Alphalisa - A New, Simple, Highly Sensitive Technology for HTP Quantification of Small Human Proteins in Matrix

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Introduction

With the aim to improve patient tailored therapy and in order to avoid deleterious drug side effects, it is crucial to understand the pharmacokinetic of small therapeutic agents like peptides. In consequence their quantification in biological matrices in a sensitive and reliable way is mandatory. System limitations often observed in traditional immunoassays include narrow analytical ranges and low sensitivity. Moreover, classical ELISAs with their multiple sample processing steps are very time-consuming and rarely transferrable on robotic systems for high throughput (HTP) analysis.

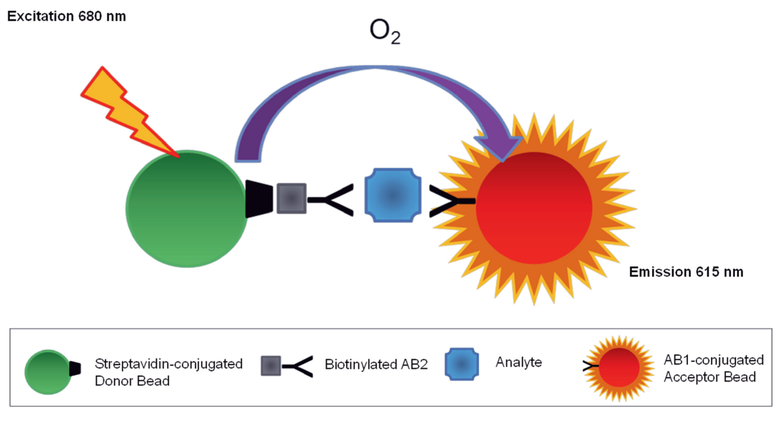
Goals

To overcome the above-mentioned limitations, we utilized a simple and robust AlphaLisa luminescence assay for the quantification of a small protein drug (hereafter referred as “Analyte”), commonly used in the treatment of diabetic patients. In order to facilitate HTP analysis, the method was successfully transferred to a liquid handling robotic system.

Analytical Method

The AlphaLisa technology is based on the proximity of two types of beads, donor and acceptor beads, which are brought together by a bridging Analyte. Upon excitation of the donor beads singlet oxygen species are produced and electrons freed by this reaction are transferred to acceptor beads which ultimately emit light. This light emission is proportional to the amount of Analyte present in the sample. (Figure 1). In detail Analyte-specific antibody (AB1) conjugated to acceptor beads and biotinylated Analyte-specific antibody (AB2) are used to capture the Analyte in the sample during incubation step over night. Next day, streptavidin-coated donor beads are added and incubated for 1 hour. In the presence of the Analyte, the acceptor beads and donor beads are brought together and after excitation, light emission is quantified.

Figure 1: Principle of AlphaLisa



Analytical Challenges and Solutions

Assay Buffer

For optimal performance, AlphaLisa assay requires special assay buffers, several of which are commercially available. Here, our cost-effective, in-house prepared buffer with carefully chosen detergent and blocking reagents enabled a fully functional assay with a broad analytical range (500 – 100 000 pM) for the detection of the Analyte (Figure 2).

Figure 2: Analytical range using in-house assay buffer

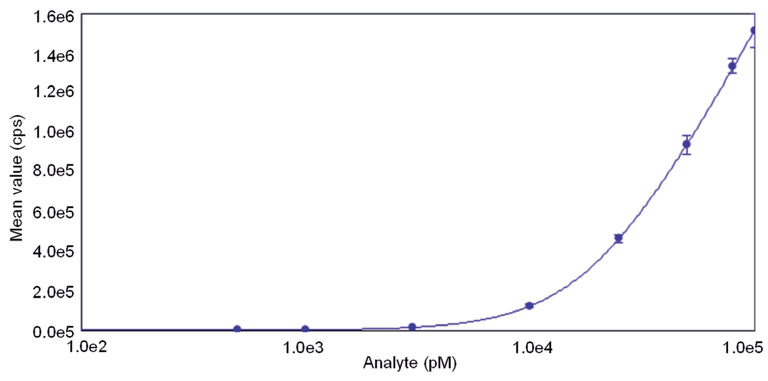
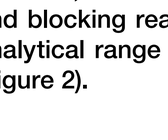


Plate drift

AlphaLisa assays are often done using special plates (for example half-area or 384-well plates) which, depending on the reader and plate type, can have signal drift-problems. Therefore, to enable accurate reading throughout the plate, it is important to check the plate geometry within the reader software. Here, adjustment of Costar plate geometry (Tecan Infinite 1000 Pro reader with Magellan 7.2 software) significantly improved signal homogeneity (Figure 2A, overall %CV 7.15) when compared to a standard plate setting (Figure 2B, overall %CV 9.81).

