



A Membrane Receptor Binding Assay Using MesoScale Electrochemiluminescence Technology

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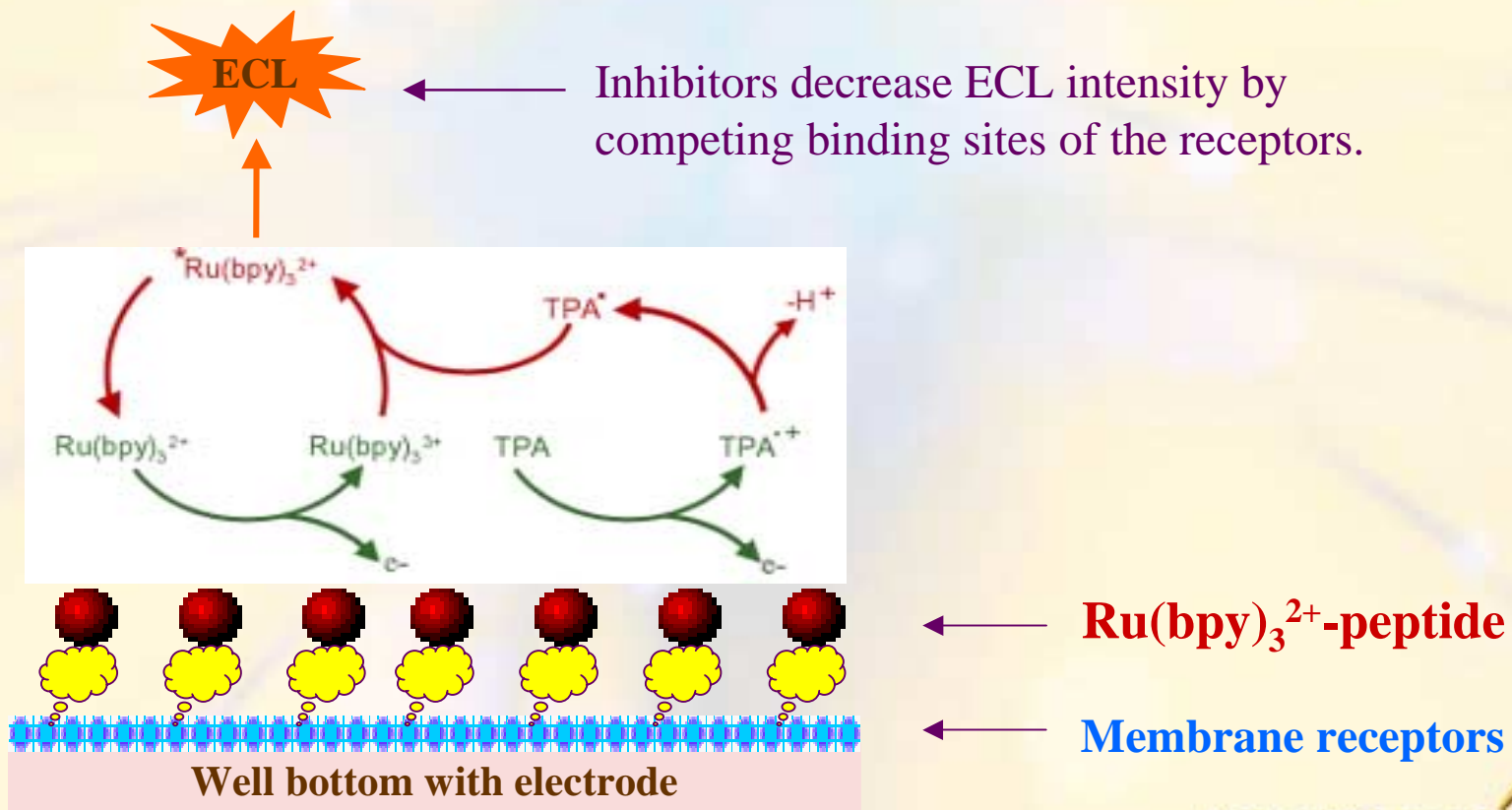
Background

- **The receptor**
 - A GPCR stably expressed in HEK293 cells
 - Membrane
- **The ligand**
 - A neuropeptide with Ruthenium labeled at the N-terminus
- **The goal**
 - To identify small molecules that bind to the target receptor

