Aβ38, Aβ40, and Aβ42 Peptide Immunoassays That Can be Multiplexed

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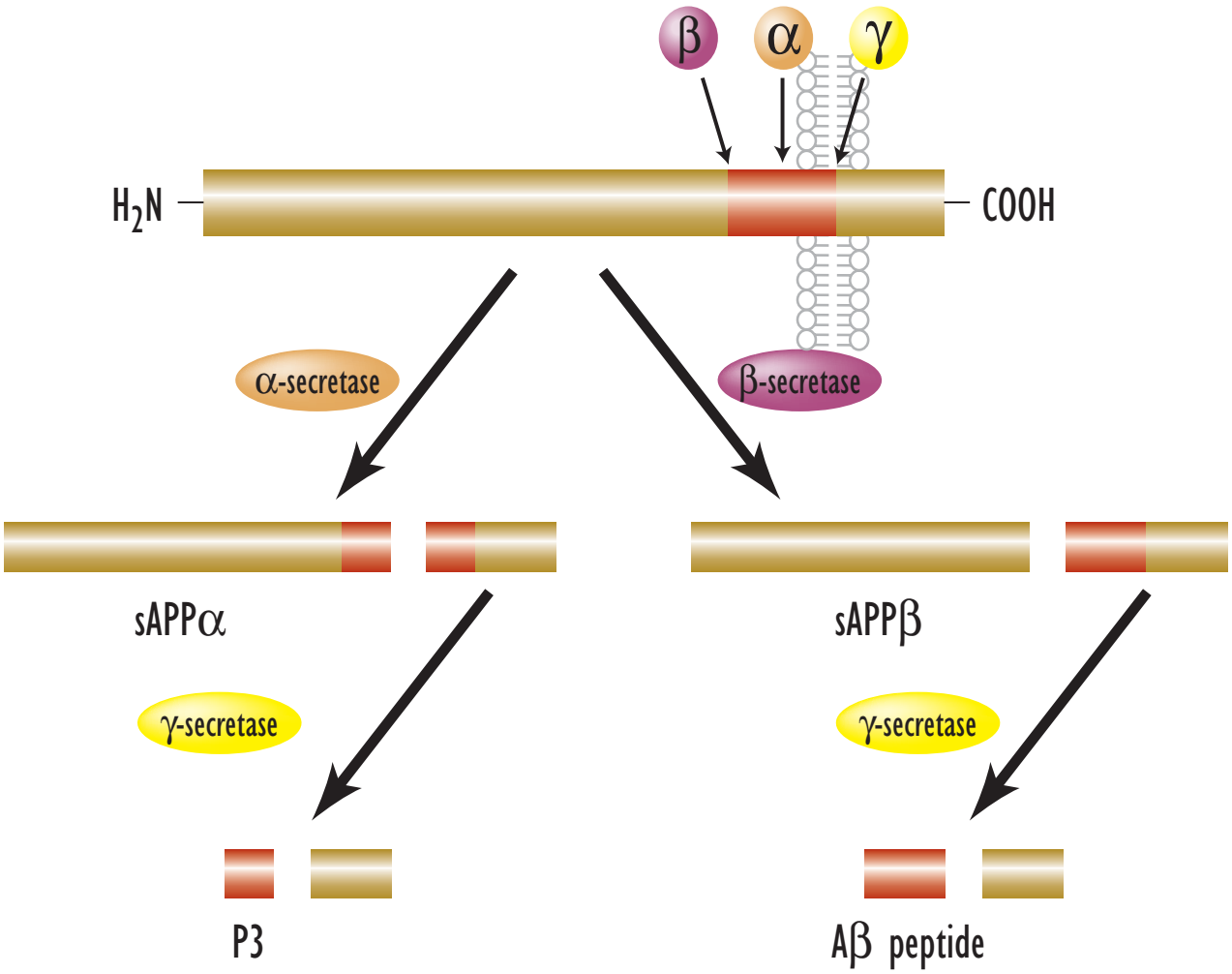
The Aβ peptides are fragments of the amyloid precursor protein (APP) formed by sequential cleavage of APP by β-secretase and γ-secretase. One of the Aβ peptides, Aβ42, is the major component of amyloid plaques, the extra-cellular protein deposits characteristically seen in the brains of patients with Alzheimer's Disease (AD). A great deal of AD research involves very sensitive measurements of different Aβ peptides in a wide range of samples, including cell culture medium, rodent brain homogenates, and human cerebrospinal fluid (CSF). In the development of novel immunoassays for Aβ38, Aβ40, and Aβ42 peptides, we first produced new peptide-specific monoclonal antibodies. The multiplexing capability of MSD technology was employed to screen hybridomas against all three peptide sequences, so that only highly specific, strongly reactive hybridomas were selected for further development. By screening for antigen reactivity and specificity simultaneously, the time and effort involved in developing clones was minimized. The new antibodies were used to develop highly sensitive immunoassays against the Aβ peptides. On MSD plates, the most sensitive human-specific assays that have been developed are singleplex assays, with 6E10 capture antibody and peptide-specific detection antibodies. The performance of these assays is not significantly affected by complex matrices, and peptide levels in human CSF can be measured with high sensitivity. Using 4G8 as a detection antibody and our new peptide-specific antibodies as capture antibodies, a triplex peptide assay has been developed that can be used to simultaneously detect Aβ38, Aβ40, and Aβ42 in a variety of sample types, including human CSF. All of the peptide assays can be multiplexed with a variety of other assays, including total and phosphorylated tau, and soluble APPs.

