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Validation of a SC5b-9 Commercial Kit for Preclinical Biomarker Analysis

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INTRODUCTION

During preclinical testing, a plethora of vital information can be uncovered by analyzing multiple complement proteins; however, the data generated must be interpreted and presented carefully. Some of the most challenging assays (single-plex or multiplex) to validate are ones that look for endogenous markers. One endogenous marker that can be examined and quantified in a preclinical testing system is SC5b-9. This poster presents the analytical method validation results for determining complement protein SC5b-9, using a Quidel® MicroVue™ SC5b-9 Plus Enzyme Immunoassay (EIA) kit with cynomolgus monkey K2EDTA plasma.

SC5b-9, known as the Terminal Complement Complex (TCC), is an assembly of proteins created by joining proteins C5 and C9 (see **Figure 1**). SC5b-9 can be formed from either the classical, lectin or alternative pathways. The advantage of this analytical method is that it provides a full picture of what is occurring in the Nonhuman Primate (NHPs) complement system. The kit contains a pre-coated plate that uses a monoclonal antibody that attaches to the TCC's C9 ring, which then captures the complex. After incubation with the samples, Horseradish Peroxidase (HRP)-conjugated antibodies are added and bind to the SC5b-9 complex antigens. The following parameters were tested for the SC5b-9 method validation: Intra/Inter-Accuracy and Precision, Dilutional Linearity, Specificity, Selectivity, Sensitivity, 4 ºC Benchtop Stability, Freeze/Thaw Stability, and Long-Term Stability. This poster details Freeze/Thaw Stability, 4 ºC Benchtop Stability, Long-Term Stability, and Selectivity parameters.

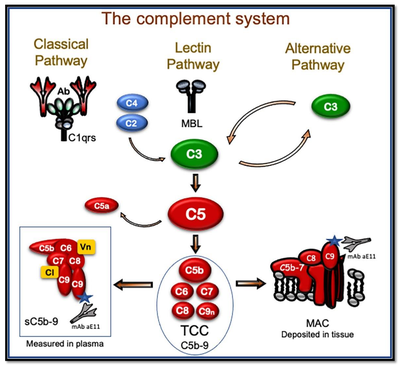

Figure 1. The Complement System With SC5b-9 Complex
https://www.frontiersin.org/articles/10.3389/fimmu.2021.738927/full

MATERIALS, METHODS, AND EQUIPMENT

NHP K2EDTA Plasma Samples (In-House and External)
Cynomolgus monkey plasma K2EDTA was obtained from an in-house animal colony, suppliers BioIVT, and Worldwide Primates. Plasma was stored in a freezer set to maintain -20 ºC or lower. All samples were removed from the freezer (-20 ºC for individual plasma, 5% hemolyzed plasma for Selectivity and Specificity, and -80 ºC for Stability samples) and then placed on dry ice before the beginning of the assay procedure. Samples were processed promptly to prevent any endogenous SC5b-9 complexes from clumping together.

Quidel® MicroVue™ SC5b-9 Plus EIA kit (Cat. #A020; #A029 (CE))
The Quidel® MicroVue™ SC5b-9 Plus EIA kit consists of an ELISA assay format with a 96 well flat bottom plate and reagents to analyze a max total of 40 samples in duplicate. The kit can use human serum, plasma, and other experimental specimens, such as NHP K2EDTA plasma samples. The SC5b-9 Plus EIA kit's reagents include five Standards of varying SC5b-9 concentrations, two Quality Controls (QCs) of both High and Low SC5b-9 concentrations, two vials of Wash Solution, and one vial each of Specimen Diluent, SC5b-9 Conjugate, TMB Substrate, and Stop Solution respectfully. All Standard and QC nominal concentrations are provided via the kit's Certificate of Analysis (see **Table 1**) and are used to format analytical runs with a BioTek Synergy H1 Hybrid Multi-Mode system.

The kit was removed from a refrigerator set to maintain 4 ºC and allowed to warm to room temperature for at least 30 minutes. A 1X Wash Solution was created using a kit-provided 20X Wash Solution Concentrate with Milli-Q deionized water. Standards and Controls were ready to use with no dilution necessary and added to the kit-provided plate at a 1:1 dilution fold. NHP plasma samples were thawed with agitation in a water bath set to 37 ºC until just thawed. Plasma samples were then diluted 1:10 in specimen diluent before being dispensed in duplicate into the provided plate. See **Figure 2** for the detailed procedure.

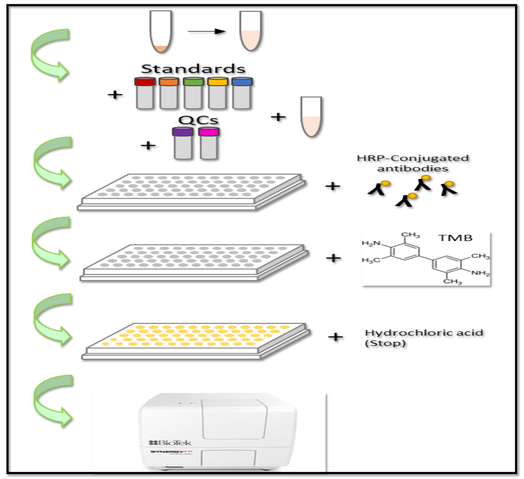

Figure 2. SC5b-9 Assay Procedure

Table 1. Nominal Concentrations (NG/ML) and Range Concentrations (NG/ML) for Quidel® MicroVue™ SC5b-9 Plus EIA Kit

Standard/Control	Nominal Concentration (ng/mL)	Range Concentration (ng/mL)
Standard A	0	------
Standard B	10	------
Standard C	33	------
Standard D	104	------
Standard E	162	------
Low Control	------	13-27
High Control	------	99-145

Synergy H1 Hybrid Multi-Mode Reader

The BioTek Synergy H1 monochromator-based optics and filter-based optics multi-mode microplate reader was used in the validation. All data were collected and exported using Gen5™ Data Analysis Software with UV-Vis absorbance wavelengths set at 450nm. Assay results were analyzed using a linear curve fit. The Standard curve is calculated using blank subtracted standards and a 4-parameter logistic curve.