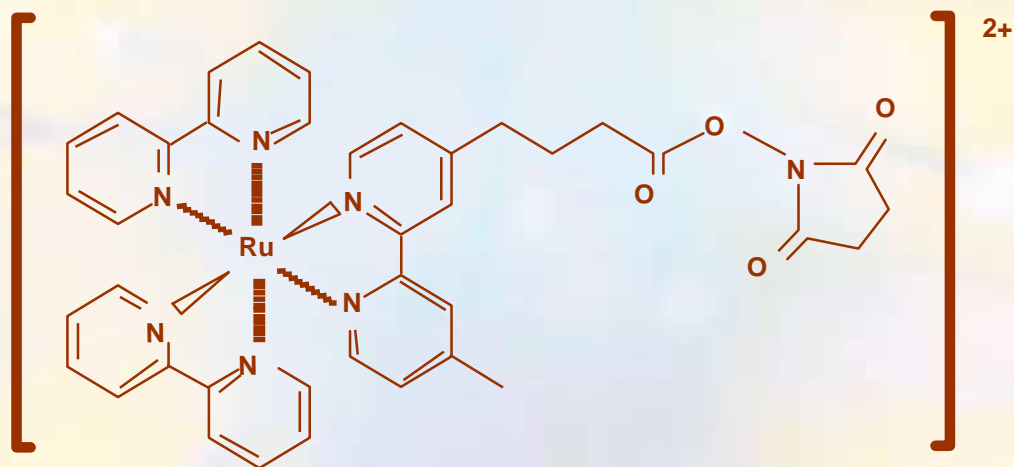


Meso Scale Technology – Assay Mechanism

- Electrochemiluminescence (ECL) signal (image by CCD camera)
- Ruthenium labeled ligand binding

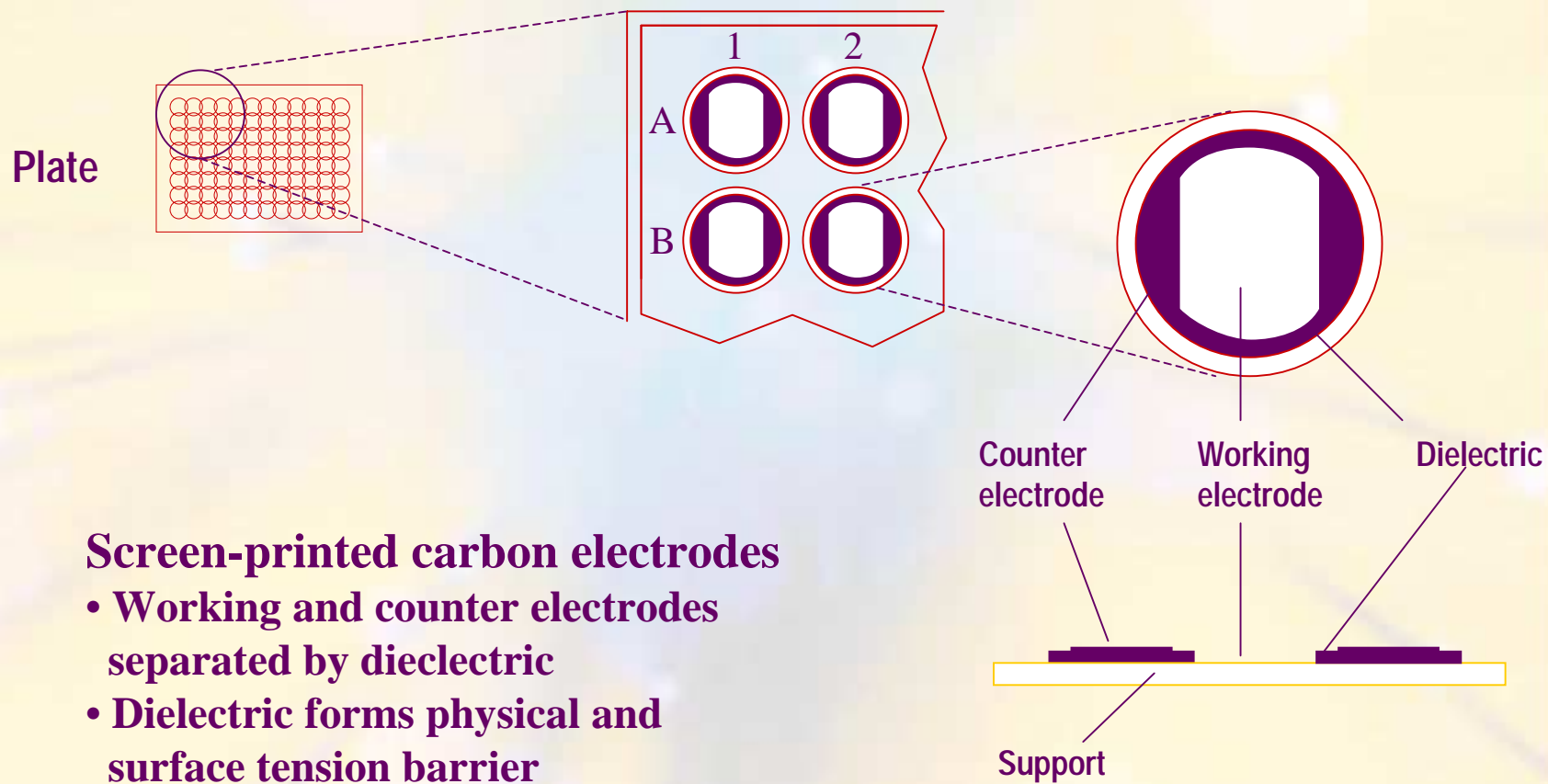


The labeling molecule



Ruthenium (II) tris-bipyridine NHS ester
 $\text{Ru}(\text{bpy})_3^{2+}$

The Plates



Screen-printed carbon electrodes

- Working and counter electrodes separated by dielectric
- Dielectric forms physical and surface tension barrier