Figure 3A & B: Adjustment of half-area plate geometry

A:



	1	2	3	4	5	6	7	8	9	10	11	12
A	914	1001	814	934	1041	749	832	883	899	957	851	962
B	912	1015	925	899	907	913	787	944	971	840	928	889
C	947	907	896	960	879	966	1025	923	843	898	847	870
D	893	768	925	815	840	901	902	880	821	954	889	960
E	898	969	819	865	776	1074	949	918	809	957	931	927
F	934	885	822	917	1010	900	962	888	747	930	888	859
G	861	857	820	920	955	826	909	832	890	863	820	956
H	911	870	775	868	853	911	942	767	894	857	928	910

Mean	895
SD	63.96
%CV	7.15

B:

	1	2	3	4	5	6	7	8	9	10	11	12
A	755	803	816	950	854	682	767	888	816	758	696	887
B	809	924	745	752	819	878	804	766	891	719	966	781
C	810	698	778	817	888	857	842	769	944	913	839	815
D	857	801	743	766	826	875	822	750	768	721	918	752
E	748	781	615	790	827	761	800	798	792	757	769	820
F	709	830	763	753	791	748	863	772	844	821	800	663
G	776	682	681	729	808	662	653	658	695	734	702	700
H	720	638	647	667	704	683	707	680	728	726	691	713

Mean	776
SD	76.16
%CV	9.81

Results

1: Precision & Accuracy

Precision and accuracy of the assay was done by analyzing standard curves (STDs) and all 6 quality control (QC) levels of the Analyte in 3 separate runs (Table 1A & 1B). Each run contained freshly prepared STDs and QCs.

Table 1A: Precision and accuracy of STDs

Nominal (pM)	STD1 500 pM	STD2 1000 pM	STD3 3000 pM	STD4 10000 pM	STD5 25000 pM	STD6 50000 pM	STD7 80000 pM	STD8 100000 pM
mean	483	1010	3077	10110	23967	48233	88433	102333
SD	30.66	17.32	95.04	155.88	416.33	2402.78	1159.02	1154.70
CV [%]	6.4	1.7	3.1	1.5	1.7	5.0	1.3	1.1
nom [%]	96.5	101.0	102.6	101.1	95.9	96.5	110.5	102.3
n	3	3	3	3	3	3	3	3

Table 1B: Precision and accuracy of QCs

Nominal (pM)	LLOQ 500 pM	LQC 1500 pM	MQC 12 000 pM	HQC 75 000 pM	ULOQ 100 000 pM	DQC 500000 pM (diluted 1:10)
n (runs)	3	3	3	3	3	3
n (replicates)	18	18	18	18	18	18
nom [%]	97.4	85.1	99.6	93.4	94.2	99.5
Between Run Precision (%CV)	4.2	4.0	4.3	4.3	8.5	6.3
Within Run Precision (%CV)	7.4	4.1	4.0	5.3	8.5	6.1
Total Variation (%CV)	8.5	5.7	5.9	6.8	12.0	8.8

2: Stability in Matrix

The stability of three duplicates of high quality control (HQC) and low quality control (LQC) samples was analyzed in different conditions against freshly prepared STDs. The Analyte showed stability at each tested condition (Table 2).

Table 2: Analyte stability

QC level	LQC 1500 pM		HQC 75000 pM	
Nominal [pM]	mean recovery [%]	status	mean recovery [%]	status
Non-diluted Benchtop 22h	113.1	3/3 acceptable	118.0	3/3 acceptable
Diluted Benchtop 6h	108.4	3/3 acceptable	113.6	3/3 acceptable
Freeze-thaw (6 cycles)	104.0	3/3 acceptable	114.8	3/3 acceptable

3: Selectivity

Recovery of the Analyte was demonstrated by spiking 10 healthy individuals (Table 3A) as well as ten type I (Table 3B) and type II (Table 3C) diabetic patient serum samples at LLOQ, HQC or at the upper level of quantitation (ULOQ). In all cases, the recovery was within acceptance. All non-spiked samples (blank) were below limit of quantitation (BLQ).

Table 3A. Analyte recovery in normal human sera

Spiking	Blank	LLOQ 500 pM	HQC 75 000 pM
Individuals	10/10 BLQ	10/10 acceptable	10/10 acceptable
Mean [pM]	N/AP	457	78 770
SD	N/AP	24.3	3495
CV [%]	N/AP	5.31	4.44
Mean recovery [%]	N/AP	91.4	105
n	10	10	10
Pool	BLQ	acceptable	acceptable

Table 3B. analyte recovery in type I diabetic patient sera

Spiking	Blank	LLOQ 500 pM	ULOQ100 000 pM
Individuals	10/10 BLQ	10/10 acceptable	10/10 acceptable
Mean [pM]	N/AP	548	96 500
SD	N/AP	28.9	4065
CV [%]	N/AP	5.28	4.21
Mean recovery [%]	N/AP	109.5	97
n	10	10	10

Table 3C. analyte recovery in type II diabetic patient sera

Spiking	Blank	LLOQ 500 pM	ULOQ100 000 pM
Individuals	10/10 BLQ	10/10 acceptable	10/10 acceptable
Mean [pM]	N/AP	540	94 810
SD	N/AP	40.7	3773
CV [%]	N/AP	7.54	3.98
Mean recovery [%]	N/AP	108.0	95
n	10	10	10

4: Assay Specificity

To examine the potential interference of various Analyte analogs normal human serum was spiked with the Analyte (LLOQ and ULOQ) and with high or low concentrations of 5 analog molecules (Table 4). None of the analogs interfered with the assay, demonstrating high assay specificity.

