Multiplex Detection of Alzheimer's Disease-related Proteins and Peptides

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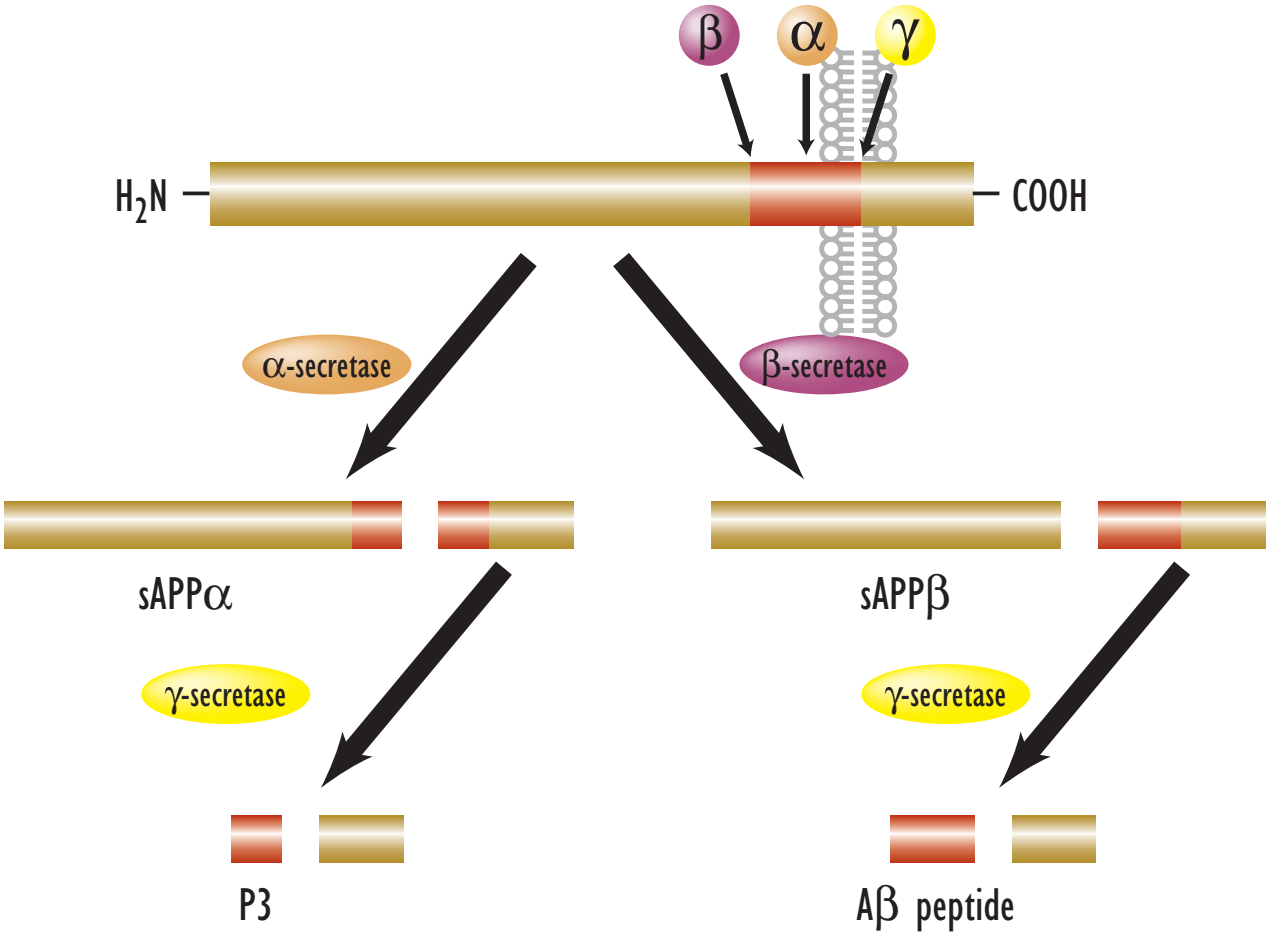
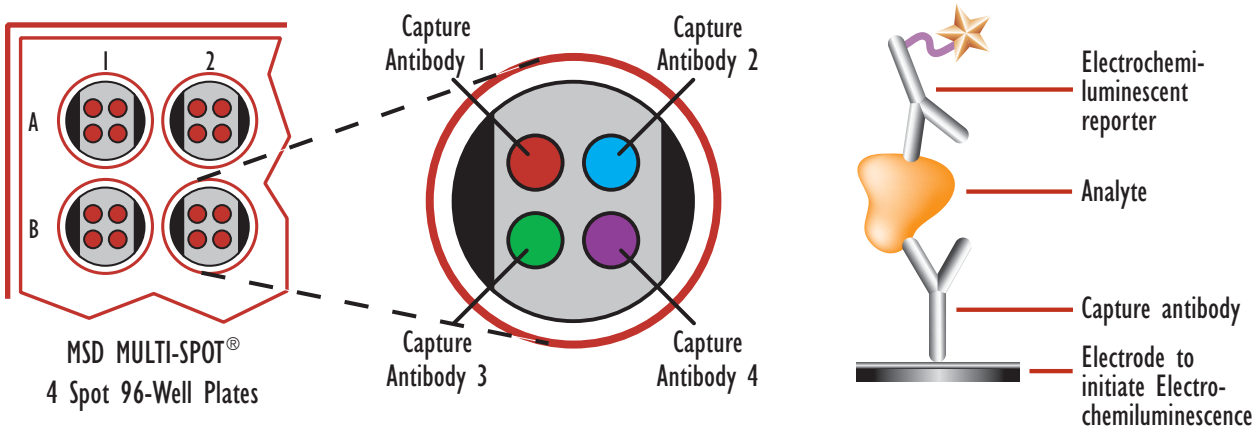
The Alzheimer precursor protein (APP) is processed into multiple fragments, some of which play a role in Alzheimer's disease (AD). APP cleavage results in the release of sAPP and multiple peptides, including Aβ40 and Aβ42. Aβ42 is a major component of amyloid plaques, the characteristic protein deposits found in AD patients. We have developed rapid and sensitive multiplex assays to analyze APP products. For example, sAPPα and sAPPβ levels can be simultaneously quantified in CSF samples, brain homogenates or cell culture supernatants in 3-4 hours. Accurate quantification of various sAPP species is afforded through the use of recently developed, recombinant sAPPs from a mammalian expression system. We also show multiplex assays that include: total Tau, phospho-Tau (T231), Aβ40 and Aβ42. The importance of BACE1 (β-secretase) in the generation of APP fragments makes it an attractive target for pharmaceutical intervention in AD. We have developed a simple, rapid assay for the identification of BACE1 inhibitors.