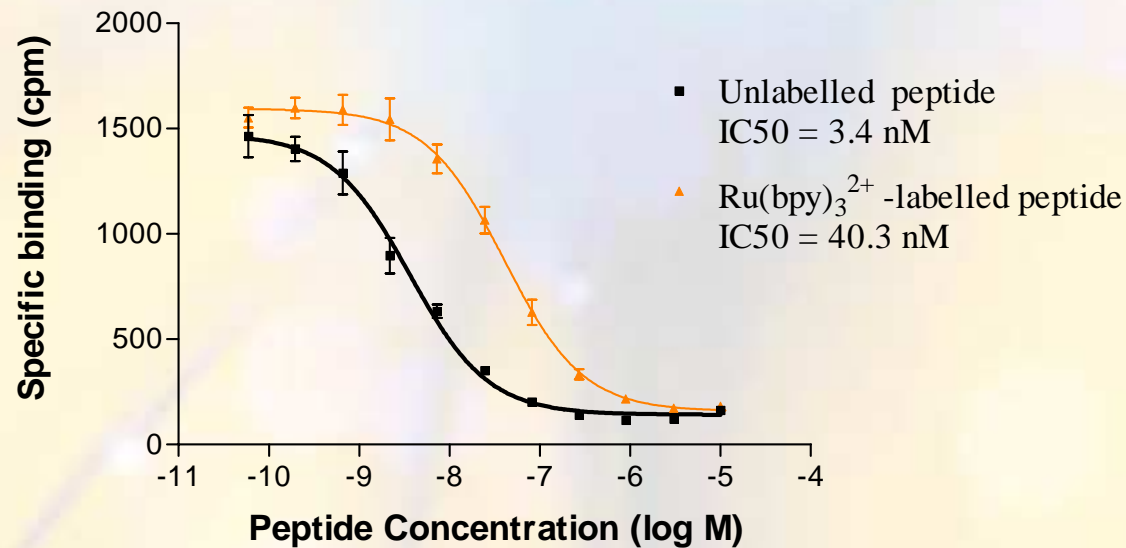
Binding assay using MSD technology

1. Deposit membranes to assay plates, incubate @ RT for 1 hr (**CyBi-well**)
2. Add Blocker to minimize non-specific binding, incubate @ RT for 30 min (**CyBi-well**)
3. Add test compounds (**CyBi-well**)
4. Add Ru-labeled ligand, incubate @ RT, 1 hr (**multidrop**)
5. Add reading buffer (TPA) (**multidrop**)
6. Read ECL intensity in MSD's instrument (**Plate crane/Sector**)

Effects of Ru-labeling on the Binding Affinity of the Peptide

^{125}I - binding SPA:

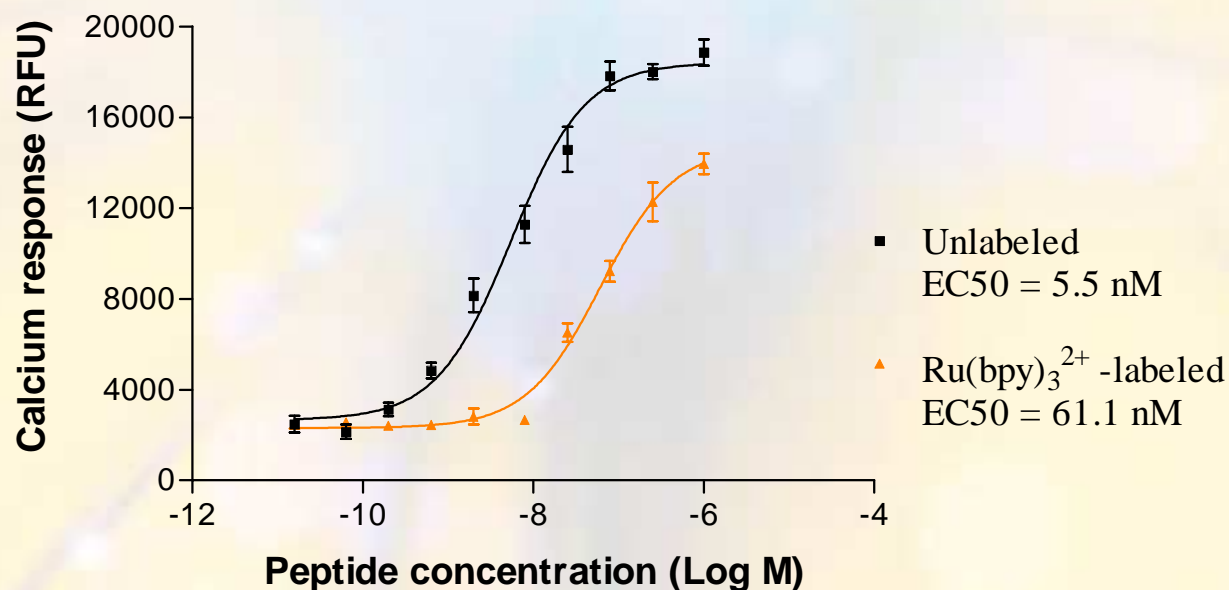
Labeling caused >10 fold increase of IC_{50}
in ^{125}I competition binding.



