Quantitative complement classical pathway activity assay

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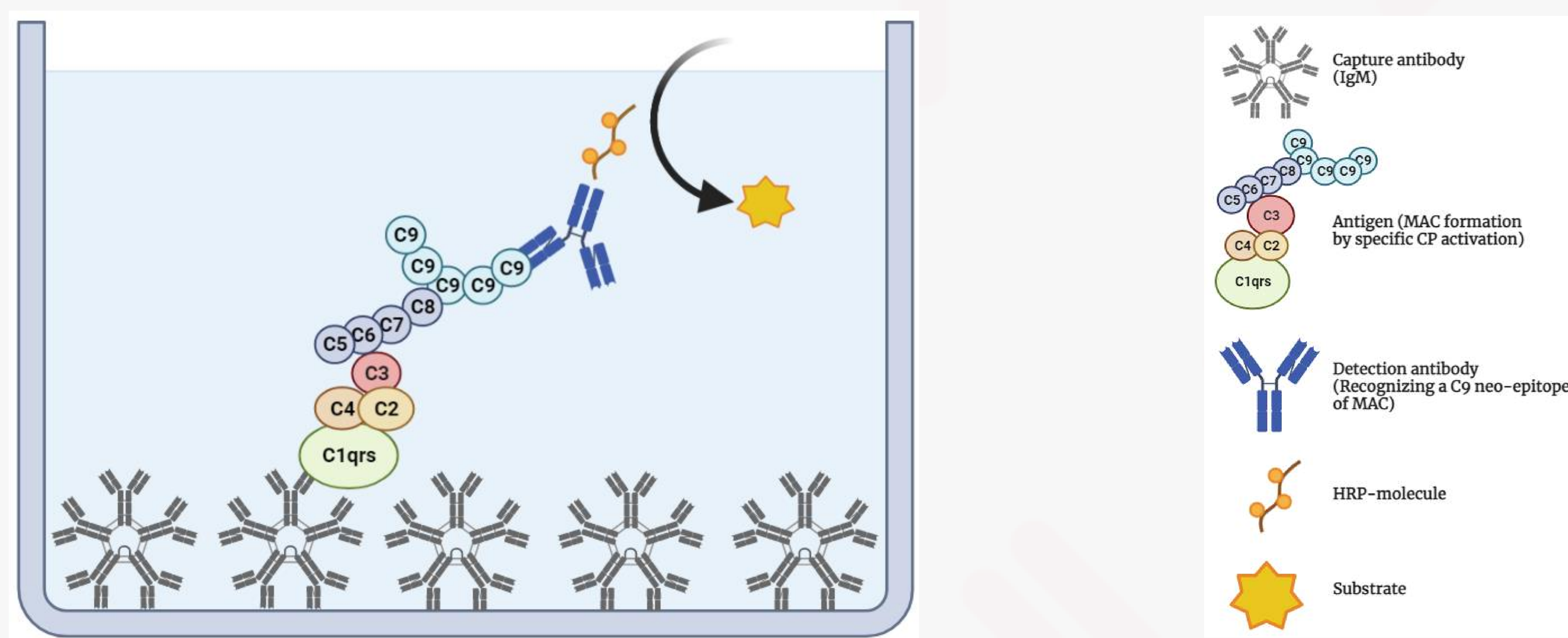
Introduction

Assessing individual activation of the complement pathways has added value for investigating complement-mediated diseases or therapeutics. Unfortunately, reliable quantitative measurement of functional pathway activation is often hampered by:

- Difficult to standardize
- Inter lab/operator variation
- Reproducibility
- Pathway interference
- False negative results

The goal of this study is to develop a robust quantitative immunoassay for measuring human classical pathway (CP) activity.