Amyloid Precursor Protein (APP) Processing

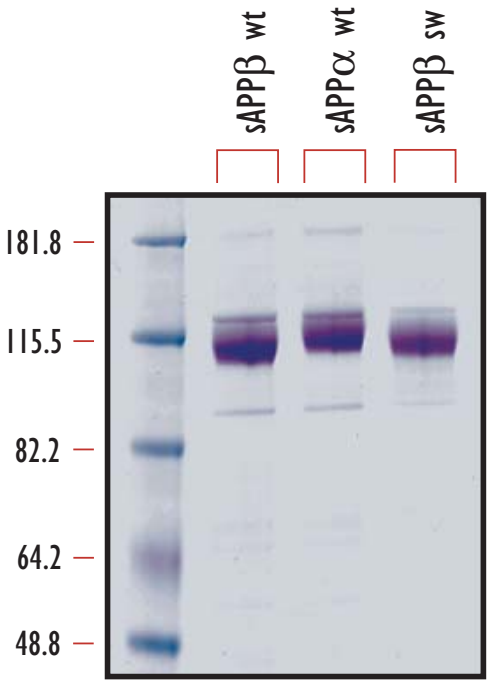


Multiplex Assay Detection Format



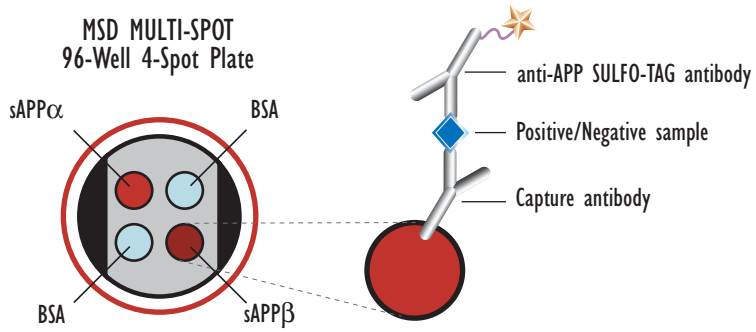
1. MULTI-SPOT 4 Spot 96-Well Plates precoated with capture antibodies are blocked with 150 µL of MSD Blocker-A for 1 hr and washed with Tris Wash Buffer.
2. 25 µL of diluted recombinant protein standards, synthetic peptide standards and/or samples are added to the wells and incubated for 1 hr with shaking, followed by washing with Tris Wash Buffer.
3. 25 µL MSD SULFO-TAG™ antibodies are added to the wells and incubated for 1 hr with shaking.
4. 150 µL MSD Read Buffer T (with surfactant) are added to the wells and analyzed on the SECTOR™ 6000 instrument.

Recombinant Human sAPP Protein Standards

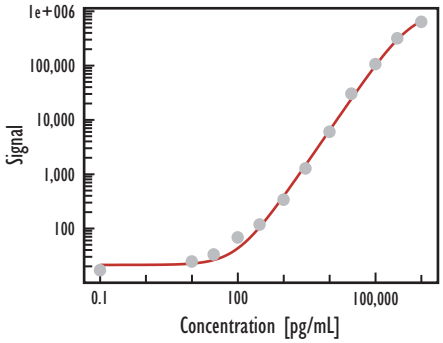


Recombinant human sAPP proteins were purified from mammalian cells. A 0.5 µg sample of each protein was run on 4-12% Bis-Tris NuPAGE gel to demonstrate purity (>95%).