Amyloid Precursor Protein (APP) Processing



MSD MULTI-ARRAY™ Technology and MULTI-SPOT® Plates

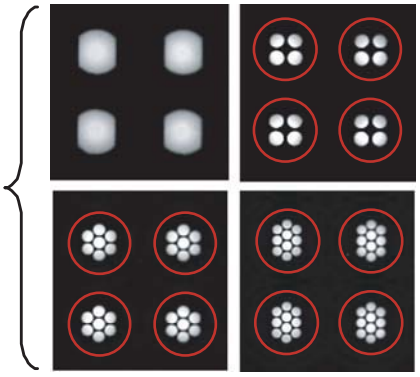
Instrument Features

- Highly sensitive
- High-speed motion control systems
- SECTOR Imager designed for high-throughput screening (HTS)
- Custom optics
- SECTOR Imager ideal for assay development
- Electrochemiluminescence (ECL) detection

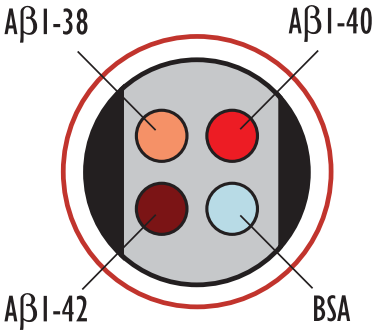
SECTOR™ Imager 6000

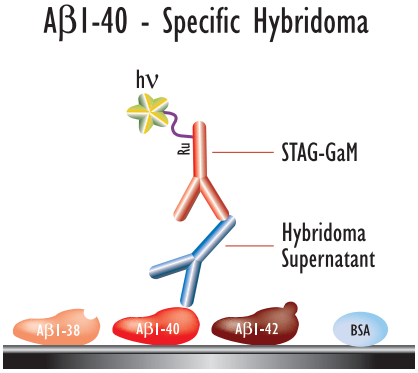
Plate Features

- Disposable plates
- Carbon electrodes with high binding capacity
- Screen printing affords easy patterning
- Suitable electrochemistry for ECL
- A variety of surface treatments, array preparations and coatings are available