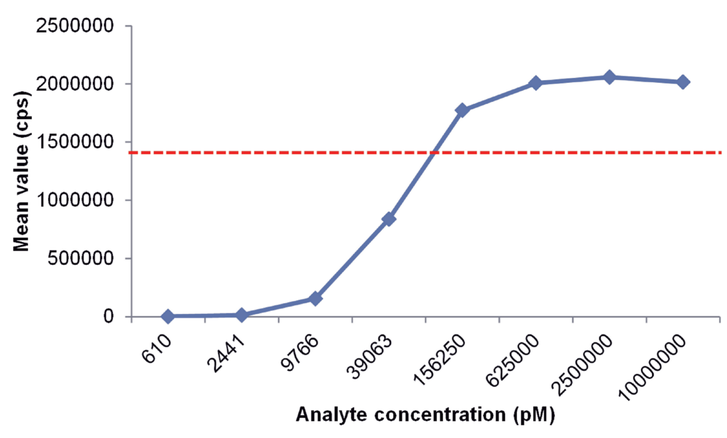
Table 4. Interference of Analyte analogs

Mean recovery [%]		Analyte		Status
		Blank 0 pM	LLOQ 500 pM	ULOQ 100 000 pM	
Analog 1	2000 pM	BLQ	102.2	107.0	acceptable
	400 pM	BLQ	89.0	90.3	acceptable
Analog 2	10 000 pM	BLQ	88.0	79.6	acceptable
	2000 pM	BLQ	98.4	85.3	acceptable
Analog 3	10 000 pM	BLQ	98.6	84.2	acceptable
	2000 pM	BLQ	92.6	92.6	acceptable
Analog 4	800 pM	BLQ	95.0	84.8	acceptable
	300 pM	BLQ	99.6	79.4	acceptable
Analog 5	500 pM	BLQ	105.0	82.9	acceptable
	200 pM	BLQ	106.0	80.2	acceptable

5: Dilution linearity

A sample with high Analyte concentration (100 x ULOQ: 10 000 000 pM) was serially diluted 1:4 in human serum to reach concentrations spanning the analytical range. The ULOQ response is indicated as a red line (Figure 4). Dilution integrity was verified up to dilution factor of 16 384.

Figure 4: Dilution Linearity



6: Assay Automation

To enable HTP analysis, the 2-day method was automated as follows:

Day 1: Sample dilutions and addition of antibody mixture (AB1-conjugated acceptor beads and biotinylated AB2) were performed using Hamilton liquid handling system with MICROLAB VENUS Two software.

Day 2: Addition of donor beads was done with Tecan Freedom EVO system (EVOware Plus software) connected to a Tecan Infinite M1000 Pro plate reader.

Precision and Accuracy (P&A) of the robot run (Table 5A) were comparable to that of manual run (Table 5B). Assay automation enables HTP analysis of up to 480 samples per day.

Table 5A. P&A of automated method

	LLOQ 500 pM	LQC 1500 pM	MQC 12 000 pM	HQC 75 000 pM	ULOQ 100 000 pM	DQC 500000 pM (diluted 1:10)
mean	458	1377	10902	71457	96646	44668
SD	55.42	114.25	1548.32	5020.28	7526.59	4967.46
CV [%]	12.1	8.3	14.2	7.0	7.8	11.1
nom [%]	91.7	91.8	90.8	95.3	96.6	89.3
n	6	6	6	6	6	6

Table 5B. P&A of manual method

	LLOQ 500 pM	LQC 1500 pM	MQC 12 000 pM	HQC 75 000 pM	ULOQ 100 000 pM	DQC 500000 pM (diluted 1:10)
mean	506	1498	12583	72667	98150	52350
SD	45.83	61.13	453.50	3647.28	8152.48	3371.50
CV [%]	9.1	4.1	3.6	5.0	8.3	6.4
nom [%]	101.2	99.9	104.9	96.9	98.2	104.7
n	6	6	6	6	6	6

Discussion and Conclusions

AlphaLisa is an innovative technology allowing for the detection of small therapeutic proteins within a large analytical range. In the present study we showed the successful implementation of this novel technology in order to detect a small protein drug used to treat diabetic patients. During development all key assay parameters (P&A, selectivity etc.) were met and lastly the assay was transferred on robotic systems for HTP analysis, making this new technology a very attractive, simply and cost effective tool for bioanalytical assay development.

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Poster presentation at EBF 2018