Multiplex Soluble APP Assay: Detection of Human sAPPα and sAPPβ in the Same Well

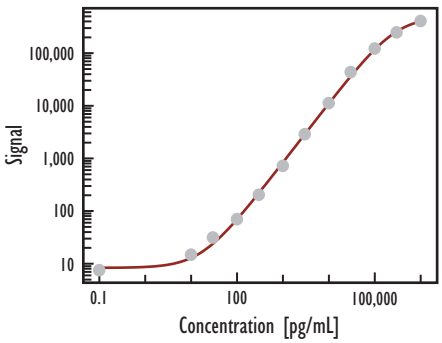


sAPPα



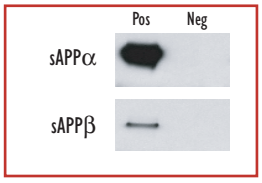
Concentration	Average	StdDev	%CV	S/B
0	17	10	62	1
10 pg/mL	32	9	28	2
30 pg/mL	33	7	22	2
100 pg/mL	68	8	12	4
300 pg/mL	118	7	6	7
1 ng/mL	337	3	1	20
3 ng/mL	1,271	49	4	75
10 ng/mL	6,012	586	10	354
30 ng/mL	30,119	491	2	1,772
100 ng/mL	105,764	17,363	16	6,221
300 ng/mL	316,441	6,268	2	18,614
1 µg/mL	634,377	50,042	8	37,316

sAPPβ

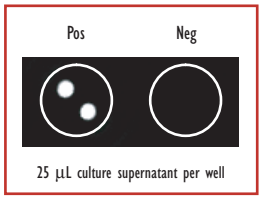


Concentration	Average	StdDev	%CV	S/B
0	10	6	62	1
10 pg/mL	20	6	31	2
30 pg/mL	31	5	16	4
100 pg/mL	70	14	20	9
300 pg/mL	203	18	9	26
1 ng/mL	720	60	8	93
3 ng/mL	2,878	219	8	371
10 ng/mL	11,193	1,596	14	1,444
30 ng/mL	43,550	3,390	8	5,619
100 ng/mL	121,938	5,558	5	15,734
300 ng/mL	246,786	16,891	7	31,843
1 µg/mL	405,610	35,490	9	52,337

Traditional Western



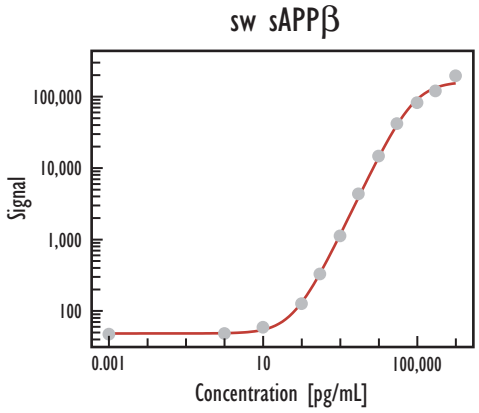
MSD Experimental

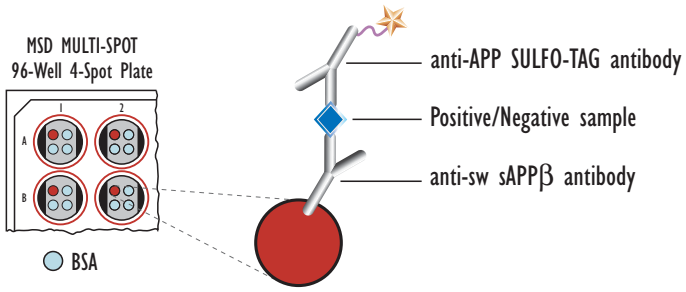


Detection Limits (3 StdDev over bkgnd)	
sAPPα	120 pg/mL
sAPPβ	52 pg/mL

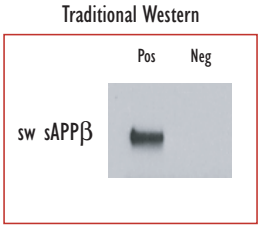
Recombinant human sAPPα and sAPPβ were purified from mammalian cells (>95% pure) and diluted in fresh culture medium (DMEM, 10% FBS, Pen/Strep). Samples were added to MSD MULTI-SPOT 4-Spot plates coated with anti-sAPPα and sAPPβ antibodies on two of the four spatially distinct electrodes per well. The sAPPα and sAPPβ proteins were detected with anti-APP antibody labeled with MSD SULFO-TAG reagent.