Antibody Screening Protocol





- MSD MULTI-SPOT 4 Spot 96 well plates were coated with 5 ng of each peptide or BSA on the four spatially distinct spots in the bottom of each well and dried for 1 hour.
- The plate was blocked with MSD Blocker A for 1 hr. and washed.
- For screening mouse bleeds, 1 µL serum and 24 µL fresh DMEM were added to each well; for screening hybridomas, 25 µL. culture supernatant was added to each well. Plates were incubated for 1 hour with shaking, then washed.
- SULFO-TAG™ labeled goat-anti-mouse secondary antibody was added and incubated for 30 minutes, then the plate was washed.
- MSD Read Buffer T (with surfactant) was added and the plate was read.

Antibody Screening Sample Results

Clone #	1	2	3	4	5	6	7	8	9	10	11	12
Aβ1-38	809	827	204	381	277	610	848	198	23	28	752	461
Aβ1-40	851	890	242	360	296	659	819	200	72	43	741	438
Aβ1-42	812	659	202	370	278	622	845	211	36	17	751	472
BSA	253	251	102	145	105	266	310	90	20	42	265	159

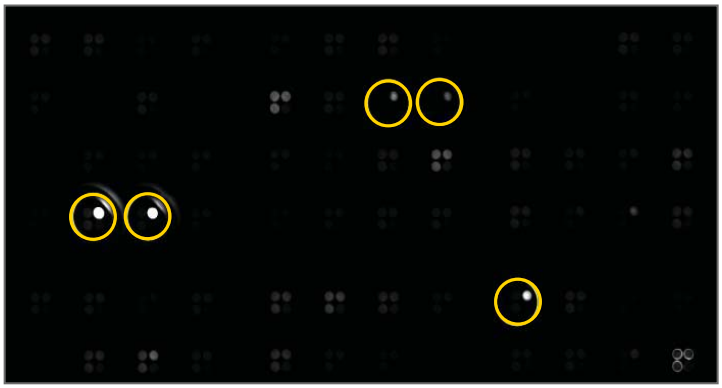
Clone #	13	14	15	16	17	18	19	20	21	22	23	24
Aβ1-38	360	31	664	40	2,787	640	90	48	272	57	358	283
Aβ1-40	400	101	665	61	2,604	605	2,352	1,736	276	88	377	304
Aβ1-42	355	40	660	32	2,578	643	91	50	267	54	344	287
BSA	134	28	190	25	538	283	20	22	101	42	158	101

Clone #	25	26	27	28	29	30	31	32	33	34	35	36
Aβ1-38	49	345	279	342	438	209	788	2,208	902	474	335	1,318
Aβ1-40	101	379	343	377	441	240	751	2,197	828	464	494	1,275
Aβ1-42	44	350	218	340	394	220	761	2,101	849	455	311	1,292
BSA	34	139	104	128	130	87	247	694	260	189	107	478

Clone #	37	38	39	40	41	42	43	44	45	46	47	48
Aβ1-38	167	1,033	458	257	230	332	531	524	839	250	214	881
Aβ1-40	221	116,748	75,578	374	217	360	513	519	800	508	1,307	847
Aβ1-42	156	974	424	236	192	317	516	499	834	228	193	887
BSA	72	418	189	98	97	126	171	156	307	98	89	259

Clone #	49	50	51	52	53	54	55	56	57	58	59	60
Aβ1-38	470	584	192	438	1,325	1,660	1,064	348	447	803	129	181
Aβ1-40	483	662	265	461	1,336	1,622	1,021	406	5,789	733	156	180
Aβ1-42	423	529	168	424	1,299	1,585	1,001	373	402	799	177	169
BSA	173	170	104	148	454	510	474	114	133	215	51	68

