Fab Based High Sensitivity PK ELISA for the Detection of a Therapeutic Antibody Administered in Neurodegenerative Disease Treatment

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Introduction

A PK assay using Fab fragments for capture and detection was set up for a therapeutic mAb targeting a neurodegenerative disease. The main challenges encountered were the targeted sensitivities (50 and 1 ng/ml in plasma and CSF respectively) as well as the adaptation to small sample volume (< 40 µL).

The use of Fab fragments in Ligand Binding Assays comprises benefits and drawbacks.

Benefits:

- Easiest access to epitopes, which are difficult to reach
- No interaction with rheumatoid factors (unspecific signal)
- Suitable to detect the Fc part of mAb
- Easier production

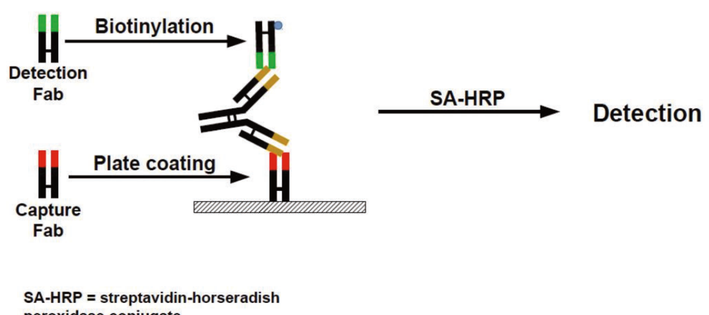
Drawbacks:

- Only affinity effects (no avidity)
- Reduced labelling efficiency

Assay Development

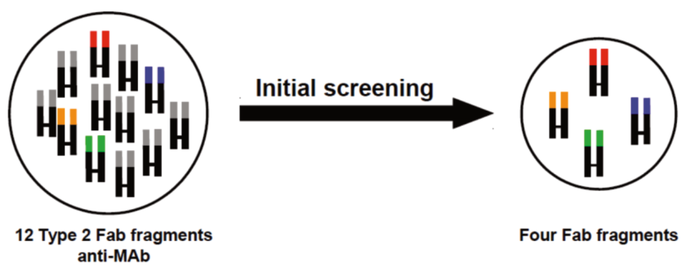
The assay format was a sandwich ELISA using Fab fragments for capture and detection (Figure 1).

Figure 1: Principle of the assay



Twelve Fab fragments were tested either for coating or directly conjugated to HRP for detection in an initial screening (Figure 2).

Figure 2: Initial screening of Fab fragments



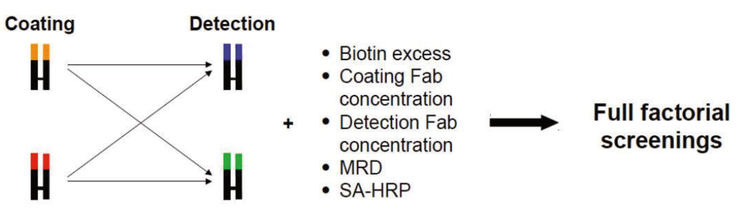
Taking into consideration signal over background ratios, two pairs of fragments were selected for further assay development: Fab 3 and 9; Fab 7 and 8, for capture and detection respectively (Figure 3).

Figure 3: Results from initial screening of Fab for capture and detection signal over background ratios are depicted.

		Detection											
		Fab 1	Fab 2	Fab 3	Fab 4	Fab 5	Fab 6	Fab 7	Fab 8	Fab 9	Fab 10	Fab 11	Fab 12
Capture	Fab 1	33	30	8	17	3	4	32	66	39	59	44	11
	Fab 2	31	62	32	21	17	18	99	52	53	46	64	20
	Fab 3	48	46	16	93	36	31	81	90	49	51	47	22
	Fab 4	23	13	5	26	2	4	65	28	12	28	12	3
	Fab 5	25	38	19	5	12	17	41	4	21	29	33	10
	Fab 6	36	48	23	20	19	23	84	42	18	33	62	12
	Fab 7	86	62	23	21	8	20	84	67	38	35	9	8
	Fab 8	4	6	6	8	2	11	14	36	78	17	42	38
	Fab 9	84	61	37	31	23	23	109	108	52	53	82	37
	Fab 10	67	43	37	59	24	39	65	74	36	22	36	14
	Fab 11	51	78	8	43	20	18	7	24	38	45	81	13
	Fab 12	50	58	44	26	18	18	50	60	31	52	59	18