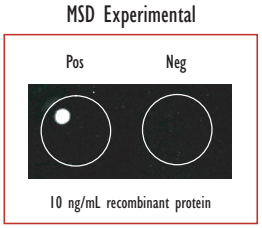
Detection of Human Swedish Soluble APPβ (sw sAPPβ)





Concentration	Average	StdDev	%CV	S/B
0	47	9	19	
1 pg/mL	48	11	24	1
10 pg/mL	59	22	37	1
100 pg/mL	126	18	14	3
300 pg/mL	329	15	4	7
1 ng/mL	1,120	37	3	24
3 ng/mL	4,345	176	4	92
10 ng/mL	14,664	356	2	310
30 ng/mL	41,785	125	0	883
100 ng/mL	81,860	20,236	25	1,729
300 ng/mL	119,720	3,060	3	2,529
1 µg/mL	194,794	19,973	10	4,115



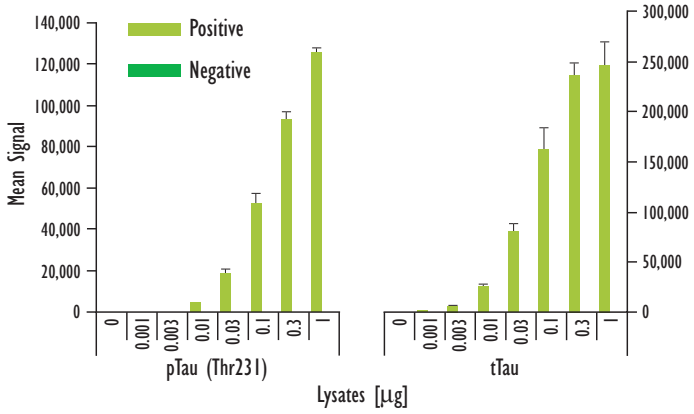


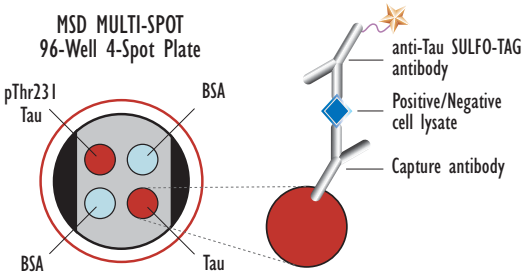
sw sAPPβ Detection Limit (3 StdDev over background)	39 pg/mL

Cross Reactivity (at 100 ng/mL)	
wt sAPPβ	≤0.07%
sAPPα	≤0.10%

Recombinant human Swedish sAPPβ was purified from mammalian cells (>95% pure) and diluted in fresh culture medium (DMEM, 10% FBS, Pen/Strep). Samples were added to MSD MULTI-SPOT 4-Spot plates coated with anti-Swedish sAPPβ antibody on one of the four spatially distinct electrodes per well. The Swedish sAPPβ protein was detected with anti-APP antibody labeled with MSD SULFO-TAG reagent.

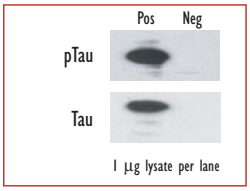
Multiplex Tau Assay: Detection of Phosphorylated (pThr231) and Total Tau in the Same Well



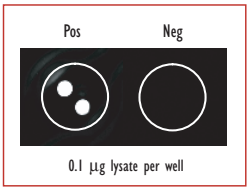


	Lysates (µg)	Positive			Negative			P/N
		Average	StdDev	%CV	Average	StdDev	%CV	
pTau	0	70	8	11	81	9	12	
	0.001	137	18	13	80	5	6	2
	0.003	570	43	8	86	16	19	7
	0.01	3,766	381	10	81	6	7	47
	0.03	17,931	3,001	17	81	6	7	222
	0.1	51,958	4,919	9	89	35	39	583
	0.3	92,761	3,328	4	96	34	36	963
	1	124,208	3,999	3	101	32	32	1,226
tTau	0	48	18	38	50	7	14	
	0.001	1,630	162	10	54	13	25	31
	0.003	5,993	730	12	47	10	22	129
	0.01	24,599	2,962	12	50	9	17	494
	0.03	79,433	8,471	11	60	32	54	1,317
	0.1	161,010	22,296	14	65	39	59	2,483
	0.3	235,688	12,904	5	70	47	67	3,375
	1	246,024	23,099	9	88	32	36	2,796

