Evaluation of an ELISA Method Using Novel NanoLuc® Reporter and Its Comparison with Traditional Electrochemiluminecence Assay for the Measurement of Ranibizumab

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INTRODUCTION

Neovascular or age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population. Abnormal angiogenesis in the retina leads to loss of vision. Anti-vascular endothelial growth factor (VEGF) treatment is an effective therapy shown to reverse the changes and visual symptoms of AMD. Ranibizumab is an approved treatment for AMD and macular edema which is administered intravitreally. Ranibizumab is an affinity matured Fab fragment based on the bevacizumab antibody, specifically designed for higher affinity to VEGF and shorter half-life.

Systemic exposure to Ranibizumab after ITV injection is very low due to elimination on reaching systemic circulation from the vitreous. Characterization of the pharmacokinetics of Ranibizumab requires a high sensitive assay (<500 pg/mL). Here we report a sensitive, robust method for the measurement of Ranibizumab in plasma using high-specificity antibody from Somru BioScience. In addition, we also developed a comparable method using unique novel technology; NanoLuc® from Promega Inc. NanoLuc is a small (19kDa) and ultrabright luciferase with stable luminescence signal that can be used as a reporter in ELISAs.

METHODS

The method is based on the ELISA kit developed by Somru Bioscience for the measurement of free Ranibizumab in human plasma. Briefly, biotinylated rhVEGF and rabbit anti-Ranibizumab antibody are added to plasma samples. During overnight incubation, these two molecules bind to Ranibizumab and form an immune complex. This immune complex is then captured by streptavidin-coated plate and detected using labelled anti-Rabbit antibody (ruthenium or NanoLuc labelled). The plate was then analyzed for electrochemiluminescence (ECL) or luminescence (NanoLuc technology) signal. Figure 1 is an example of a standard curve in ECL discovery workbench software.

RESULTS

The precision and accuracy of the assay was determined by running a standard curve from 625 pg/mL up to 20,000 pg/mL Ranibizumab with an anchor point of 325 pg/mL. Quality controls at five levels, LLOQ of 625 pg/mL Ranibizumab, Low QC at 1000 pg/mL, Mid QC at 8,000 pg/mL, High QC at 15,000 pg/mL, and the ULOQ at 20,000 pg/mL were evaluated over multiple days with two operators to generate intra- and inter-assay accuracy and precision values (Data shown in Tables 1 and 2 for ECL and NanoLuc respectively). The inter-assay accuracy and precision were within ± 20% for all QCs except ± 25% at LLOQ. The recovery of QCs in both ECL and NanoLuc platform is presented in Figures 2-6.

Matrix effect/ selectivity of the method was evaluated by fortifying each 10 K2EDTA plasma samples at levels equivalent to the low and high QC samples of Ranibizumab at 1000 pg/mL and 15,000 pg/mL (Table 3 and 4). For ECL method, eight out of ten lots has recovery of within ± 25% at each level. For NanoLuc method, eight out of ten at low QC level and seven out of ten at high QC level. The mean recovery for both methods at both levels was within 80%-120%.

Interference of VEGF in the assay was evaluated by the addition of free rhVEGF into the plasma prior to addition of Ranibizumab. A dose range from 500 – 5,000 pg/mL free VEGF showed no inhibition of the Ranibizumab signal at 1000 pg/mL (Table 5). No linearity assessment was performed as the Cmax for Ranibizumab is expected to be well below the upper limit of the quantitation (20,000 pg/mL) for expected clinical trial samples.

Figure 1. Standard Curve ECL Platform

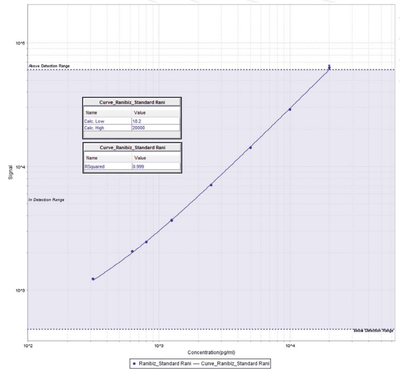

Table 1. Intra- & Inter-Batch Precision and Accuracy of QC Samples on ECL platform