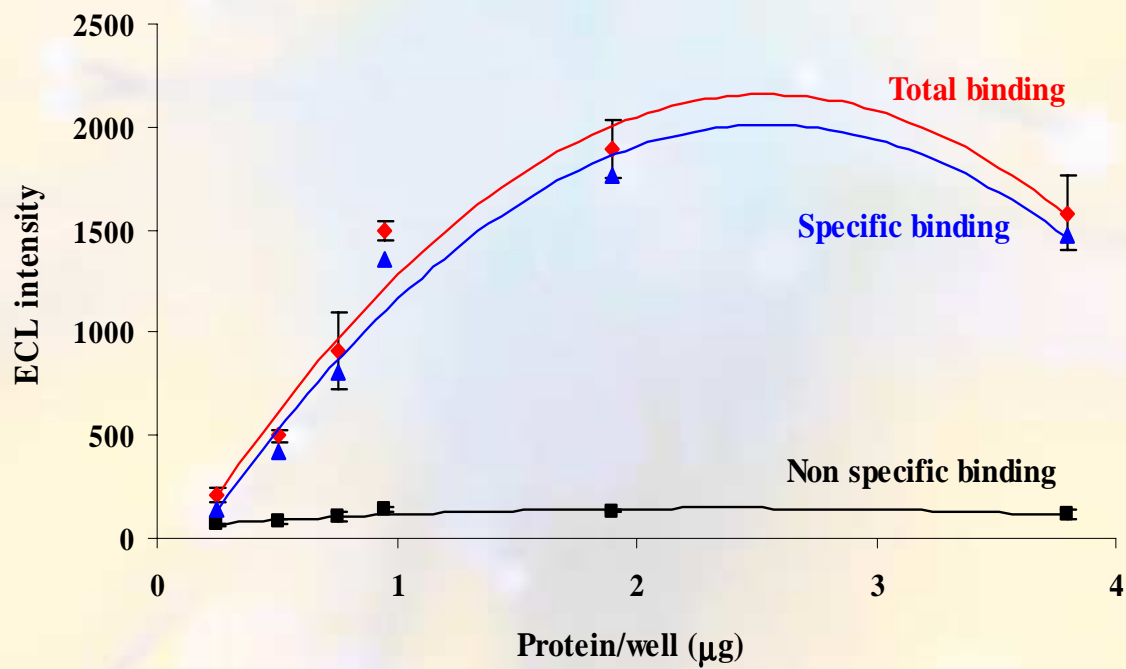
Effects of Ru-labeling on Functional Activity

FLIPR assay:

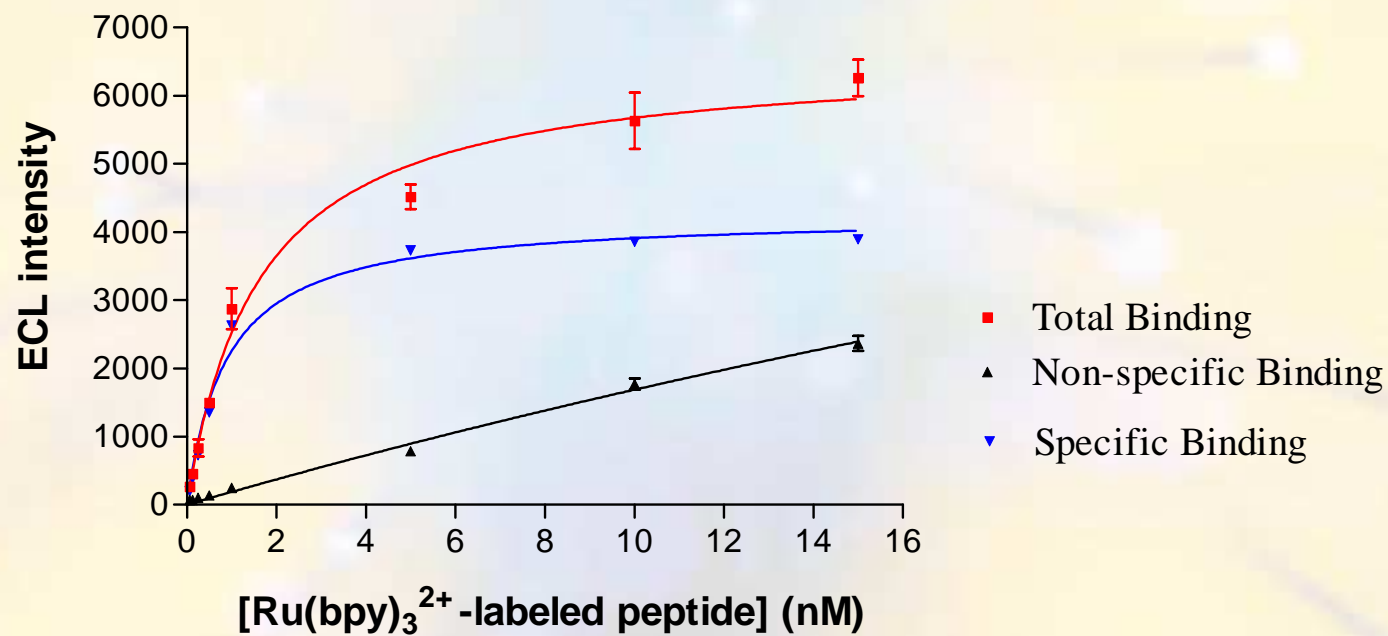
Labeling caused >10 fold increase of EC₅₀ in FLIPR assay