Traditional Western

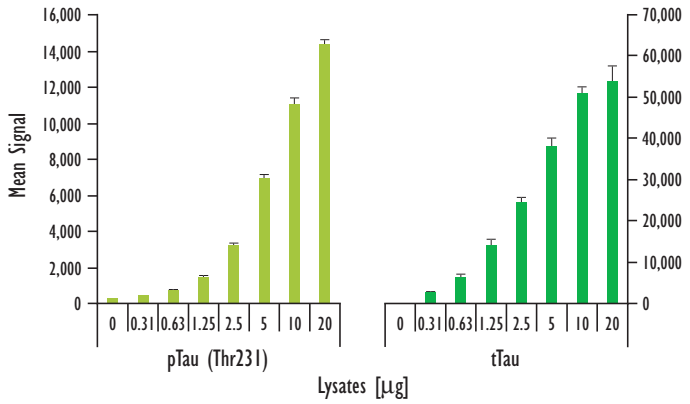


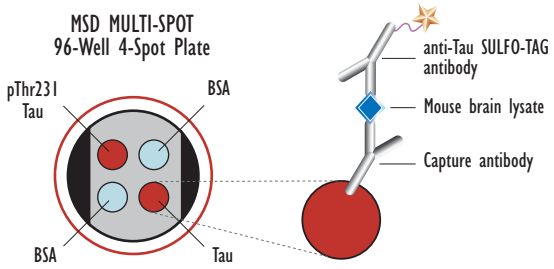
MSD Experimental



Logarithmically growing CHO cells were transfected with Tau expression plasmid (positive) or mock transfected (no plasmid)(negative), and harvested after 48 hr. Whole cell lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total-Tau and anti-phospho-Tau antibodies on two of the four spatially distinct electrodes per well. Phosphorylated and total Tau were detected with anti-total-Tau antibody labeled with MSD SULFO-TAG reagent.

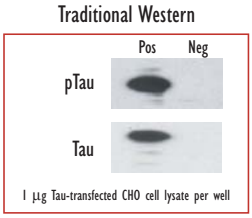
Multiplex Tau Assay: Detection of Phosphorylated (pThr231) and Total Tau in Mouse Brain Extract

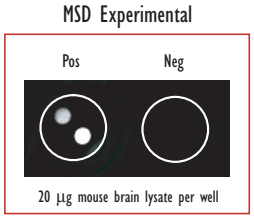




	Lysates (µg)	Average	StdDev	%CV
pTau	0	249	9	4
	0.31	442	12	3
	0.63	721	33	5
	1.25	1,440	80	6
	2.5	3,230	127	4
	5	6,983	207	3
	10	11,053	383	3
	20	14,380	254	2

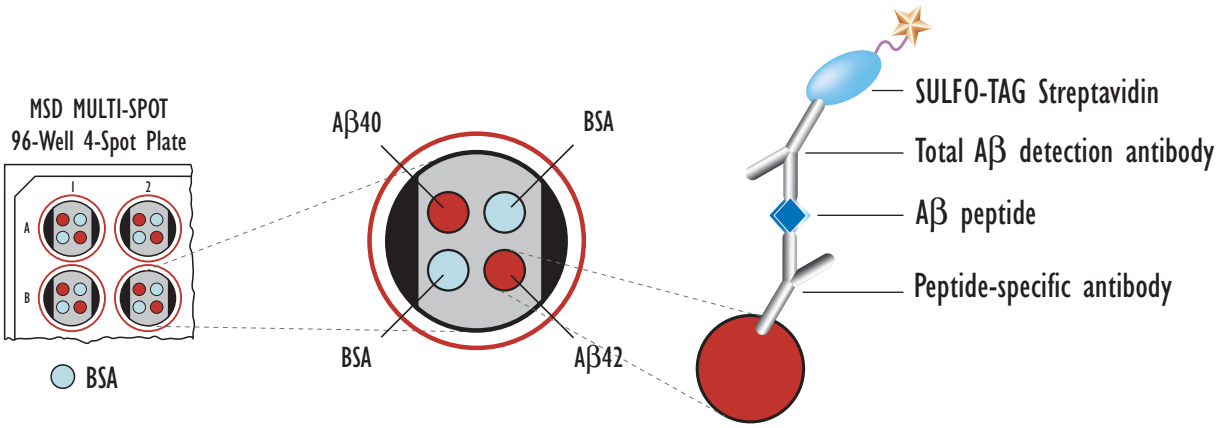
	Lysates (µg)	Average	StdDev	%CV
tTau	0	121	12	9
	0.31	2,681	220	8
	0.63	6,348	595	9
	1.25	14,218	1,258	9
	2.5	24,556	2,031	4
	5	38,150	993	5
	10	50,822	1,775	3
	20	53,922	3,596	7