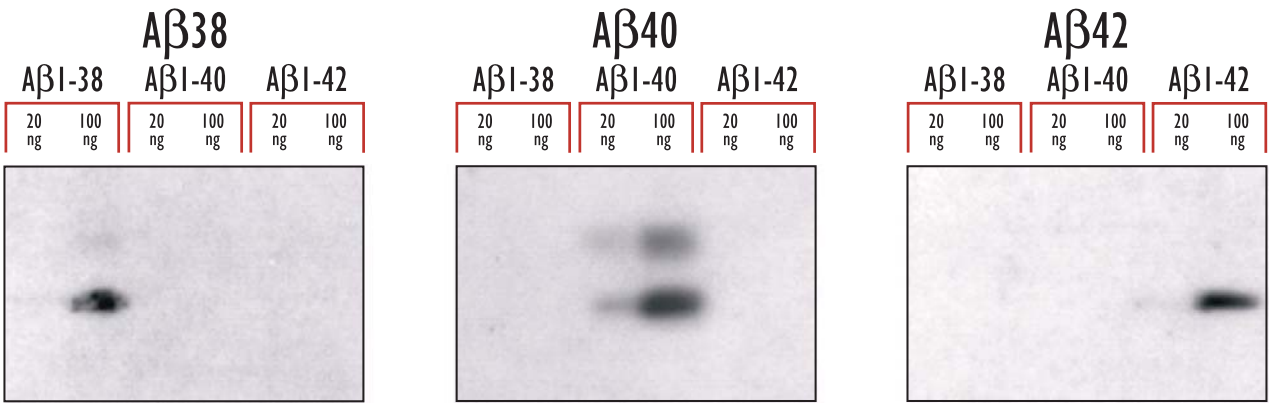
Clone #	61	62	63	64	65	66	67	68	69	70	71	72
Aβ1-38	50	1,074	1,512	465	1,347	366	269	28	440	467	206	2,556
Aβ1-40	95	1,107	2,393	497	1,246	374	305	59	396	459	830	2,533
Aβ1-42	42	1,000	1,363	429	1,218	357	255	35	400	456	190	2,336
BSA	37	389	497	183	493	110	99	27	169	190	105	517



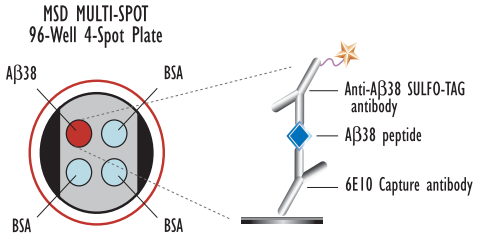
- 72 clones were simultaneously screened for affinity to the Aβ1-40 peptide, cross-reactivity with Aβ1-38 and Aβ1-42 peptides, and non-specific signal on a BSA-coated spot.

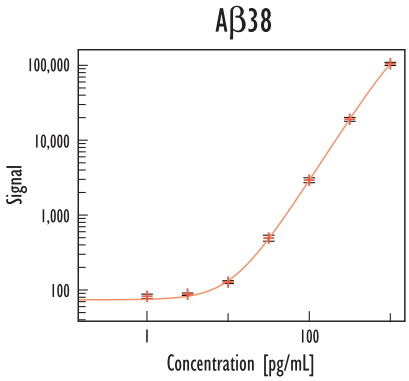
- The clones that are specific for Aβ1-40 are indicated in blue on the table, and are circled on the image of the plate.

Western Blot Demonstrating Specificity of Monoclonal Peptide Antibodies Selected by Multiplex Screening Method