	Batch	QC LLOQ 625 pg/mL	QC A 1000 pg/mL	QC B 8000 pg/mL	QC C 15000 pg/mL	ULOQ 20000 pg/mL
	1	680	953	7930	15800	20300
		681	922	7860	15700	21700
		689	989	8030	15300	18300
Intrarun Mean		683	955	7940	15600	20100
Intrarun SD		4.93	33.5	85.4	265	1710
Intrarun %CV		0.7	3.5	1.1	1.7	8.5
Intrarun %Bias		9.3	-4.5	-0.8	4	0.5
n		3	3	3	3	3
	2	655	984	8230	15300	21000
		652	968	7930	15500	21000
		609	1050	8050	14400	20800
Intrarun Mean		639	1000	8070	15100	20900
Intrarun SD		25.7	43.5	151	586	115
Intrarun %CV		4	4.4	1.9	3.9	0.6
Intrarun %Bias		2.2	0	0.9	0.7	4.5
n		3	3	3	3	3
	3	654	1000	8000	15200	20200
		635	1020	8190	15200	20600
		629	1030	7690	*No Value	*No Value
Intrarun Mean		639	1020	7960	15200	20400
Intrarun SD		13.1	15.3	252	0	283
Intrarun %CV		2.1	1.5	3.2	0	1.4
Intrarun %Bias		2.2	2	-0.5	1.3	2
n		3	3	3	2	2
Mean Concentration Found (pg/mL)		654	991	7990	15300	20500
Inter-run SD		26.6	39.9	165	428	1000
Inter-run %CV		4.1	4	2.1	2.8	4.9
Inter-run %Bias		4.6	-0.9	-0.1	2	2.5
n		9	9	9	8	8

* not aliquoted

Table 2. Intra- & Inter-Batch Precision and Accuracy of QC Samples on NanoLuc Platform

	Batch	QC LLOQ 625 pg/mL	QC Low 1000 pg/mL	QC Mid 8000 pg/mL	QC High 15000 pg/mL	ULOQ 20000 pg/mL
	1	~1000	1080	7470	16800	20600
		770	1200	7020	17400	22400
		606	820	6660	16800	21000
Intrarun Mean		792	1030	7050	17000	21300
Intrarun SD		198	194	406	346	945
Intrarun %CV		25	18.8	5.8	2	4.4
Intrarun %Bias		26.7	3	-11.9	13.3	6.5
n		3	3	3	3	3
	2	629	965	7550	14700	18100
		667	1110	7640	15100	19100
		610	1170	7800	14600	20400
Intrarun Mean		635	1080	7660	14800	19200
Intrarun SD		29	105	127	265	1150
Intrarun %CV		4.6	9.7	1.7	1.8	6
Intrarun %Bias		1.6	8	-4.3	-1.3	-4
n		3	3	3	3	3
	3	~402	~~777	6480	14500	21900
		600	~~1220	~~6260	15100	21200
		618	1010	7460	14500	20300
Intrarun Mean		540	1000	6730	14700	21100
Intrarun SD		120	222	639	346	802
Intrarun %CV		22.2	22.2	9.5	2.4	3.8
Intrarun %Bias		-13.6	0	-15.9	-2	5.5
n		3	3	3	3	3
	4	645	1190	8560	16500	24200
		726	1180	6870	15700	22000
		684	1090	8450	16800	19800
Intrarun Mean		685	1150	7960	16300	22000
Intrarun SD		40.5	55.1	946	569	2200
Intrarun %CV		5.9	4.8	11.9	3.5	10
Intrarun %Bias		9.6	15	-0.5	8.7	10
n		3	3	3	3	3
Mean Concentration Found (pg/mL)		663	1070	7350	15700	20900
Inter-run SD		139	148	725	1090	1610
Inter-run %CV		21	13.8	9.9	6.9	7.7
Inter-run %Bias		6.1	7	-8.1	4.7	4.5
n		12	12	12	12	12
~ > 25%Bias						
~~ > 20%Bias						

