Challenges in Development and Validation of a Multiplex Assay to Detect Biomarkers in Human Serum and Cerebrospinal Fluid

Darshana Jani[1], Tatiana Plavina[1],Sri Laxmanan[1], Nicholas Messinese[1], Laurie Stephen[2], and Jaya Goyal[1]

Translational Sciences, Biogen Idec Inc. Cambridge, MA[1] and Myriad RBM, Lake Placid, NewYork[2]

Abstract

Purpose: To develop and validate a multiplex assay in order to 1) evaluate the pharmacological activity of a biotherapeutic in human serum and cerebrospinal fluid (CSF) and 2) monitor disease activity and efficacy trends upon treatment with the biotherapeutic.

Method: Using Luminex xMAP platform, a custom multiplex assay was developed to measure the concentrations of selected Biotherapeutic regulated analytes and inflammation markers (BAFF, BLC/CXCL13, MIG/CXCL9, IP-10/CXCL10, MCP-1/CCL2, MIP-3 beta/CCL19, 6Ckine/CCL21, SDF-1/CXCL12, LIGHT, ITAC/CXCL11) in human serum and CSF. For each analyte a panel of antibodies was conjugated to beads (capture) and biotin (detection). Each capture:detection antibody pair was screened by a single Luminex immunoassay using recombinant protein as a standard and human serum or CSF as assay matrix, and the best pairs selected based on the achieved sensitivity, specificity, and the ability to detect the analyte at biologically-relevant concentrations. Performance of each assay was then further optimized as a part of a 10-plex. Blinded clinical samples (serum n=24, CSF n=54) previously tested in individual ELISAs were analyzed in the developed multiplex, and based on the concordance, nine of the ten analytes were included in the final panel. The assay was then validated using standard parameters.

Results: Analysis of blinded samples in a 10-plex assay demonstrated that the results obtained for all analytes correlated well with those obtained in ELISA, with the exception of LIGHT. Efforts to optimize the LIGHT assay in a multiplex format were not successful resulting in the removal of LIGHT from the panel. 9-plex assay was subsequently validated in human serum and CSF demonstrating robust performance. The sensitivity and range of the individual assays were shown to be equal or superior as compared to those of corresponding ELISAs.

Conclusions: A custom multiplex assay to measure concentrations of nine selected analytes was successfully developed and validated using Luminex xMAP platform. The fit-for-purpose approach to assay validation was utilized to achieve a balance between assay parameters and multiplexing capabilities, to create a practical tool for clinical studies. The assay proved to be useful in conserving assay matrices, saving time, resources and costs, and be a valuable tool for several biotherapeutic development programs.

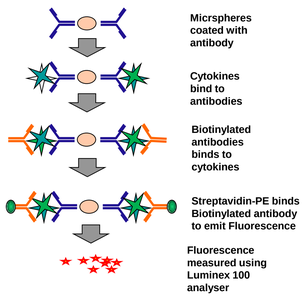
Methods

- 10-plex Assay Development consisting of the following 10 analytes in Human Serum and Cerebrospinal fluid:

1. BAFF
2. BLC/CXCL13
3. MIG/CXCL9
4. IP-10/CXCL10
5. MCP-1/CCL2
6. MIP-3 beta/CCL19
7. 6Ckine/CCL21
8. SDF-1/CXCL12
9. LIGHT
10. ITAC/CXCL11

- Antibodies for each analyte were purchased and conjugated to beads or biotinylated (detection) using standard protocols. Each bead and detection was screened by Luminex assay with a recombinant protein and serum samples. Pairs were selected based on sensitivity, linearity of dilution and the ability to detect the samples at expected levels, followed by multiplexing in a 10-plex assay. Assay verification for MIP-3 beta/CCL19, 6Ckine/CCL21, SDF-1/CXCL12, LIGHT BLC/CXCL13 was performed by running blinded samples in the 9-plex with known values. BAFF and ITAC/CXCL11 were correlated to ELISA. MIG/CXCL9, IP-10/CXCL10 and MCP-1/CCL2 were previously used in multiplex assay and did not undergo further verification.
- Nine of ten analytes where then scaled up for final validation. The assay was validated for Least Detectable Dose(LDD), lower limit of quantitation (LLOQ), spike-recovery, linearity and precision. Validation was performed for serum and CSF.