Capture fragments were tested for coating at 0.5 and 5.0 µg/mL. Detection fragments were labelled with 3:1 and 10:1 biotin:Fab molar excess and tested at 0.05 and 0.25 µg/mL. SA-HRP was tested 1:10'000 and 1:100'000 diluted. Plasma sample dilutions MRD50 and MRD100 were compared (Figure 4).

Figure 4: Development approach



Two 2-level full factorials screenings were performed sequentially to optimize the assay conditions.

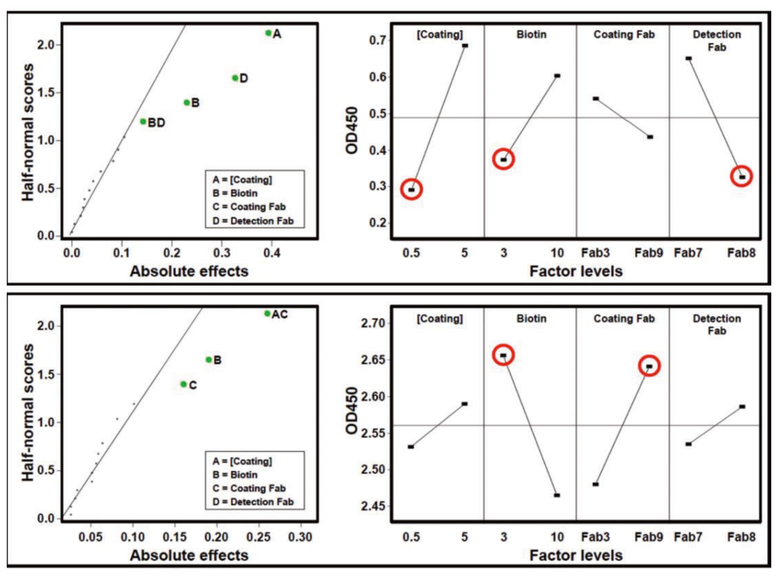
Results

The first screening analyzed the effect of the concentration of coating Fab ([Coating]), the molar excess of biotin (Biotin), the coating Fab and the detection Fab on the background and on the signal of the assay (Figure 5).

Half-normal plots identified [Coating], Biotin and Detection Fab as important factors affecting the background, and Biotin and Coating Fab, the signal (Figure 5, left panels).

Based on the main effects plots, the following factor levels were selected for further optimization of the assay: Coating Fab 9, [Coating] = 0.5 µg/mL and Detection Fab 8, biotinylated with a 3:1 molar excess (Figure 5, right panels).

Figure 5: 2-level full factorial analysis of coating concentration, biotin excess for labelling, choice of fragments for capture and detection. Upper panel: analysis on blanks, lower panel: analysis on spiked samples. Red circles indicate optimal levels of each factor identified as significant in the half-normal plots.