Method



Quantification of Complement Activation

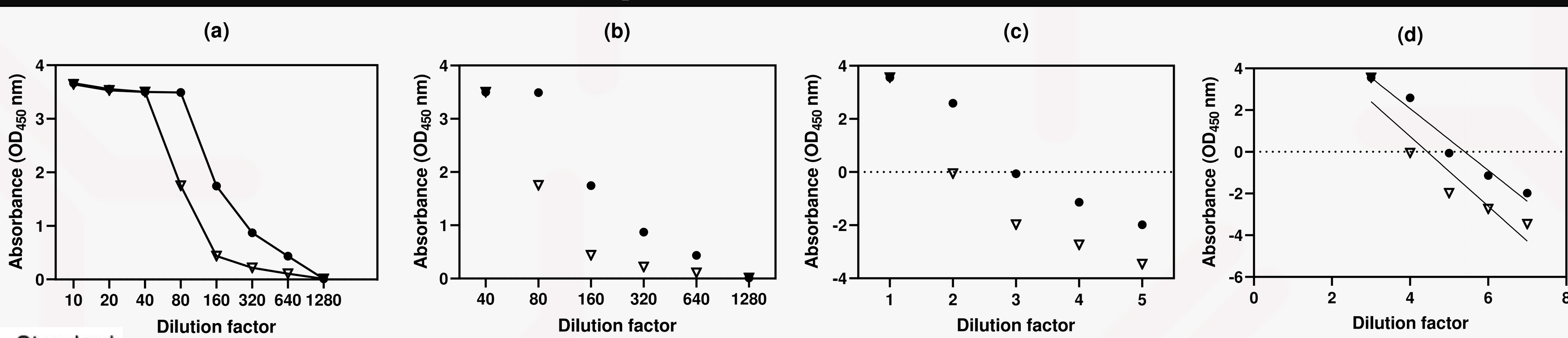


Figure 1: (a-d) Regression analysis on logistically transformed optical density (OD) values. Pathway activity expressed as percentage of activity of calibrator. (a) OD values of a calibrator serum and a donor serum. (b) Max OD is established and dilutions with similar OD or higher are omitted. (c) Transformation 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. Activity is compared with dilutions of 50% absorbance. [1]

Results – Buffer Optimization

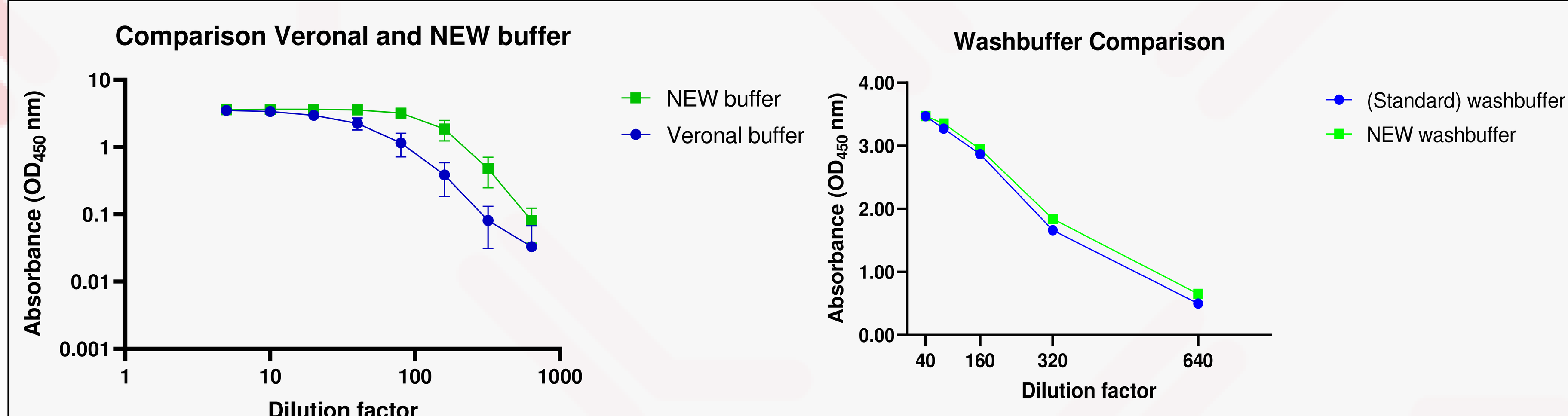


Figure 2: *Buffer comparison Veronal and NEW buffer.*

Figure 3: *Wash buffer comparison standard Hycult used wash buffer and NEW wash buffer.*

The use of Barbital based buffers is limited in many countries. Therefore, a substitute was selected. In *Figure 2* is visible that the reproducibility is improved and more consolidated data is obtained.

It is preferred to have the same buffer system within one assay. Therefore, a new wash buffer is selected in comparison to existing pathway assays. In *Figure 3* is visible that both buffers provide the same results.

Results – Pathway Interference

Interference with other pathways is eliminated. It is established that the AP is activated by the IgM-coated wells. An inhibitor was added to the dilution buffer in which samples are diluted. The difference between no inhibitor, inhibitor and a placebo inhibitor can be seen in *Figure 4*.