Assay Format



- Add 5ul capture antibody beads + 5ul blocking buffer + 10uL standard/control/sample in 96 well plate
- Incubate 1 hr at ambient temp
- Add 10uL biotinylated detection antibody
- Incubate 1 hr at ambient temp
- Add 10uL Streptavidin-PE
- Incubate 1 hr at ambient temp
- Transfer contents to Pre wetted filter membrane 96 well plate
- 2x wash with 100uL wash buffer
- FL intensity read in Luminex 100 analyser
- Data analysis by Plateviewer software using Best fit option
- MFI interpolated using standard curve

Assay Development

Reagent Selection

- ELISA Kits vs Singleplex and multiplex Luminex reagents
- Optimization of assay matrix
- Selection of assay format
- Selection of assay controls

Development

- Determination of assay performance parameters
- Evaluation of assay sensitivity
- Evaluation of assay specificity
- Optimization of data reduction to express the concentration of analyte in each matrix

Validation

- Assay range
- Precision
- Selectivity
- Sensitivity
- LDD & LLOQ
- Dilution linearity
- Martix Interference
- Sample Stability

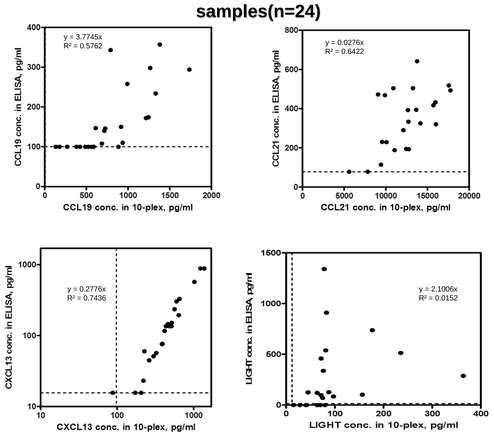
Results and Discussion

Analysis of blinded samples in a 10-plex assay demonstrated that the results obtained for:

- CCL19, CCL21 and CXCL13 analytes gave good correlations to in-house ELISA results, with CCL19 exhibiting better sensitivity
- MIP-3 beta/CCL19, 6Ckine/CCL21 and SDF-1/CXCL12 analytes showed good correlation to the ELISA with similar or better sensitivity
- ITAC/CXCL11 development was also successful, having samples in the expected range
- LIGHT did not correlate well in a subset of samples, possibly due to differences in binding sites in the Luminex assay compared to the ELISA. A comparable pair was not found and LIGHT was removed from the multiplex
- No correlation between serum LIGHT concentrations derived from ELISA and 10-plex
- Adequate sensitivity to evaluate analytes in CSF matrix

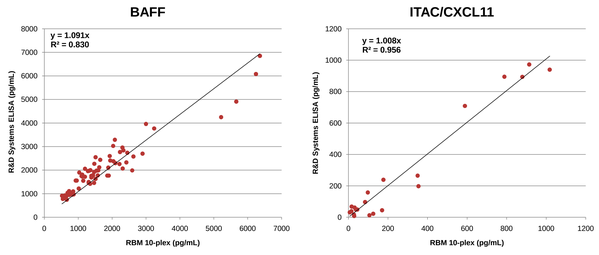
Challenges faced during multiplex validation were that endogenous levels in serum or CSF prevented use of blank serum for specificity testing. This was mitigated by testing samples with single detection antibodies compared to multiplex and spiking a range of standard concentrations for selectivity.

Verification of CCL19, CCL21, CXCL13 and LIGHT Concentrations in the 10-plex with known blinded serum samples(n=24)



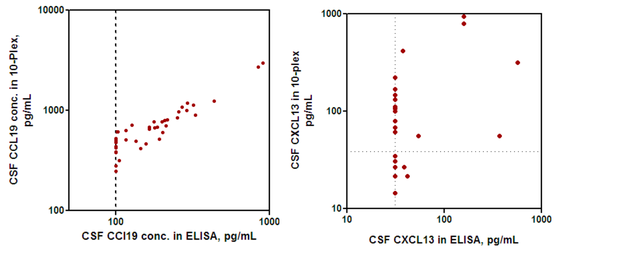
- CCL19, CCL21 and CXCL13 analytes showing correlation of R2 value >0.5 to ELISA results, with CCL19 exhibiting better sensitivity
- No correlation between serum LIGHT concentrations derived from ELISA and 10-plex

Verification of BAFF and ITAC/CXCL11 in the 10-plex with known blinded serum samples(n=24)



- Excellent correlation of 10-plex to the ELISA with similar sensitivity

Correlation between ELISA and 10-plex derived CCL19 & CXCL13 concentrations in human CSF



Greater sensitivity in 10-plex compared to ELISA

CXCL13 was not detected in the ELISA but was detected in the multiplex assay

- Nine of ten analytes were scaled up for method validation.
- Validation was performed for plasma, serum and CSF matrices.