Human (6E10) Ultra-Sensitive Singleplex Aβ38 Assay



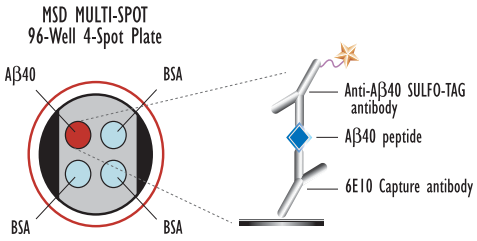


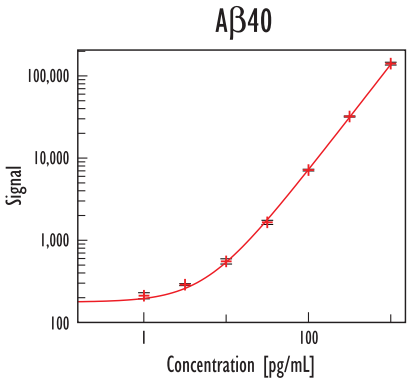
Peptide (pg/mL)	Aβ38			S/B
	Average	StdDev	%CV	
0	68	11	16.5	
1	82	6	7.0	1
3	88	3	3.9	1
10	127	6	4.5	2
32	494	46	9.3	7
100	2,929	222	7.6	43
316	19,054	1,135	6.0	282
1,000	104,752	5,151	4.9	1,552

Aβ38 Lower Limit of Detection	6.4 pg/mL

Synthetic Aβ38 peptides were diluted in 1% Blocker A in Tris Wash Buffer. Samples were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with 6E10 antibody on one of the four spatially distinct electrodes per well. Aβ38 was detected with MSD SULFO-TAG labeled anti-Aβ38.

Human (6E10) Ultra-Sensitive Singleplex Aβ40 Assay



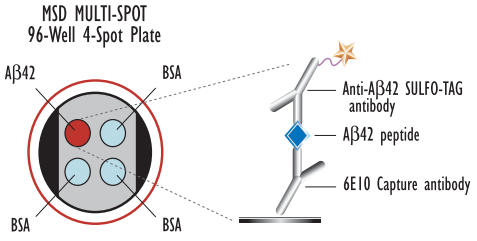


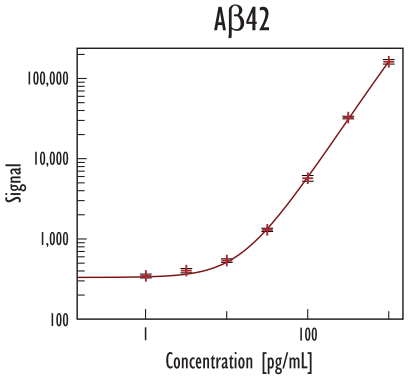
Peptide (pg/mL)	Aβ40			S/B
	Average	StdDev	%CV	
0	160	11	6.8	
1	212	18	8.5	1
3	289	8	2.8	2
10	555	41	7.3	3
32	1,656	97	5.8	10
100	7,157	175	2.4	45
316	32,098	465	1.4	201
1,000	141,338	5,527	3.9	885

Aβ40 Lower Limit of Detection	1.3 pg/mL

Synthetic Aβ40 peptides were diluted in 1% Blocker A in Tris Wash Buffer. Samples were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with 6E10 antibody on one of the four spatially distinct electrodes per well. Aβ40 was detected with MSD SULFO-TAG labeled anti-Aβ40.

Human (6E10) Ultra-Sensitive Singleplex Aβ42 Assay



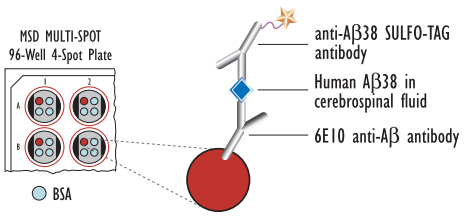


Peptide (pg/mL)	Aβ42			S/B
	Average	StdDev	%CV	
0	299	27	9.1	
1	346	19	5.6	1
3	403	25	6.1	1
10	539	27	5.0	2
32	1,300	63	4.9	4
100	5,715	468	8.2	19
316	32,667	1,225	3.8	109
1,000	161,962	10,397	6.4	541