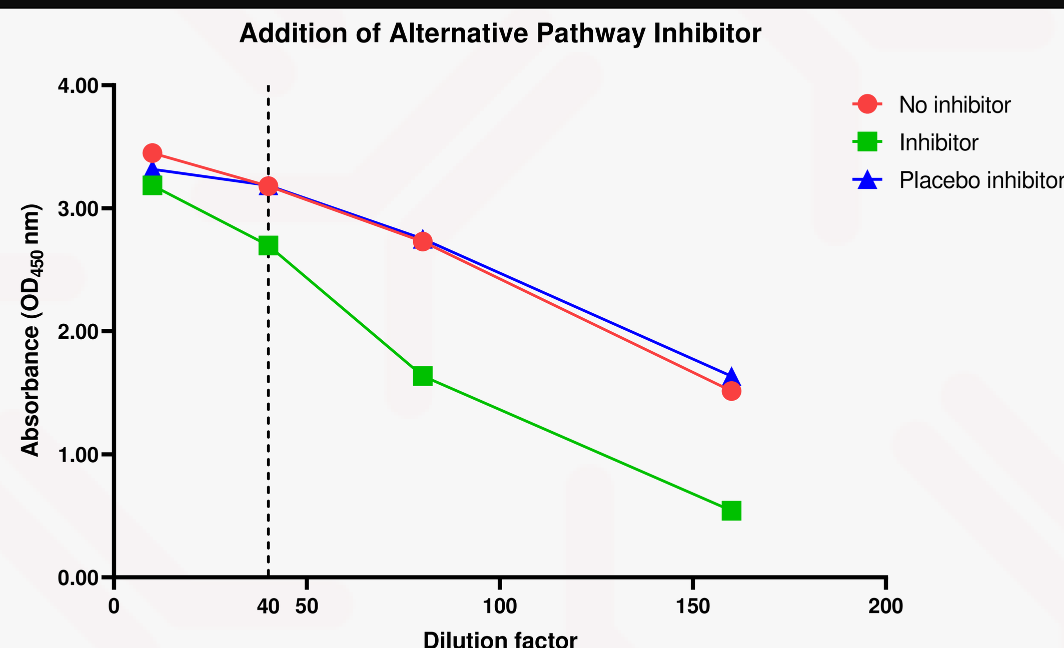


Figure 4: *Calibrator comparison with and without addition of pathway inhibitor.*

Results – Optimal Sample Dilution & Quantification

In order to establish the optimal sample dilution, in which the activity of the classical pathway is still 100%, the so-called 'key-point' is determined. At this dilution (40x, *Figure 5*) none of the complement components are rate limiting. From the optimal sample dilution, five dilutions are chosen ranging from 0-100% activity.