RESULTS

Freeze/Thaw (F/T) Stability
F/T stability samples within 3 different tubes at Trending QC nominal concentration were prepared and stored in a freezer at -80 °C for 24 hours. Then, the F/T stability samples were rapidly thawed in a 37 ºC water bath before being placed on wet ice for at least 2 hours. F/T stability samples were then re-frozen under the same conditions for more than 12 hours, followed by an additional rapid thaw with an immediate placement on wet ice. After 3 F/T cycles, the F/T stability samples were analyzed.

Table 2. Freeze/Thaw Stability for Trending QCs

Freeze Thaw Stability	Trending QC		
Nominal Concentration (ng/mL)	126		
Plate ID, Cycles	Conc. (ng/mL)	%CV	%RE
Plate 6, 3 F/T Cycles	117.39	1.10	-6.9
	113.06	0.80	-10.3
	116.17	4.50	-7.8
N	3		
Mean	115.54		
SD	2.23		
%CV	1.9		
%RE	-8.3		

4 ºC Benchtop Stability
Benchtop stability samples within 3 different tubes were prepared and stored under frozen conditions in a freezer set to -80 °C for 24 hours. Then the benchtop stability samples were rapidly thawed before being placed on wet ice for at least 4.5 hours.

Table 3. Benchtop Stability for Trending QCs

Benchtop Stability	Trending QC		
Nominal Concentration (ng/mL)	126		
Plate ID, Time on Wet Ice	Conc. (ng/mL)	%CV	%RE
Plate 6 4.5 Hours	116.28	2.90	-7.7
	121.56	4.70	-3.5
	124.44	0.10	-1.3
N	3		
Mean	120.76		
SD	4.14		
%CV	3.4		
%RE	-4.2		

Long-Term Stability (LTS)
Long-Term Stability samples in 3 different tubes at Trending QC concentration level were prepared and stored under frozen conditions in a freezer ser to -80 °C for 63 days. After this storage period was finished, the stability samples were analyzed.

Table 4. LTS Stability for Trending QCs

Long Term Stability	Trending QC		
Nominal Concentration (ng/mL)	146		
Plate ID, Time Stored at -80 °C	Conc. (ng/mL)	%CV	%RE
Plate 11 63 Days	172.30	0.5	18.3
	173.99	7.1	19.4
	170.90	1.1	17.3
N	3		
Mean	172.40		
SD	1.55		
%CV	0.9		
%RE	18.3		

Selectivity
Selectivity was tested by spiking individual cynomolgus monkey plasma on wet ice at two different concentrations (in the range of HQC and at LLOQ level). At least 10 sources of non-hemolyzed plasma and 1 source of 5% hemolyzed plasma were tested.

Table 5. Selectivity for LLOQ and HQC

Selectivity Name	Sel-LLOQ			Sel-HQC		
Nominal or Range Concentrations (ng/mL)	10			99-145		
Plasma Number	Conc. after blank subtracted	%CV	%RE	Conc. after blank subtracted	%CV	In Range?
1	2.54	0.8	-74.6	113.19	3.3	Yes
2	13.83	2.3	38.3	92.71	0.3	No
3	14.40	2.9	44.0	121.75	4.9	Yes
4	8.52	2.7	-14.8	136.91	3.4	Yes
5	11.87	0.8	18.7	126.82	0.4	Yes
6	8.48	10.0	-15.2	99.98	1.2	Yes
7	10.37	2.2	3.7	129.21	1.7	Yes
8	10.92	0.7	9.2	142.85	0.5	Yes
9	16.63	4.9	66.3	120.43	2.7	Yes
10	12.45	3.3	24.5	145.66	0.1	No
Hemolyzed	6.69	10.0	-33.1	100.59	0.5	Yes

Red values: Above Nominal/Out of Range

CONCLUSIONS

The intra/inter accuracy (%RE) and precision (CV%) were within the +/- 25% Bias acceptance criteria for ULQC and LLQC. The HQC/LQCs were in the range provided by the Certificate of Analysis for 4 ºC benchtop at 4.5 hours, 3 freeze/thaw cycles, and 63 days of stability parameters. The stability samples themselves met acceptance criteria for 4 ºC benchtop at 4.5 hours, 3 freeze/thaw cycles, and 63 days when stored in a freezer set to maintain -80 ºC.

As expected during the execution of a validation looking at an endogenous biomarker, Selectivity was the most difficult parameter to validate, as the plasma samples were not consistent in meeting acceptance criteria for both LLOQ and HQC parameters, i.e., less than 80% of the Selectivity Samples met acceptance criteria for LLOQ, including 5% Hemolyzed. During the validation, the samples that failed Selectivity still failed to meet acceptance criteria, and more Selectivity Samples will need to be tested. Parallelism will also be assessed if a native sample can be found with high enough SC5b-9 concentrations. Validating endogenous markers should not be shied away from, as overall, they can be very successful, even if one parameter is difficult.