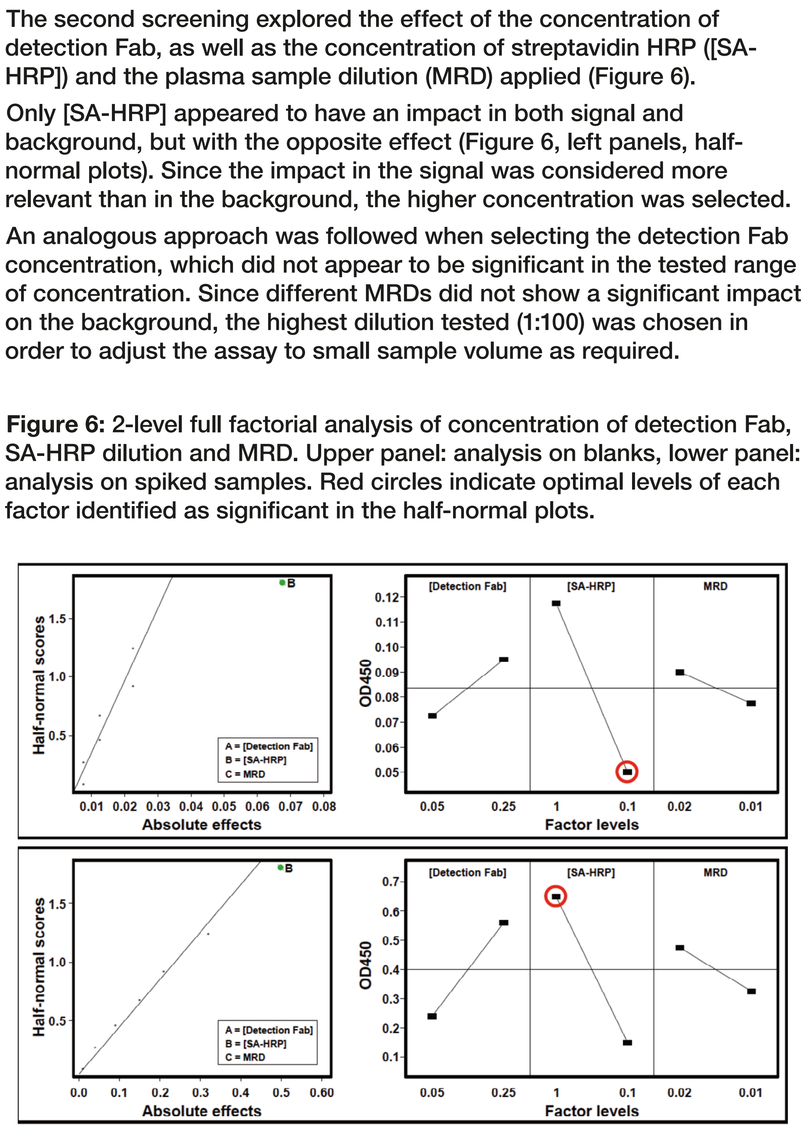


The second screening explored the effect of the concentration of detection Fab, as well as the concentration of streptavidin HRP ([SA-HRP]) and the plasma sample dilution (MRD) applied (Figure 6).

Only [SA-HRP] appeared to have an impact in both signal and background, but with the opposite effect (Figure 6, left panels, half-normal plots). Since the impact in the signal was considered more relevant than in the background, the higher concentration was selected.

An analogous approach was followed when selecting the detection Fab concentration, which did not appear to be significant in the tested range of concentration. Since different MRDs did not show a significant impact on the background, the highest dilution tested (1:100) was chosen in order to adjust the assay to small sample volume as required.

Figure 6: 2-level full factorial analysis of concentration of detection Fab, SA-HRP dilution and MRD. Upper panel: analysis on blanks, lower panel: analysis on spiked samples. Red circles indicate optimal levels of each factor identified as significant in the half-normal plots.



Based on these results, the following parameters were chosen for further development and assay qualification:

Coating	Fab fragment for capture: Fab 9
	Biotin excess for labelling: 3:1
	Concentration of Fab fragment for capture in coating buffer: 0.5 µg/mL
Detection	Fab fragment for detection: Fab 8
	Concentration of Fab fragment for detection: 0.25 µg/mL
	SA-HRP dilution: 1:10'000
MRD	MRD100

Further improvement was attained by comparing two different substrates of HRP: a classical colorimetric reagent (TMB), and a fluorometric reagent (QuantaBlu™). The fluorometric assay showed a significant improvement in its sensitivity (2x improvement, LOD from 15 to 7 ng/mL), as well as in its dynamic range (Figure 7).

Figure 7: Comparison QuantaBlu™ vs TMB. Signal over background ratios values are shown.

Reference item [ng/ml]	QuantaBlu™	TMB
200000	93.9	19.9
100000	94.6	19.9
50000	94.8	20.2
25000	92.0	19.8
2500	88.9	20.0
1250	83.6	18.6
1000	68.4	15.0
500	45.3	12.6
250	31.1	11.5
125	18.3	8.19
62.5	10.6	5.44
31.3	5.76	3.56
15.6	3.28	2.21
7.81	2.10	1.53
3.91	1.58	1.34
0	1.00	1.00

The assay developed in plasma was further adapted to CSF to improve the sensitivity. The method adapted to each biological matrix is summarized in Figure 8.

