38x diluted) and thereby decreasing the change of false negative results.

If you want to test our assay, please contact us!

Sample handling

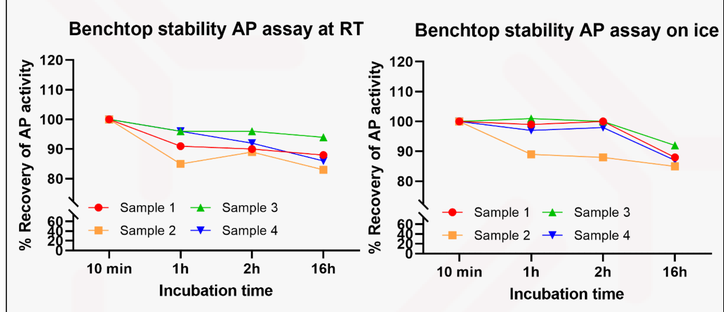

Figure 5: *Benchtop stability at room temperature and on ice of preserved serum samples.*

The benchtop and freeze-thaw stability of four different samples was determined. A recovery% between 80-120% of the AP activity was considered stable. Samples remained stable after 16 hours at room temperature or O/N on ice (figure 5). Furthermore, samples were stable after four freeze-thaw cycles (figure 6). Nevertheless, it is advised to minimize freeze-thaw cycles and to store samples on ice to prevent complement activation.

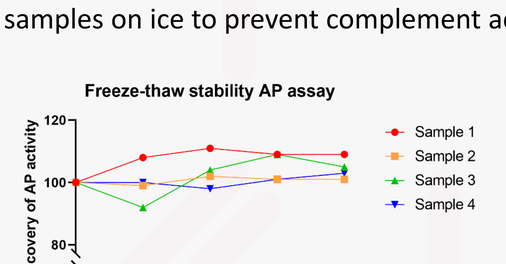

Figure 6: *Freeze-thaw stability of preserved serum samples.*

Intra- and inter-assay variation

The reproducibility of the assay was tested using three identical samples across 12 experiments. Both intra- and inter-assay variation meet the requirements of a CV% <15% and a CV% <20% respectively (table 1), meaning that the AP assay is reproducible.

Table 1: *Intra and inter assay variation of the AP assay.*

Intra-assay variation			
Sample	1	2	3
Average activity (%)	119	94	79
CV%	7.3	8.9	9.6
Sample	**1**	**2**	**3**
Operator 1			
Average activity (%)	119	96	77
CV%	4.7	4.4	4.3
Operator 2			
Average activity (%)	130	105	89
CV%	10.3	3.9	10.3
Inter-assay variation			
Average activity (%)	123	101	83
CV%	8.1	6.1	11.2

Influence of incubation temperature and time

The robustness of the assay was assessed by increasing and decreasing the incubation temperature and time. A higher signal is obtained when incubating at 40°C, while a decrease in incubation temperature prevents pathway activation completely (figure 3). The complement cascade relies on enzymatic activity and complex formation, which is most optimal at 37°C. Shortening the protocol is also not advised, as this leads to a strong decrease in complement activity.

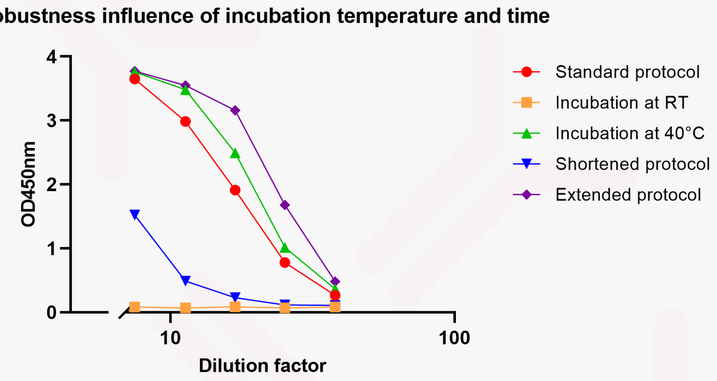

Figure 3: *Robustness of the AP assay. Incubation temperatures of room temperature and 40°C were tested as well as a shortened (30 minutes sample incubation) and extended (90 minutes sample incubation) protocol.*

Inhibitor testing

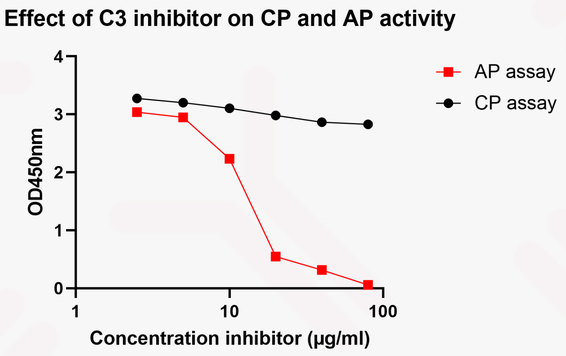

Figure 7: *Inhibiting effectiveness of an AP C3 specific inhibitor in the AP assay.*

One of the applications of the AP assay is to test efficacy of complement inhibitors. An AP specific C3 inhibitor was tested in the AP assay. A decrease in complement activity is observed with increasing inhibitor concentration, eventually leading to complete blockage of the AP activity. The results (figure 7) confirm that the AP assay is suitable for screening for new complement inhibitors.

References: [1] Palarasah, Y. et. al., Clinical and Experimental Immunology 2011, 164(3):388-95

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