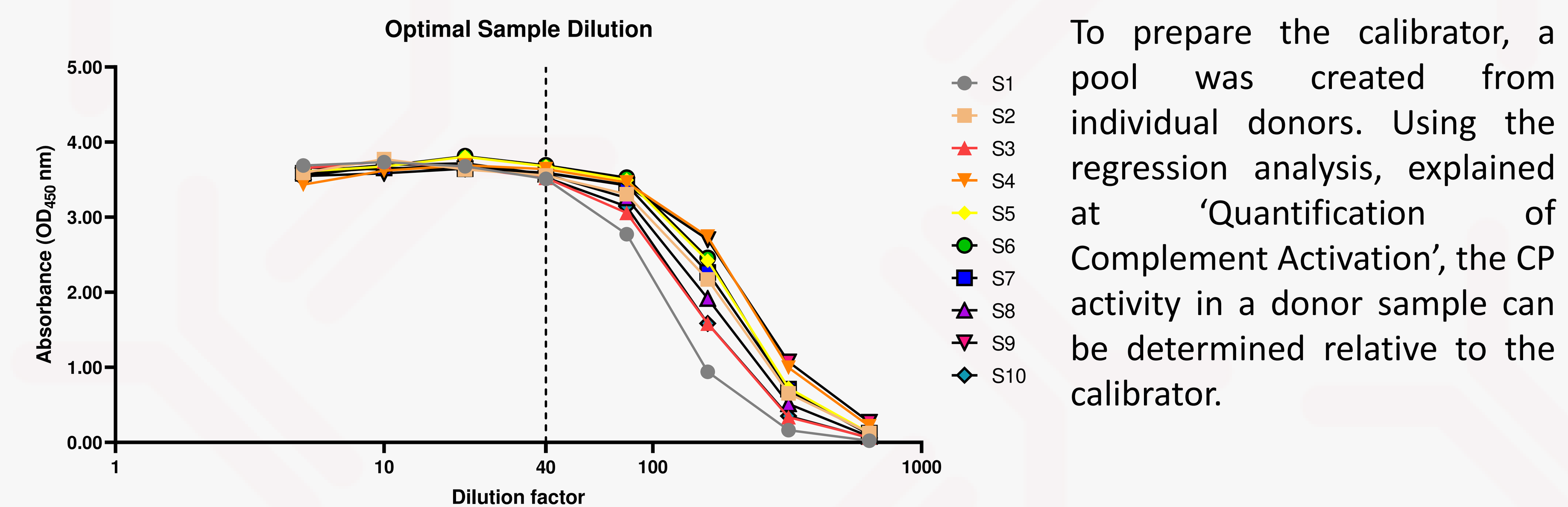


Figure 5: *Determination of the optimal sample dilution of ten individual donors.*

To prepare the calibrator, a pool was created from individual donors. Using the regression analysis, explained at 'Quantification of Complement Activation', the CP activity in a donor sample can be determined relative to the calibrator.

Results – Handling

Four preserved serum samples of individual donors were tested on freeze-thaw stability. Samples were frozen and thawed for several cycles. *Figure 6* shows samples are stable for at least four cycles. Within this range deviation in activity meets requirements of 80-120%.

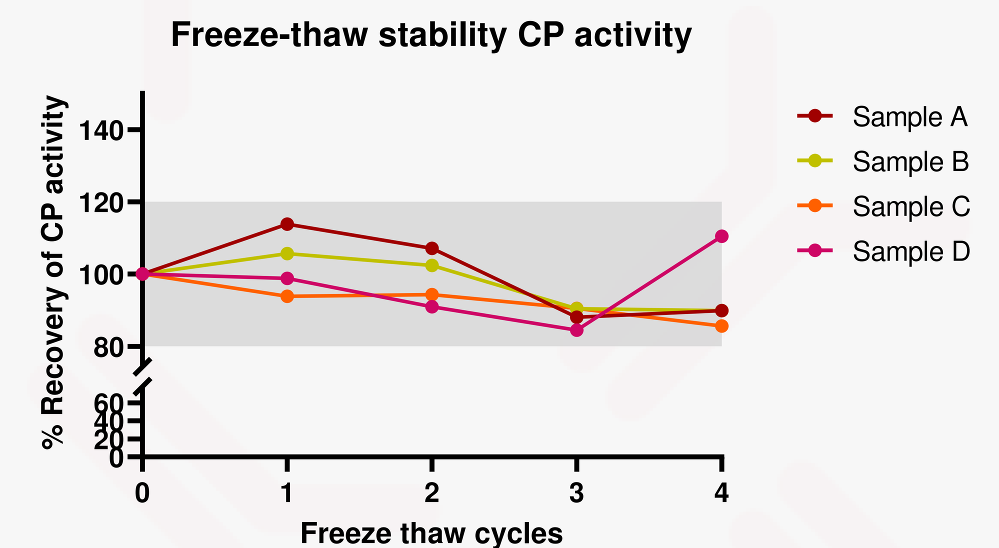


Figure 6: *Freeze-thaw stability of preserved serum samples.*

Robustness of the assay was tested. In *Figure 7* is visible that incubation at RT is decreasing the signal (A-B). In addition, rough pipetting/mechanical stress is causing a signal drop after dilution >160x (C-E). Remarkable, there is no difference in standard handling performance on ice or RT (D-F).