Figure 8: Method summary for plasma and CSF samples

	Plasma	CSF
Sample Volume (µL)	10	17
MRD	100	10
Analytical range (ng/mL)	10 - 500	1 - 40

Results from precision and accuracy (P&A) runs performed during Qualification in plasma and CSF are shown in Figure 9 and 10, respectively.

Figure 9: Results from qualification P&A runs in plasma. Averages of duplicate measurements are reported.

Nominal [ng/ml]	QC LLOQ 10.0	Nom %	QC Low 30.0	Nom %	QC Med 125	Nom %	QC High 350	Nom %	QC ULOQ 500	Nom %
Run 01	9.8	98.1	29.7	98.8	124	99.3	336	95.9	494	98.9
	11.5	115	28.4	94.8	131	105	344	98.3	466	93.2
mean [ng/ml]	10.7		29.0		127		340		480	
SD	1.22		0.86		4.80		6.01		19.9	
CV [%]	11.4		3.0		3.8		1.8		4.2	
Nominal [%]	107		96.8		102		97.1		96.1	
n	2		2		2		2		2	
Run 02	10.2	102	27.6	92.0	118	94.4	312	89.3	437	87.4
	10.5	105	28.1	93.7	113	90.3	322	91.9	449	89.9
mean [ng/ml]	10.4		27.9		115		317		443	
SD	0.21		0.36		3.66		6.59		8.90	
CV [%]	2.0		1.3		3.2		2.1		2.0	
Nominal [%]	104		92.9		92.3		90.6		88.6	
n	2		2		2		2		2	
Run 03	9.6	95.7	34.0	113	134	107	332	94.9	459	91.9
	8.6	86.2	34.2	114	136	109	334	95.4	481	96.3
mean [ng/ml]	9.1		34.1		135		333		470	
SD	0.67		0.14		0.94		1.37		15.6	
CV [%]	7.3		0.4		0.7		0.4		3.3	
Nominal [%]	90.9		114		108		95.2		94.1	
n	2		2		2		2		2	

Figure 10: Results from qualification P&A runs in CSF. Averages of duplicate measurements are reported.

Nominal [ng/ml]	QC LLOQ 1.00	Nom %	QC Low 3.00	Nom %	QC Med 15.0	Nom %	QC High 28.0	Nom %	QC ULOQ 40.0	Nom %
Run 04	0.8	82.1	3.0	101	14.4	96.2	26.3	94.0	38.0	95.1
	0.9	88.5	3.4	113	16.1	107	28.4	102	39.2	97.9
mean [ng/ml]	0.9		3.2		15.3		27.4		38.6	
SD	0.05		0.24		1.16		1.50		0.80	
CV [%]	5.3		7.5		7.6		5.5		2.1	
Nominal [%]	85.3		107		102		97.8		96.5	
n	2		2		2		2		2	
Run 05	1.0	97.4	2.9	97.7	13.9	93.0	26.4	94.4	35.2	88.1
	0.8	81.7	2.4	81.2	13.6	90.9	26.3	93.8	37.8	94.4
mean [ng/ml]	0.9		2.7		13.8		26.3		36.5	
SD	0.11		0.35		0.22		0.12		1.79	
CV [%]	12.4		13.1		1.6		0.5		4.9	
Nominal [%]	89.6		89.5		91.9		94.1		91.3	
n	2		2		2		2		2	
Run 06	0.9	93.4	3.0	98.4	15.4	103	28.8	103	37.6	93.9
	1.0	95.4	3.0	101	15.8	105	28.6	102	37.6	94.1
mean [ng/ml]	0.9		3.0		15.6		28.7		37.6	
SD	0.01		0.05		0.26		0.16		0.05	
CV [%]	1.5		1.7		1.6		0.6		0.1	
Nominal [%]	94.4		99.6		104		103		94.0	
n	2		2		2		2		2	

Discussion and Conclusion

In the present work

- Bioanalytical challenges defined during the feasibility phase were assessed
- Fine method tuning applied throughout development lead to improved reliability, maximal reproducibility and robustness
- State-of-the-art platforms were compared to fulfill sponsors expectations
- Customized assays were successfully qualified

Poster presentation at EBF 2017