Titration of Membrane Proteins



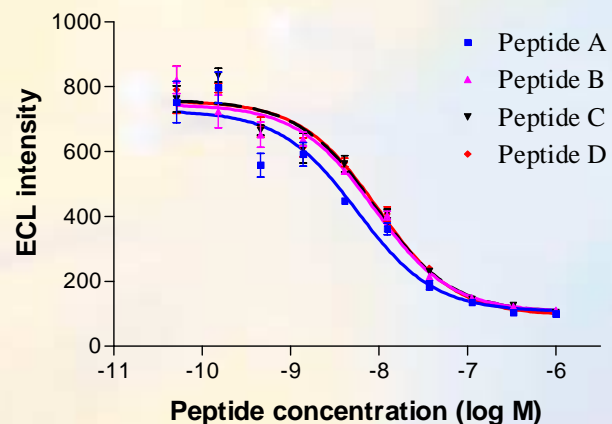
Saturation Binding



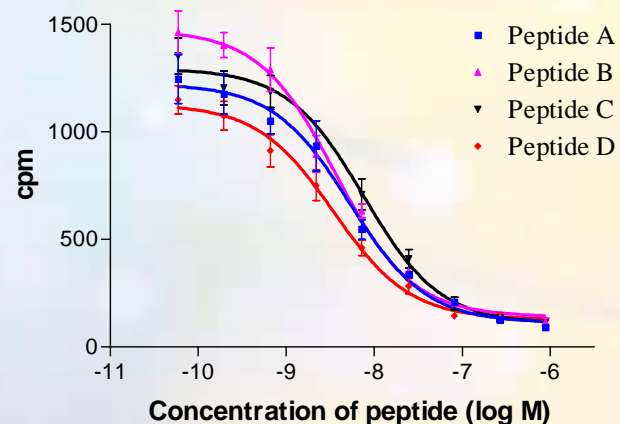
$K_d = 0.89 \pm 0.14$ nM
 $B_{max} = 4.0$ pmol/mg protein

Competition Binding

ECL binding assay



¹²⁵I-SPA assay

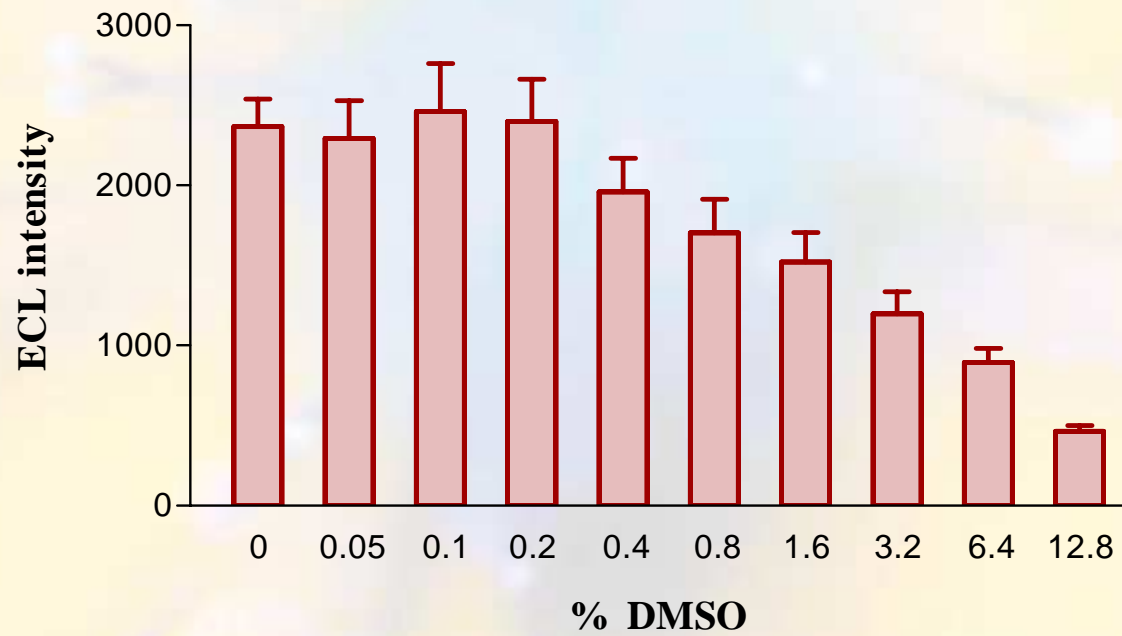


Ki values (nM) of peptide ligands

	Peptide A	Peptide B	Peptide C	Peptide D
Meso Scale	3.01	4.95	5.26	3.67
SPA	4.38	3.01	6.31	2.87