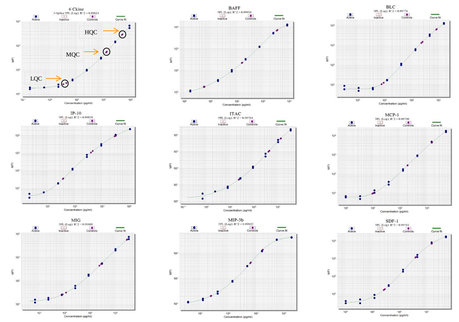
Validation Results

Assay Range

Analyte	Mean MFI	n	SD	Mean + 3SD (MFI)	Serum/Plasma		CSF	
					LDD* (pg/mL)	LLOQ (pg/mL)	LDD (pg/mL)	LLOQ (pg/mL)
6Ckine	14	20	1.7	19	5.8	10	2.3	10
BAFF	9.1	20	1.6	14	20	15	8.1	13
BLC	6.3	20	1.0	9.2	33	28	13	22
IP-10	4.7	20	1.1	7.9	53	51	21	29
ITAC	2.3	20	1.0	5.3	34	35	14	13
MCP-1	5.0	20	0.90	7.8	36	21	15	23
MIG	21	20	1.6	25	36	91	15	151
MIP-3β	8.5	20	1.0	12	37	63	15	26
SDF-1	4.2	20	1.1	7.4	53	40	21	31

*Least Detectable Dose (LDD)
The LDD characterizes the sensitivity of the kit and is determined by adding three standard deviations to the average signal of 20 replicate determinations of standard curve blanks and calculating the corresponding concentration.

Representative Standard Curves



Precision of Serum-based Controls

	LOW QC				MED QC				HIGH QC			
	Range (pg/mL)	Obs (pg/mL)	Intra CV	Inter CV	Range (pg/mL)	Obs (pg/mL)	Intra CV	Inter CV	Range (pg/mL)	Obs (pg/mL)	Intra CV	Inter CV
6Ckine	9.4-25	17	7%	17%	460-884	644	4%	13%	2127-3367	2830	4%	10%
BAFF	20-45	34	10%	13%	680-986	816	7%	9%	14662-20773	19930	3%	5%
BLC	25-61	41	13%	21%	1024-1410	1287	3%	4%	3430-4888	4623	3%	4%
IP-10	167-283	210	5%	10%	1577-2764	2113	5%	13%	11041-18359	15400	4%	4%
ITAC	102-164	135	7%	10%	1538-2898	2149	4%	16%	4617-8928	7750	3%	8%
MCP-1	33-79	51	7%	18%	914-1210	1100	2%	6%	5873-8432	8098	6%	7%
MIG	303-668	503	9%	17%	12246-21541	14630	5%	15%	77390-131287	104660	12%	3%
MIP-3β	109-222	141	6%	11%	2371-4032	3162	3%	12%	7099-11035	9500	3%	8%
SDF-1	102-181	143	4%	19%	1590-2244	1962	2%	10%	5569-8734	8103	5%	8%

High QC - Serum (97%) spiked with all recombinant analytes

Medium QC - Serum (98%) spiked with recombinant analytes except 6Ckine, BAFF, and SDF-1

Low QC - Serum (5%) spiked with recombinant analytes, except 6Ckine

Controls were measured in duplicate

Precision of CSF-based Controls

	LOW QC				MED QC				HIGH QC			
	Range (pg/mL)	Obs (pg/mL)	Intra CV	Inter CV	Range (pg/mL)	Obs (pg/mL)	Intra CV	Inter CV	Range (pg/mL)	Obs (pg/mL)	Intra CV	Inter CV
6Ckine	8.5-14	11	9%	20%	33-58	42	6%	21%	132-212	169	5%	17%
BAFF	99-121	108	3%	7%	311-417	354	4%	10%	2280-2630	2428	2%	5%
BLC	47-62	54	5%	8%	128-157	142	5%	7%	313-357	333	4%	4%
IP-10	180-230	196	2%	9%	437-486	457	2%	4%	1220-1630	1442	3%	9%
ITAC	32-49	38	7%	15%	121-146	133	2%	7%	1040-1170	1095	3%	4%
MCP-1	32-64	47	16%	20%	106-144	122	6%	9%	431-531	475	4%	7%
MIG	830-1180	969	8%	13%	2670-3560	3119	5%	10%	17300-22500	19760	2%	8%
MIP-3β	462-675	540	7%	11%	761-887	825	2%	5%	1370-1850	1577	6%	9%
SDF-1	77-98	88	7%	8%	295-336	315	3%	4%	1330-1450	1398	3%	3%