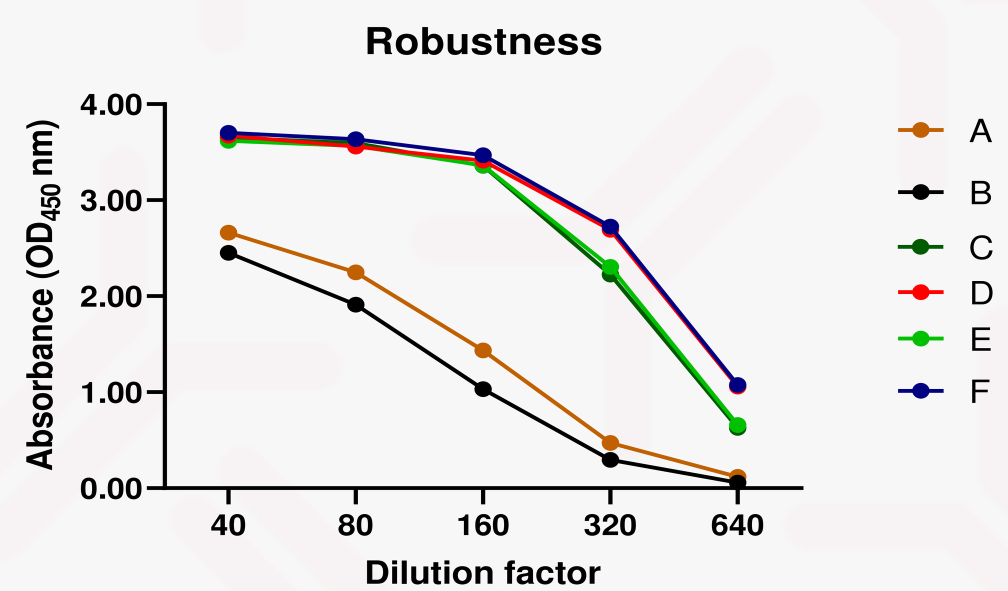


Figure 7: *Stress experiment;*
(A) Ms, RT inc., performance at RT
(B) Ms, RT inc., performance on ice
(C) Ms, 37°C inc., performance at RT
Ms = Mechanical stress, inc.= incubation
(D) 37°C inc., performance at RT
(E) Ms, 37°C inc., performance on ice
(F) Standard handling, 37°C inc., performance on ice

Results – Validation (preliminary results)

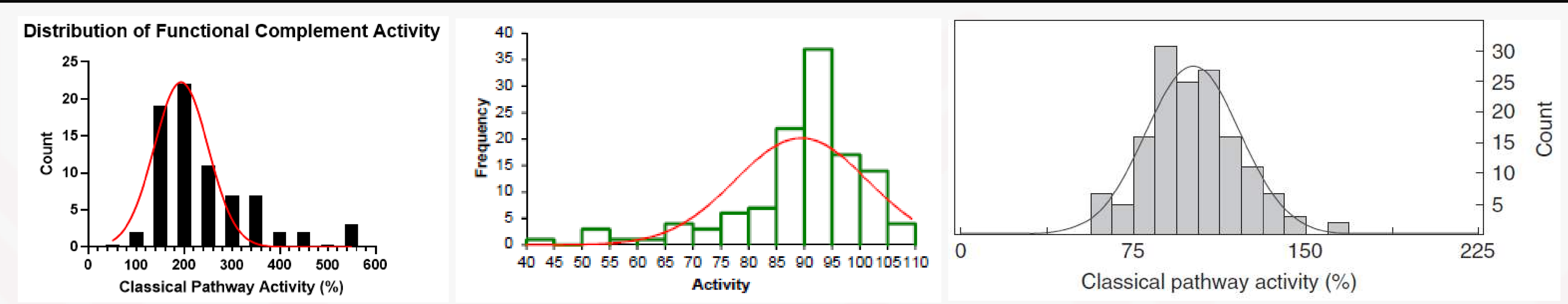


Figure 8: *Normal distribution of functional complement activity in Hycult Biotech assay prototype (75 healthy blood donors).*

Figure 9: *Normal distribution of complement activity in SVAR WIESLAB assay (120 healthy blood donors) [2].*

Figure 10: *Normal distribution of complement activity in Y. Palarasah assay (150 healthy blood donors) [1].*

	Test 1 (%)	Test 2 (%)	Test 3 (%)	Average	CV%
Sample 1	69.6	71.9	73.5	71.7	2.7
Sample 2	151.4	175.5	171.9	166.3	7.8
Sample 3	217.9	288.8	288.2	265.0	15.4
Sample 4	112.2	115.9	112.2	113.4	1.9

Table 1: *Intra-assay precision for quantitative application was determined by testing four samples in three replicates at one occasion.*

Conclusion

- Complement activity can be reliably quantified;
- New buffer formulations improves reproducibility and sample dilution;
- Interference of the alternative pathway is effectively blocked;
- Sample handling can affect the outcome of the assay.

Future Development

- Optimization of calibrator and detection antibody stability, inter/intra and batch to batch variation;
- Testing cohort and patient samples with known deficiencies for determination of normal activity of classical pathway;
- **Request: If you want to test our assay, please contact us!**

References: [1] Palarasah, Y. et. al., *Novel assays to asses the functional capacity of the classical, the alternative and the lectin pathways of the complement system.* Clinical and Experimental Immunology, 2011. [2] SVAR, WIESLAB Complement system Classical pathway Qualitative and Semi-Quantitative test instructions for use, December 2018

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