Effects of DMSO

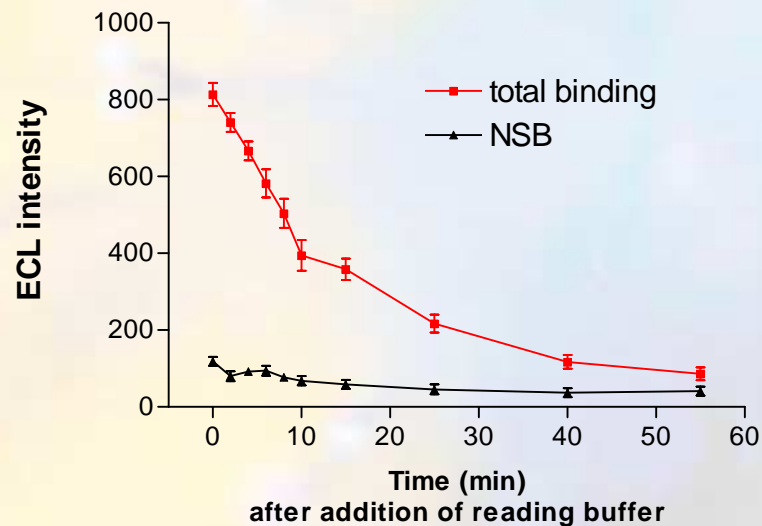




Technical Challenge 1

- Signal drops after the addition of reading buffer

**ECL Signal Declination
after Addition of Reading Buffer**



Time (min)	% signal left
0	100.0
2	95.1
4	82.6
6	70.2
8	61.4
10	47.0
15	43.1
25	24.6
40	11.5
55	6.5

- Reason:
Reading buffer caused irreversible dissociation of receptor-ligand complex.
- Solution:
Read 1 min after addition of reading buffer.



HTS Assay Conditions

- **Total reaction volume = 25 μ l**
- **Total membrane protein = 0.9 μ g/well**
- **Final labeled ligand concentration = 0.5 nM**
- **Final Compound concentration = 10 μ M**
- **Final BSA concentration = 0.2%**
- **Final DMSO concentration = 0.2%**
- **Positive control peptide = 1 μ M**
- **Reading buffer (TPA) = 1 X**



Signal Declination during Read Time



	Bottom left	Top left	Whole plate
Avg. ECL	2773.6	2302.7	2483.1
SD	176.4	230.0	254.2
% CV	6.4	10.0	10.2

Overall Assay window: 19.2
Z' factor: 0.7

Technical Challenge 2: Membrane deposition

- Shape matters
- Volume matters





Bad Membrane Deposition

Plate AZW04008: 7 hits in row A. None confirmed.