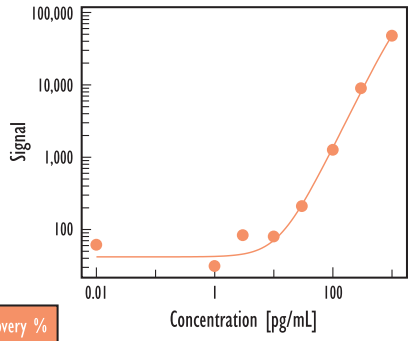
Aβ42 Lower Limit of Detection	5.2 pg/mL

Synthetic Aβ42 peptides were diluted in 1% Blocker A in Tris Wash Buffer. Samples were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with 6E10 antibody on one of the four spatially distinct electrodes per well. Aβ42 was detected with MSD SULFO-TAG labeled anti-Aβ42.

Detection of Aβ38 in Human CSF with MSD Human Ultra-Sensitive Singleplex Assay



Aβ38 Calibration Curve



Peptide (pg/mL)	Aβ38			S/B
	Average	StdDev	%CV	
0	62	5	9	1
1	31	20	63	1
3	84	14	16	1
10	80	4	5	1
30	211	19	9	3
100	1,264	42	3	21
300	8,964	311	3	146
1,000	47,722	2,322	5	776

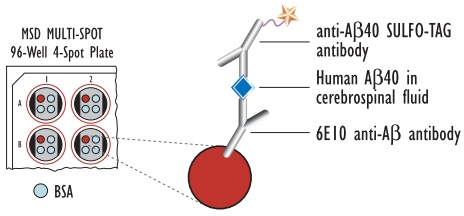
LLOD (2.5 StdDev over 0)	13 pg/mL
LOQ	<25 pg/mL

Spike Recovery

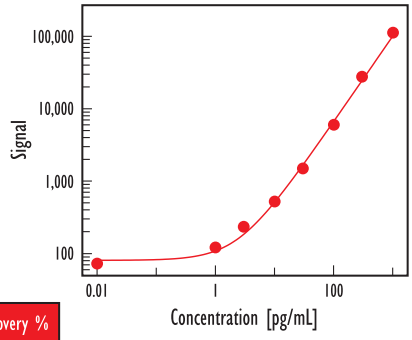
Sample	Amount Spiked (pg/mL)	Expected (pg/mL)	Measured (pg/mL)	Recovery %
human CSF	400	697	663	95%
	200	497	476	96%
	125	422	400	95%
	50	347	341	98%
	25	322	324	101%
	0		297	

Aβ38 peptide was diluted in 10% MSD Blocker A in 1X MSD wash buffer to construct a standard curve, and peptides were spiked into human cerebrospinal fluid to calculate spike-recoveries. All samples were added to an MSD MULTI-SPOT 4 Spot 96-well plate coated with 6E10 anti-human Aβ antibody on one of the four spatially distinct electrodes per well, which had been pre-incubated with an equal volume of 10% MSD Blocker A in 1X MSD wash buffer. Aβ38 was detected with anti-Aβ38 antibody labeled with MSD SULFO-TAG reagent.

Detection of Aβ40 in Human CSF with MSD Human Ultra-Sensitive Singleplex Assay



Aβ40 Calibration Curve



Peptide (pg/mL)	Aβ40			S/B
	Average	StdDev	%CV	
0	72	18	24	1
1	121	10	8	2
3	233	26	11	3
10	520	41	8	7
30	1,489	161	11	21
100	5,972	601	10	83
300	27,546	4,341	16	381
1,000	111,728	12,412	11	1,546

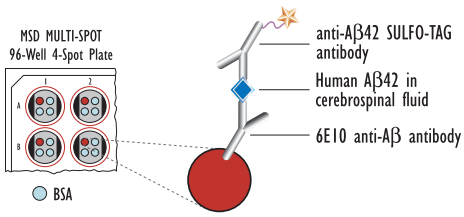
LLOD (2.5 StdDev over 0)	1.2 pg/mL
LOQ	~50 pg/mL