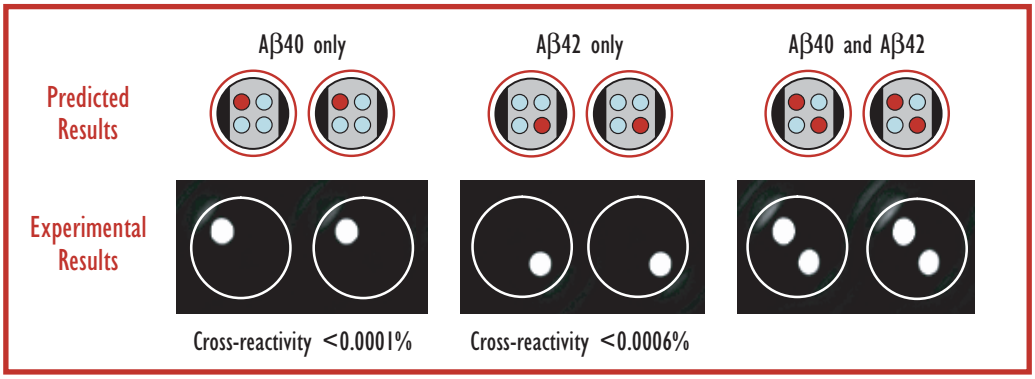


Mouse brain lysate was prepared by pulverizing frozen tissue, then homogenizing through a syringe with a 25 gauge needle. The debris was cleared by centrifugation and the lysates were added to MSD MULTI-SPOT 4-Spot plates coated with anti-total-Tau and anti-phospho-Tau antibodies on two of the four spatially distinct electrodes per well. Phosphorylated and total Tau were detected with anti-total-Tau antibody labeled with MSD SULFO-TAG reagent.

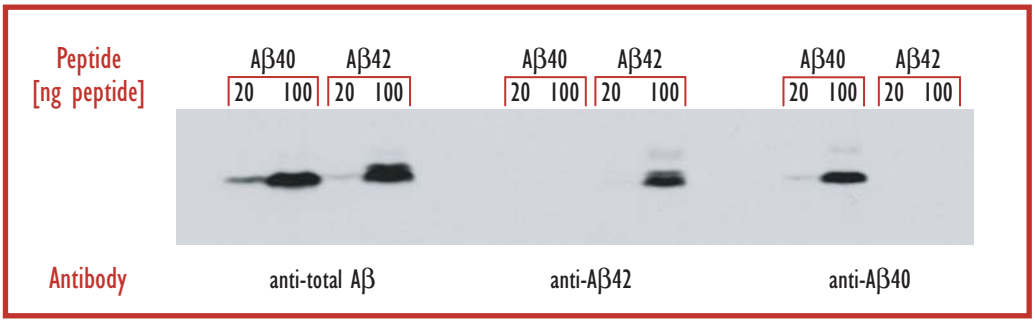
Detection of Aβ Peptides: β-amyloid Peptide Duplex



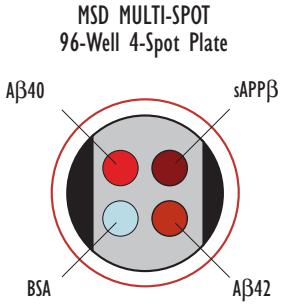
MSD Experimental

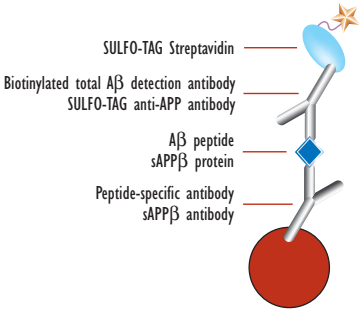


Traditional Western

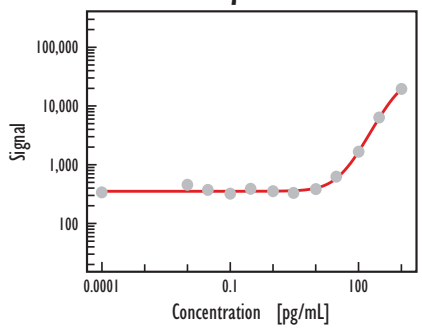


Multiplex Aβ40, Aβ42, and sAPPβ Assay





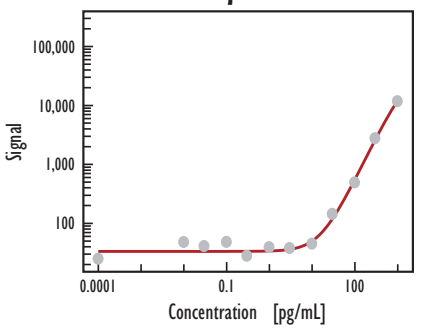
Aβ40



Concentration	Aβ40			S/B
	Average	StdDev	%CV	
0	337	41	12	1
0.01 pg/mL	532	519	97	2
0.03 pg/mL	371	81	22	1
0.1 pg/mL	320	30	9	1
0.3 pg/mL	388	91	24	1
1 pg/mL	353	74	21	1
3 pg/mL	329	33	10	1
10 pg/mL	383	34	9	1
30 pg/mL	624	79	13	2
100 pg/mL	1,659	269	16	5
300 pg/mL	6,330	557	9	19
1 ng/mL	19,451	2,158	11	58

Aβ40 Detection Limit (3 StdDev over background)	18 pg/mL

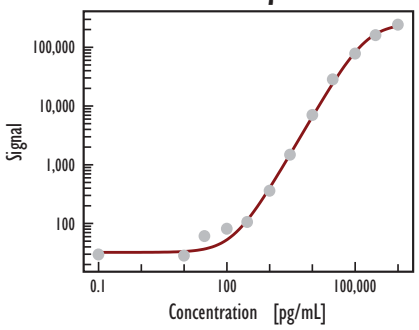
Aβ42



Concentration	Aβ42			S/B
	Average	StdDev	%CV	
0	25	12	48	1
0.01 pg/mL	48	13	26	2
0.03 pg/mL	41	14	35	2
0.1 pg/mL	48	10	21	2
0.3 pg/mL	28	9	33	1
1 pg/mL	39	9	23	2
3 pg/mL	38	21	56	2
10 pg/mL	45	6	13	2
30 pg/mL	144	18	12	6
100 pg/mL	493	70	14	20
300 pg/mL	2,770	368	13	111
1 ng/mL	11,754	1,305	11	470