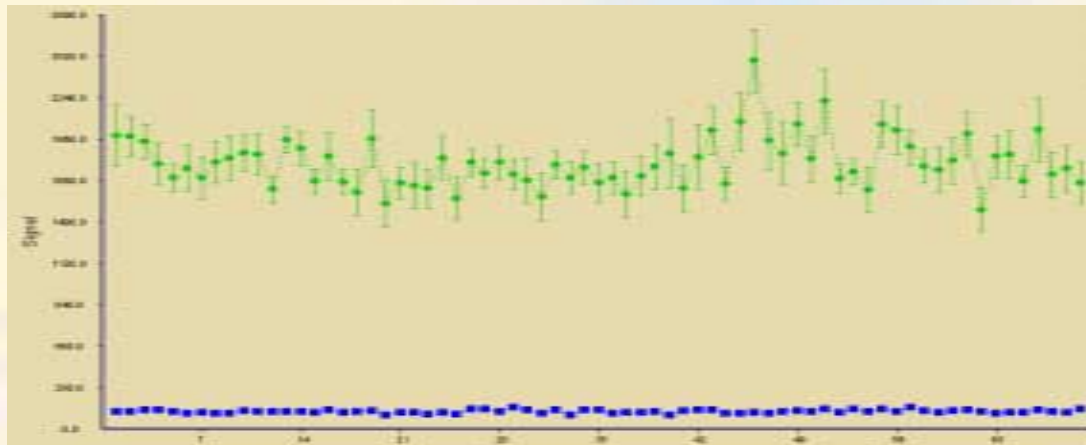
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1290	9129	1364	1445	1362	1435	1434	1478	1351	1370	1459	1343	821	1449	993	944	901	914	928	965	949	962	195	195
B	1515	9179	1412	1434	1504	1399	1545	1451	1440	1440	1534	1403	1547	1312	1523	1507	1467	1374	1470	1260	1445	1303	95	195
C	1672	7941	1381	1435	1559	1405	1515	1554	1487	1513	1356	1554	1280	1411	1409	1535	1308	1550	1305	1462	1467	1260	94	130
D	1567	1562	1197	1294	1435	1266	1414	1446	1430	1492	1493	1362	1382	1531	1501	1569	1579	1540	1534	1469	1399	1367	92	195
E	1480	1544	1338	1275	1514	1424	1568	1478	1495	1570	1479	1495	1572	1643	1718	1584	1538	1510	1581	1531	1537	1574	89	111
F	1520	9179	1573	1372	1373	1422	1292	1506	1405	1563	1484	1605	1471	1509	1182	1606	1475	1645	920	1500	1645	1409	189	189
G	1647	1799	1551	1484	1526	1553	1413	1434	1593	1435	1692	1601	1552	1602	1508	1571	1494	1619	1640	1594	1419	1543	92	195
H	1573	1728	1472	1377	1443	1434	1580	1375	1488	1572	1357	1520	969	1728	1541	1728	1609	1672	1336	1574	1676	1651	102	130
I	1640	1791	1629	1590	1581	1751	1682	1723	1680	1552	1532	1602	1334	1644	1720	1689	1699	1699	1690	1770	1464	1519	109	139
J	1625	1719	1617	1593	1714	1880	1687	1632	1664	1499	1647	1610	1217	1680	1585	1719	1748	1695	1683	1547	1651	1493	109	117
K	1794	1594	1579	1595	1794	1683	1539	1577	1617	1560	1719	1723	1680	1797	1681	1571	1681	1609	1604	1509	1671	1539	106	114
L	1540	1599	1658	1525	1525	1564	1658	1574	1676	1636	1674	1709	1602	1708	1458	1644	1708	1606	1699	1621	1662	1672	100	100
M	1676	1599	1545	1671	1726	1681	1571	1644	1435	1522	1562	1506	1749	1603	1769	1722	1712	1597	1686	1557	1532	1418	100	100
N	1427	1672	1532	1617	1672	1688	1625	1589	1623	1724	1661	1640	1679	1736	1624	1717	1706	1623	1581	1575	1383	1508	89	195
O	1688	1772	1694	1673	1670	1706	1714	1533	1703	1495	1495	1579	1700	1691	1719	1692	1560	1479	1562	1414	1380	1304	86	195
P	1570	1758	1736	1542	1573	1405	1537	1492	1536	1496	1370	1647	1372	1643	1460	1569	1538	1495	1482	1402	1376	1353	100	110

11 plates of 977 plates screened had this phenomenon
88 of 267 hits were from these wells



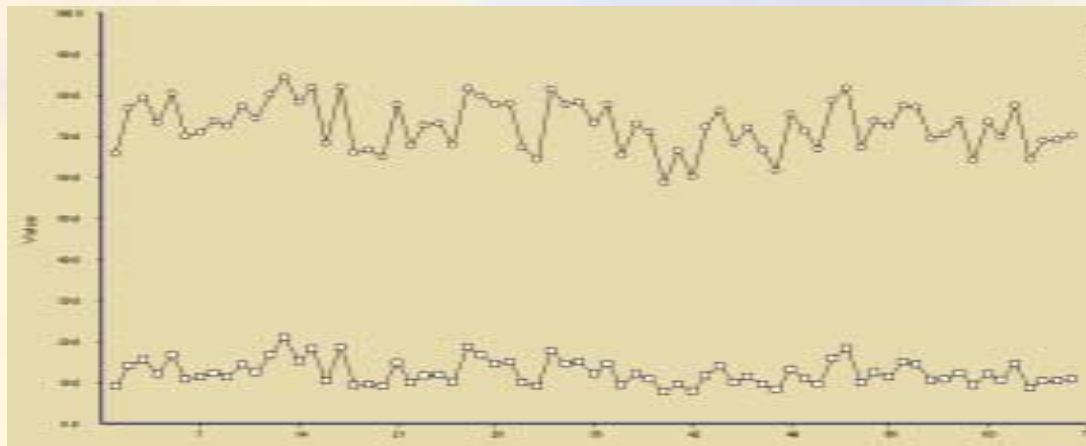
Screen Performance

A typical daily results summary (70 plates)