Spike Recovery

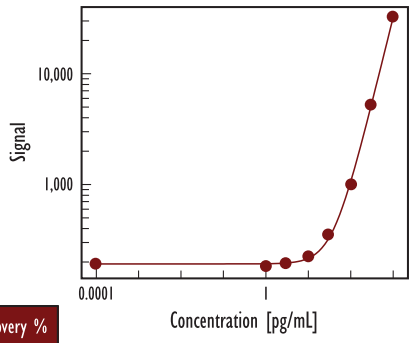
Sample	Amount Spiked (pg/mL)	Expected (pg/mL)	Measured (pg/mL)	Recovery %
human CSF	400	2,787	2,741	88%
	200	2,587	2,602	107%
	125	2,512	2,508	97%
	50	2,437	2,433	92%
	25	2,412	2,429	168%
	0		2,387	

Aβ40 peptide was diluted in 10% MSD Blocker A in 1X MSD wash buffer to construct a standard curve, and peptides were spiked into human cerebrospinal fluid to calculate spike-recoveries. All samples were added to an MSD MULTI-SPOT 4 Spot 96-well plate coated with 6E10 anti-human Aβ antibody on one of the four spatially distinct electrodes per well, which had been pre-incubated with an equal volume of 10% MSD Blocker A in 1X MSD wash buffer. Aβ40 was detected with anti-Aβ40 antibody labeled with MSD SULFO-TAG reagent.

Detection of Aβ42 in Human CSF with MSD Human Ultra-Sensitive Singleplex Assay



Aβ42 Calibration Curve



Peptide (pg/mL)	Aβ42			S/B
	Average	StdDev	%CV	
0	192	22	12	1
1	184	14	8	1
3	195	16	8	1
10	224	22	10	1
30	350	11	3	2
100	999	17	2	5
300	5,242	14	0	27
1,000	32,542	1,197	4	169

LLOD (2.5 StdDev over 0)	17 pg/mL
LOQ	~35 pg/mL

Spike Recovery

Sample	Amount Spiked (pg/mL)	Expected (pg/mL)	Measured (pg/mL)	Recovery %
human CSF	500	868	870	100%
	250	618	632	105%
	125	493	528	128%
	62.5	431	431	101%
	31	399	392	77%
	0		368	

Aβ42 peptide was diluted in 10% MSD Blocker A in 1X MSD wash buffer to construct a standard curve, and peptides were spiked into human cerebrospinal fluid to calculate spike-recoveries. All samples were added to an MSD MULTI-SPOT 4 Spot 96-well plate coated with 6E10 anti-human Ab antibody on one of the four spatially distinct electrodes per well, which had been pre-incubated with an equal volume of 10% MSD Blocker A in 1X MSD wash buffer. Aβ42 was detected with anti-Aβ42 antibody labeled with MSD SULFO-TAG reagent.

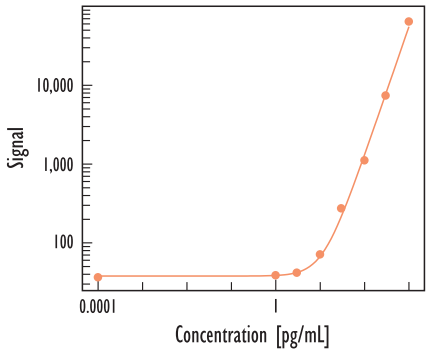
Calculation of Endogenous CSF Levels of each Peptide in Individual Patient Samples Using the Ultra-Sensitive Singleplex Peptide Assays

Calculated Concentrations (pg/mL)

Sample #	Sex - Age	Aβ42	Aβ38	Aβ40
71045	Female - 56	341	373	2,696
71048	Female - 30	611	420	4,062
71043	Male - 49	370	354	2,355
71042	Female - 4	718	451	4,231
71041	Male - 16	382	425	3,223
71040	Female - 10	458	373	3,456
71039	Female - 12	696	895	5,662
71032	Female - 18	322	245	2,444
71036	Female - 54	371	408	2,973
71035	Female - 35	258	245	2,167
71034	Female - 28	115	130	1,079