Aβ42 Detection Limit (3 StdDev over background)	14 pg/mL

sAPPβ

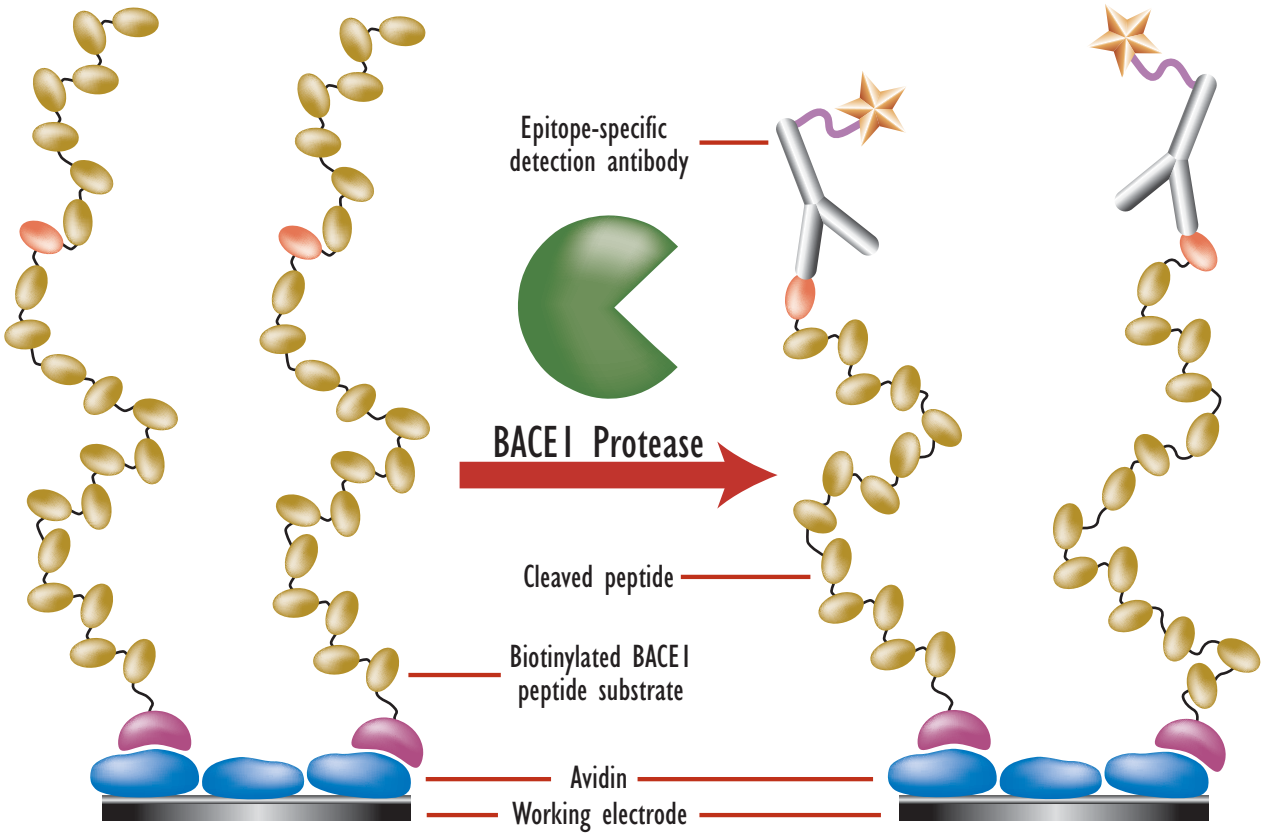


Concentration	sAPPβ			S/B
	Average	StdDev	%CV	
0	30	11	39	1
10 pg/mL	40	6	15	1
30 pg/mL	61	7	11	2
100 pg/mL	81	15	19	3
300 pg/mL	106	4	4	4
1 ng/mL	360	19	5	12
3 ng/mL	1,477	112	8	50
10 ng/mL	7,010	940	13	238
30 ng/mL	28,329	2,970	10	960
100 ng/mL	77,963	2,924	4	2,643
300 ng/mL	160,193	16,952	11	5,430
1 µg/mL	241,516	20,923	9	8,187

sAPPβ Detection Limit (3 StdDev over background)	140 pg/mL

Recombinant human sAPPβ, purified from mammalian cells (>95% pure), and synthetic Aβ peptides were combined and diluted in fresh culture medium (DMEM, 10% FBS, Pen/Strep). Samples were added to MSD MULTI-SPOT 4-Spot plates coated with anti-Aβ40, anti-Aβ42 and anti-sAPPβ antibodies on three of the four spatially distinct electrodes per well. The sAPPβ proteins were detected with anti-APP antibody labeled with MSD SULFO-TAG reagent. The Aβ peptides were detected with biotinylated antibody 4G8 and MSD SULFO-TAG labeled streptavidin.

