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Development and Validation of a Novel ECL Method for the Quantitative Detection of Complement C3 in Human Serum

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PURPOSE

The complement system plays critical roles in both innate and adaptive immune responses. The third complement factor (C3) is situated at the junction of all three complement pathways and is essential for activating the complement system (Law S.K.A. and Reid K.B.M., 1995). However, accurate measurement of C3 level in human body fluids can be challenging due to the abundance of C3 and its sensitivity to heat, which can trigger the proteolytic reaction to convert C3 into downstream substrates (Seya T, Nagasawa S., 1988). To overcome the difficulties, a novel electrochemiluminescence (ECL) immunoassay method was developed and validated to quantitatively detect C3 in human serum.

OBJECTIVE(S)

To develop and validate an electrochemi-luminescence (ECL) immunoassay method to quantitatively detect C3 in human serum.

METHOD(S)

Comparing with traditional ELISA method, ECL immunoassay platform provides better sensitivity and broader dynamic range and was chosen for the method. Due to the high endogenous level of C3 in human serum, assay buffer was used as surrogate matrix. A normal base pool (NBP) made with gender pooled normal human serum samples was included in the method as dilution quality control (DQC) to monitor the assay performance when analyzing human serum samples. A native C3 protein purified from normal human serum was chosen as reference standard. The standard stock was spiked into surrogate matrix to prepare for standard curve and high, middle, and low-quality controls (HQC, MQC and LQC). A pair of anti-human C3 antibodies, including a biotinylated capture antibody that binds to streptavidin coated assay plate and a SULFO-TAG conjugated detection antibody, were used to develop a sandwich immunoassay (Figure 1A) and applied for fit-for-purpose validation.

RESULT(S)

A novel ECL method was successfully developed and validated to measure C3 level in human serum. A representative reference standard curve is shown in Figure 1B. The validated quantitation range of the method is 655 pg/mL (Lower Limit of Quantification, LLOQ) to 400,000 pg/mL (Upper Limit of Quantification, ULOQ). The quality controls, including buffer QCs and DQC, were used to assess the accuracy and precision of the method and the stability of the samples. All assessed samples show less than 20% inter-assay precision (%CV), inter-assay accuracy (%RE) within ± 20% and less than 20% total error (Figure 2A). The samples are stable for up to 16 hours when stored at 4°C or up to 4 hours if kept at room temperature. In addition, up to 5 times freeze-thaws of samples can be tolerated (Figure 2B). Two potential interference reagents, complement C3a and C3a desArg, were assessed at a native ratio of C3 vs C3a/C3a desArg at 2000:1 according to previous report (Hubens WHG, et al. 2021). Both reagents show no significant impact on assay performance at MQC level (Figure 3A). Normal human serum samples were used to assess selectivity and parallelism to further investigate possible interfering substances present in the serum matrix. Reference standard was spiked into 10 normal individual samples, the NBP/DQC, and a C3 depleted serum sample (C3 dpl) at LLOQ level pre-sample dilution. All tested samples show ± 20% of %RE from un-spiked nominal controls, with only one exception at 22% (Figure 3B). Finally, all six tested samples show parallelism within 1:5000 to 1:1000000 dilution range (Figure 3C), which embraces the sample dilution factor at 1:50000, when %RE is limited to ± 30%.

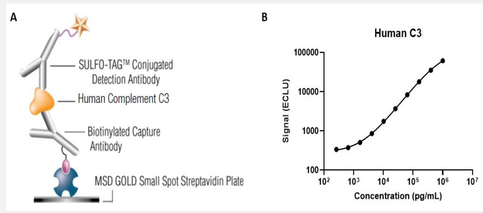

Figure 1: *(A) Schematic of the sandwich ECL immunoassay. (B) A representative reference standard curve was fitted with a 4-parameter logistic regression model with a weighting factor of $1/Y^2$.*

A

Table 1. Accuracy and Precision Summary

Control	Nominal Concentration (pg/mL)	n	Mean Concentration (pg/mL)	StdDev (pg/mL)	Inter-Assay Precision (%CV)	Inter-Assay Accuracy [a] (%RE)	Total Error [b] (%TE)
LLOQ	655	17	676	105	16.1	3.3	19.3
LQC	1,000	14	997	76	15.2	-1.6	16.8
MQC	16,000	21	16,379	898	5.6	3.9	9.5
HQC	300,000	21	290,167	16,837	5.6	-3.3	8.9
ULOQ	400,000	21	389,505	20,995	5.2	-2.6	7.9
DQC (1:50,000)	62,053	20	62,053	3,171	5.1	0.0	5.1

[a] %RE: Bias compared to nominal concentration.
[b] Total Error = |%RE| + %CV.