High QC - Pooled human CSF (25%) spiked with recombinant analytes except IP-10 and MCP-1

Medium QC - 1:3.4 dilution of high QC

Low QC - Pooled human CSF (5%) spiked with recombinant analytes except IP-10 and MCP-1

Controls were measured in duplicate

Selectivity

	Serum (n=3)			Plasma (n=3)			CSF (n=3)		
	Spike Level								
Analytes	1	2	3	1	2	3	1	2	3
6Ckine	85	92	68	118	69	36	96	87	74
BAFF	115	119	102	103	108	97	102	96	104
BLC	122	124	118	94	113	108	81	88	96
IP-10	82	85	83	52	62	59	101	105	104
ITAC	94	92	94	58	66	65	103	104	101
MCP-1	88	89	87	82	97	88	105	104	93
MIG	86	87	77	86	84	86	97	93	100
MIP-3β	132	128	115	92	102	93	81	88	85
SDF-1	63	67	70	41	50	53	116	112	106

Results: CSF recovered all the spiked in concentrations for all the analytes. Serum passed for all recoveries except for SDF-1. Plasma had low recovery when spiking for 6Ckine, IP-10, ITAC, and SDF-1. h, 2- Mid, 3-

Dilutional Linearity

	Matrix	Dilution	Exp (pg/mL)	Obs (pg/mL)	Recov
6Ckine	Serum	1:10	216	203	107%
		1:20	104	101	103%
		1:40	52	51	103%
	CSF	1:4	58	58	100%
		1:8	29	34	116%
		1:16	15	17	114%
BAFF	Serum	1:10	1034	1030	100%
		1:20	517	549	106%
		1:40	258	249	96%
	CSF	1:4	54	48	90%
		1:8	27	22	82%
		1:16	13	12	91%
BLC	Serum	1:10	95	112	117%
		1:20	48	63	132%
		1:40	<LOW>	<LOW>	N/A
	CSF	1:4	58	65	112%
		1:8	<LOW>	<LOW>	N/A
		1:16	<LOW>	<LOW>	N/A
IP-10	Serum	1:10	1079	1265	117%
		1:20	539	690	128%
		1:40	270	350	130%
	CSF	1:4	1079	1265	117%
		1:8	539	690	128%
		1:16	270	350	130%
ITAC	Serum	1:10	862	862	100%
		1:20	431	467	108%
		1:40	216	262	122%
	CSF	1:4	4.6	3.9	86%
		1:8	<LOW>	<LOW>	N/A
		1:16	<LOW>	<LOW>	N/A
MCP-1	Serum	1:10	8325	9075	109%
		1:20	4163	4470	107%
		1:40	2081	2315	111%
	CSF	1:4	159	191	120%
		1:8	80	89	111%
		1:16	40	43	107%
MIG	Serum	1:10	18350	19850	108%
		1:20	9175	10170	111%
		1:40	4588	4615	101%
	CSF	1:4	418	360	86%
		1:8	209	200	96%
		1:16	104	111	106%
MIP-3β	Serum	1:10	788	808	103%
		1:20	394	357	91%
		1:40	197	161	82%
	CSF	1:4	259	255	99%
		1:8	129	133	102%
		1:16	65	53	81%
SDF-1	Serum	1:10	3848	3845	100%
		1:20	1924	1945	101%
		1:40	962	977	102%
	CSF	1:4	453	492	109%
		1:8	226	287	127%
		1:16	113	158	140%

Results:

- Clinical samples will be run at an MRD of 1:5 for serum/plasma and 1:2 for CSF, unless a High reading is obtained for an analyte and it requires further dilution (up to the maximal dilution).
- Serum and plasma samples can be maximally diluted up to 1:20-1:40 depending upon the analyte, whereas CSF samples can be diluted up to 1:4-1:16.

Conclusion

Analytical validation supported the use of multiplex assay as a valuable tool for clinical studies to evaluate the pharmacological activity of biotherapeutic in human serum and CSF or monitor disease activity and efficacy trends upon treatment with biotherapeutic.

Results from the multiplex assay development matched reference ELISA assay results with equal or greater sensitivity for 9 out of 10 assays

The 9-plex assay was shown to be accurate and specific in both serum and CSF matrices

The assay is rapid and reproducible and allows for the quantitative measure of 9 analytes in a single 50 µl sample