Table 3. Matrix Effect Data with ECL Platform

		Low		High	
Batch	Lot#	1000 pg/mL	% Dev.	15000 pg/mL	% Dev.
4	1	1390	+39.0	19200	+28.0
	2	1190	+19.0	16800	+12.0
	3	1270	+27.0	16700	+11.3
	4	977	-2.3	15300	+2.0
	5	1160	+16.0	17300	+15.3
	6	1130	+13.0	16800	+12.0
	7	1250	+25.0	16600	+10.7
	8	1110	+11.0	11200	-25.3
	9	1110	+11.0	15400	+2.7
	10	1130	+13.0	15000	+0.0
Mean		1170		16000	
% CV		9.6		13.0	
% Theoretical		117.0		106.7	
n		10		10	

Figure 2. LLOQ QC (625 pg/mL): Comparison of Recovery in Both ECL and NanoLuc Platform

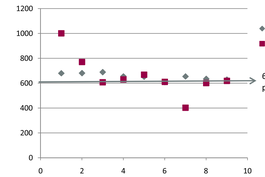

Figure 3. Low QC (1000 pg/mL): Comparison of Recovery in Both ECL and NanoLuc Platform

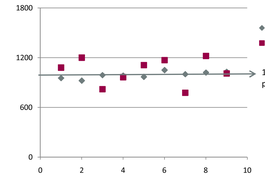

Figure 4. Mid QC (8000pg/mL): Comparison of Recovery in Both ECL and NanoLuc Platform

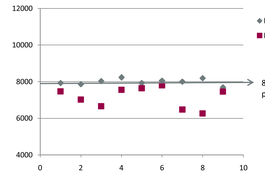

Figure 5. High QC (15,000pg/mL): Comparison of Recovery in Both ECL and NanoLuc Platform

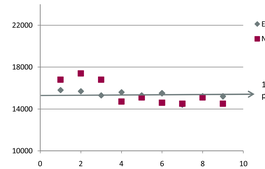

Figure 6. ULOQ QC (20,000pg/mL): Comparison of Recovery in Both ECL and NanoLuc Platform

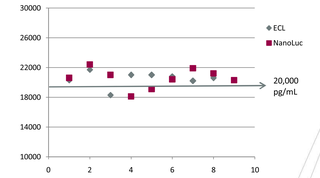

Table 4. Matrix Effect Data with NanoLuc Platform

		Low		High	
Batch	Lot#	1000 pg/mL	% Dev.	15000 pg/mL	% Dev.
11	1	1550	+55.0	15400	+2.7
	2	805	-19.5	12600	-16.0
	3	912	-8.8	14000	-6.7
	4	646	-35.4	13700	-8.7
	5	877	-12.3	15300	+2.0
	6	827	-17.3	10500	-30.0
	7	969	-3.1	14300	-4.7
	8	1070	+7.0	10900	-27.3
	9	1230	+23.0	14700	-2.0
	10	1030	+3.0	10600	-29.3
Mean		992		13200	
% CV		25.5		14.6	
% Theoretical		99.2		88.0	
n		10		10	

Table 5. Interference of Free VEGF in ECL Assay

(Low QC=1000 pg/mL Ranibizumab)	Mean ECLU	Calculated Concentration pg/mL	% CV	%Bias
Low QC + 0 VEGV	4316	1020	1.05	2
Low QC + 500 pg/mL VEGF	4467	1060	1.27	6
Low QC + 1000 pg/mL VEGF	4306.5	1020	1.3	2
Low QC + 5000 pg/mL VEGF	4157	978	0.92	-2

CONCLUSION & FUTURE WORK

The results from both platforms were comparable. The results from qualification experiments indicate that the assay is "validatable" and meets FDA Bioanalytical Guidance for pharmacokinetic assays and industry best practices.

The assay sensitivity is reported to be 625 pg/mL. We intend to directly conjugate the rabbit anti-Ranibizumab antibody to further optimize the method and achieve less than 500 pg/mL sensitivity. Further optimization will be performed to minimize crosstalk and plate washing effects for the NanoLuc method.

NOVEL ASPECT

Here we demonstrate the use of a novel reporter, NanoLuc, and its application in development of sensitive bioanalytical assays to support pharmacokinetic studies. We also demonstrated that the transition from routine ELISA or ECL method to NanoLuc is simple, cost effective and easy to execute.

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