B

Table 2. Stability Results Summary

Short-term Stability Results at 2 to 8°C in Human Serum

Sample	Nominal Concentration (pg/mL)	Condition	Observed Concentration (pg/mL)			n	Mean (pg/mL)	Standard Deviation (pg/mL)	Precision (%CV)	Accuracy Result 1 (%RE)	Accuracy Result 2 (%RE)	Accuracy Result 3 (%RE)
			Result 1	Result 2	Result 3							
LQC	1,000.00	16 hours	684.00	942.00	1,236.00	3	954.00	276.20	29.0	-31.6	-5.8	23.6
HQC	300,000.00	24 hours	232,140.00	250,288.00	257,767.00	3	246,731.67	13,178.44	5.3	-22.6	-16.6	-14.1
DQC	3,102,650,000.00	24 hours	2,260,400,000.00	2,010,350,000.00	2,411,400,000.00	3	2,227,383,333.33	202,553,328.37	9.1	-27.1	-35.2	-22.3

Short-term Stability Results at 15 to 30°C in Human Serum

Sample	Nominal Concentration (pg/mL)	Condition	Observed Concentration (pg/mL)			n	Mean (pg/mL)	Standard Deviation (pg/mL)	Precision (%CV)	Accuracy Result 1 (%RE)	Accuracy Result 2 (%RE)	Accuracy Result 3 (%RE)
			Result 1	Result 2	Result 3							
LQC	1,000.00	4 hours	942.00	1,069.00	1,083.00	3	1,031.33	77.68	7.5	-5.8	6.9	8.3
HQC	300,000.00	4 hours	291,241.00	287,818.00	302,990.00	3	294,016.33	7,957.65	2.7	-2.9	-4.1	1.0
DQC	3,102,650,000.00	4 hours	1,573,400,000.00	2,543,600,000.00	2,822,200,000.00	3	2,313,066,666.67	655,541,435.25	28.3	-49.3	-18.0	-9.0

Freeze-thaw Stability Results in Human Serum

Sample	Nominal Concentration (pg/mL)	Condition	Observed Concentration (pg/mL)			n	Mean (pg/mL)	Standard Deviation (pg/mL)	Precision (%CV)	Accuracy Result 1 (%RE)	Accuracy Result 2 (%RE)	Accuracy Result 3 (%RE)
			Result 1	Result 2	Result 3							
LQC	1,000.00	5X F/T	854.00	877.00	949.00	3	893.33	49.56	5.5	-14.6	-12.3	-5.1
HQC	300,000.00	5X F/T	270,280.00	286,755.00	294,851.00	3	283,962.00	12,521.35	4.4	-9.9	-4.4	-1.7
DQC	3,102,650,000.00	5X F/T	2,967,174,433.00	2,904,770,202.00	2,738,079,310.00	3	2,870,007,981.67	118,437,548.17	4.1	-4.4	-6.4	-11.8

Figure 2: *(A) Summary table of fit-for-purpose validation accuracy and precision assessment results. (B) Short-term stability and Freeze-thaw stability results summary. Accuracy results above ± 30% of %RE are highlighted in red.*

A

Table 3. Specificity Results (C3:C3a/desArg)~2000:1)

Sample	Nominal Concentration (pg/mL)	Mean Response (ECLU)	Concentration (pg/mL)	Accuracy (%RE)
C3	16,000	2,726	13,459	-15.9
C3 + C3a	16,000	2,787	13,813	-13.7
C3 + C3a desArg	16,000	2,610	12,779	-20.1
Sample ID	**LLOQ (pg/mL)**	**Mean Response (ECLU)**	**Concentration (pg/mL)**	**Below LLOQ**
C3a	655	397	229	YES
C3a desArg	655	396	226	YES

B

Table 4. Normal Human Serum Selectivity Results

Sample	Unspiked Samples (PreMRD)			LLOQ Spiked Samples (PreMRD)		
	Mean Response (ECLU)	Concentration (pg/mL)	Corrected Nominal Concentration (pg/mL)	Mean Response (ECLU)	Concentration (pg/mL)	Accuracy (% RE)
Individual 1	9,627	51,491	52,146	9,057	47,954	-8.0
Individual 2	10,143	54,715	55,370	10,229	55,258	-0.2
Individual 3	9,875	53,038	53,693	8,689	45,685	-14.9
Individual 4	7,825	40,405	41,060	8,345	43,575	6.1
Individual 5	8,238	42,923	43,578	8,011	41,537	-4.7
Individual 6	7,928	41,032	41,687	9,525	50,856	22.0
Individual 7	11,517	63,411	64,066	9,712	52,020	-18.8
Individual 8	8,871	46,809	47,464	8,380	43,792	-7.7
Individual 9	9,066	48,013	48,668	9,465	50,486	3.7
Individual 10	6,922	34,943	35,598	7,832	40,447	13.6
NBP	7,483	38,327	38,982	8,177	42,547	9.1
C3 Dpl	934	295	950	1,070	1,055	11.1

C

Table 5. Normal Human Serum Parallelism Results Summary

Sample	% RE ± 30%	
	Min Dilution	Max Dilution
Sample 1	1:2,500	1:1,000,000
Sample 2	1:5,000	1:1,000,000
Sample 3	1:5,000	1:1,000,000
Sample 4	1:2,500	1:1,000,000
Sample 5	1:5,000	1:1,000,000
Sample 6	1:1,000	1:1,000,000

Figure 3: *Fit-for-purpose validation results of (A) Specificity Assessment; (B) Normal Human Serum Selectivity; (C) Normal Human Serum Parallelism.*

CONCLUSION(S)

We have successfully developed and validated a novel ECL method with superior sensitivity and broad dynamic range for the quantitative determination of C3 in human serum. This method demonstrates highly consistent performance and accuracy and can be reliably utilized for complement studies. Furthermore, the method can be potentially adapted for quantification of C3 in other types of human body fluids.

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