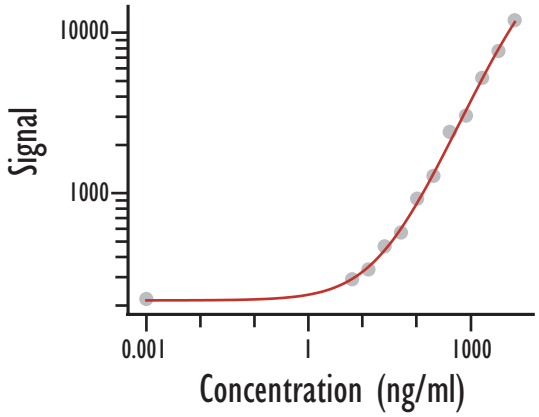
β-secretase Activity Assay: Finding Inhibitors of BACE1



1. MULTI-ARRAY™ 96-Well Plates precoated with avidin are incubated with Biotinylated BACE1 peptide substrate for 30 min and then washed.
2. BACE1 enzyme and/or other samples are added to the wells and incubated for 1 hr, followed by washing.
3. MSD SULFO-TAG detection antibody is added to the wells and incubated for 30 min, followed by washing.
4. 150µL MSD Read Buffer T (with surfactant) are added to the wells and the plate is analyzed on the SECTOR 6000 instrument.

β-secretase Activity Assay: Finding Inhibitors of BACE1 (continued)

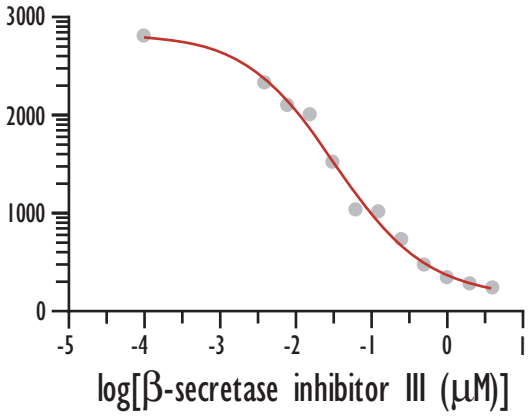
Titration of BACE1 Enzyme



ng/ml enzyme	Average	S.D.	CV%	S/B
6670	11940	1709	14	54.6
3335	7681	519	7	35.1
1667	5223	768	15	23.9
834	3040	347	11	13.9
417	2406	122	5	11.0
208	1281	185	14	5.9
104	923	115	12	4.2
52	568	109	19	2.6
26	466	14	3	2.1
13	334	15	5	1.5
6.5	290	37	13	1.3
0	219	28	13	

Detection Limit: 7.6 ng/ml

Inhibition of BACE1 Enzyme with Inhibitor III (GL-189)



μM inhibitor	Average	S.D.	CV%	S-B	Activity(%)
0	2822	114	4	2507	100
0.0039	2355	416	18	2040	81
0.0078	1820	79	4	1505	60
0.0156	2031	164	8	1716	68
0.0313	1554	376	24	1239	49
0.0625	1078	241	22	763	30
0.125	1048	270	26	733	29
0.25	764	182	24	449	18
0.5	516	67	13	201	8
1	398	30	8	83	3
2	334	25	8	19	1
4	316	97	31	1	0

Calculated IC50=0.034 μM, in good agreement with published value of 0.04 μM (Tung et al. (2002) J. Med. Chem. 45: 259).

Conclusions

1. We have developed highly specific multiplexed assays for simultaneously measuring sAPPα and sAPPβ, as well as an assay for sw sAPPβ. For all three assays, we have novel, recombinant mammalian-expressed, protein standards for calibration.

2. Total Tau and Tau p231 can also be measured simultaneously in multiplex assays. These assays will recognize human protein as well as mouse/rat Tau.

3. A very rapid and simple *in vitro* assay for BACE1 activity has been developed.

4. A multiplex assay for the Aβ40 and Aβ42 peptides has the versatility to measure peptides with either 4G8, which will also recognize rodent peptides, or with 6E10, which does not recognize rodent Aβ peptides or the P3 peptide.

5. Multiple species important in Alzheimer's disease drug development can be assayed simultaneously in a single well by using specific antibodies immobilized on MSD MULTI-SPOT plates. The MULTI-ARRAY technology-based assay can be readily adapted to any protein for which antibodies are available.

6. These assays are specific and afford higher throughput replacements to gold-standard methods like ELISAs. Assaying multiple species in the same well reduces the labor involved and the amount of sample required.

7. The assays can be easily automated, and are suitable for HTS.