0% activity control
Avg. ECL = 1795.8 ± 224.1

100% activity control
Avg. ECL = 114.2 ± 15.8



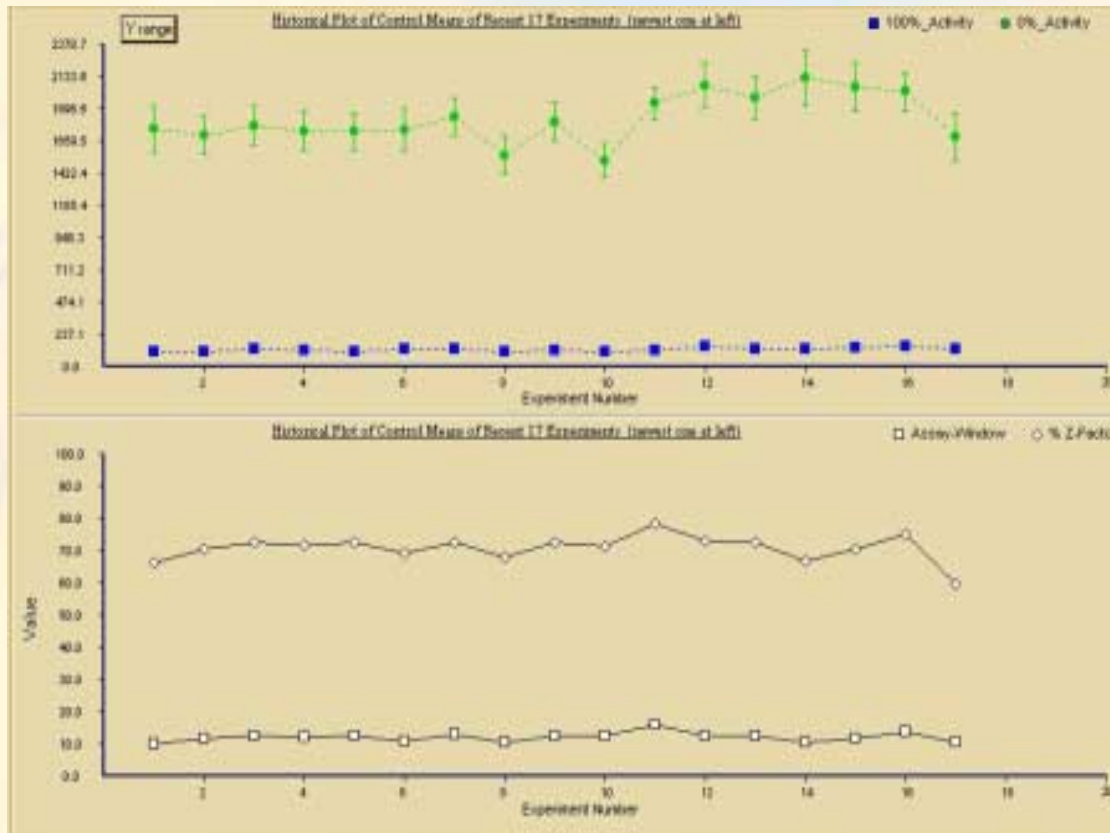
Z' factor (x 100)
Avg. Z' = 0.73

Assay window
Avg. = 12.4



Screen Performance

Overall screen performance (summary of ~1000 plates)



0% activity control
Avg. ECL = 1818.4 ± 152.6

100% activity control
Avg. ECL = 119.9 ± 12.6

Z' factor (x 100)
Avg. Z' = 0.70

Assay window
Avg. = 11.9



HTS Results

- Screened a total of 977 plates, 308,613 compounds.
- Throughput was 100 plates (384-well) per day, one person, one Sector HTS reader.
- Confirmed Hits

Compound	Meso Scale % Activity	% Light Quench	SPA % Activity
A	51.21	< 1	< 40
B	48.35	10	53.68
C	52.12	18.1	< 40
D	73.51	27.4	< 40
E	56.37	8.6	< 40
F	68.65	25.7	66.34
G	44.38	13.7	< 40
H	42.13	20.3	< 40
I	48.22	9.5	< 40
J	55.88	13.5	< 40

- Data shown were averages of triplicates.
- All hits showed minimal interference with ECL intensity.

Comparison of Binding Assay Methods

	Non Radioactive			Radioactive	
	DELFLIA**	FP	MSD	SPA, LeadSeeker	SPA, TopCount
Labeling molecule	Europium	Bodipy-TMR	Ru(bpy) ₃ ²⁺	¹²⁵ I	¹²⁵ I
Effects of labeling*	lost activity	10 times less active	10 times less active	no effect	no effect
Assay Window	N/A	1.4	> 10	5	> 10
Z'	N/A	< 0	0.7	0.5	0.7
Protein (μg/well)	N/A	10	1	5	10
Throughput (reading time: min/plate)	N/A	2	2	5	40

* Effects of labeling were measured using FLIPR assay for functions (EC₅₀) and ¹²⁵I competition binding (IC₅₀).

** DELFLIA: Dissociation-Enhanced Lanthamide FluoroImmuno Assay



Summary

- **Best binding assay amenable to high throughput screening for this membrane bound receptor**
- **Assay sensitivity rivals that of radioligand binding**
 - **Needed only 1 ug membrane protein compared to 5 ug for SPA**
 - **Could detect binding to < 0.5 fmole receptor**
- **Good assay performance with assay window > 10 and $Z' > 0.7$.**
- **Throughput was 100 plates (384-well) per day, one person, one Sector HTS reader. Can be improved with automation.**