Use of the Amyloid Peptide Triplex Assay for Determination of Peptide Levels in a Human CSF Sample - 4G8 Detection

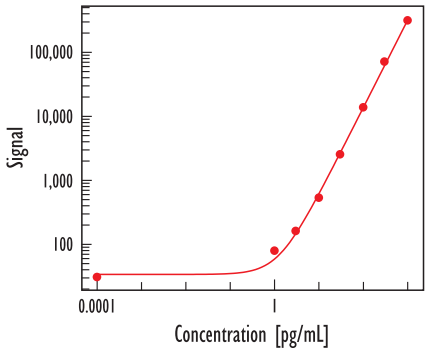
Aβ38



Peptide (pg/mL)	Aβ38			S/B
	Average	StdDev	%CV	
0	37	7	20	1
1	39	17	43	1
3	42	29	70	1
10	71	20	28	2
30	274	16	6	7
100	1,113	91	8	30
300	7,327	450	6	201
1,000	64,374	5,391	8	1,764

Aβ38 Lower Limit of Detection	8.69 pg/mL

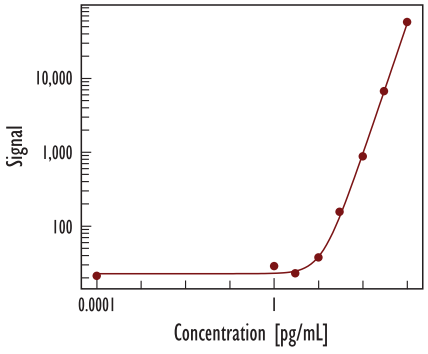
Aβ40



Peptide (pg/mL)	Aβ40			S/B
	Average	StdDev	%CV	
0	31	15	50	1
1	79	5	6	3
3	161	24	15	5
10	534	26	5	17
30	2,540	154	6	83
100	13,763	1,104	8	448
300	76,697	11,260	15	2,494
1,000	314,571	32,273	10	10,230

Aβ40 Lower Limit of Detection	1.28 pg/mL

Aβ42



Peptide (pg/mL)	Aβ42			S/B
	Average	StdDev	%CV	
0	21	5	22	1
1	29	14	48	1
3	23	21	93	1
10	38	17	44	2
30	156	17	11	7
100	879	57	6	41
300	6,787	206	3	319
1,000	57,583	4,720	8	2,710

Aβ42 Lower Limit of Detection	12.37 pg/mL

Human Aβ peptides were diluted in 10% BSA to construct a standard curve, and CSF samples were tested for endogenous peptide content. The standards and samples were pre-incubated with biotinylated 4G8 antibody while the MSD MULTI-SPOT 4 Spot 96-well plate was blocked. After 1 hour incubation, the standards and samples were transferred to the MSD plate, where anti-human Aβ peptide-specific antibodies have been coated onto three of the four spatially distinct electrodes. The final detection reagent for this assay is streptavidin labeled with MSD SULFO-TAG reagent.

Calculated CSF Concentrations	
Aβ38	307 pg/mL
Aβ40	2,037 pg/mL
Aβ42	278 pg/mL

Conclusions

- We have used MSD's multiplexing capabilities to develop monoclonal antibodies to Aβ40, Aβ42, and Aβ38 antibodies that have high affinity and specificity.
- Using our new antibodies, we have developed immunoassays to Aβ peptides which are extremely sensitive and resistant to the effects of complex matrices.
- The most sensitive peptide assays that we have developed are singleplexes which use 6E10 as a capture antibody, and are, therefore human-specific.
- The three amyloid peptides can be measured in a multiplex assay using 4G8 detection antibody; this multiplex is sensitive enough to quantify the endogenous peptide levels in human CSF.