

Ferring Pharmaceuticals A/S

Case Study: Neutralization Assay Design and Qualification

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Case Study

Objective: Discussion on the development, design and qualification of a full-curve neutralization assay for a drug agonist using cAMP as measurable endpoint

Drug: Recombinant Hormone

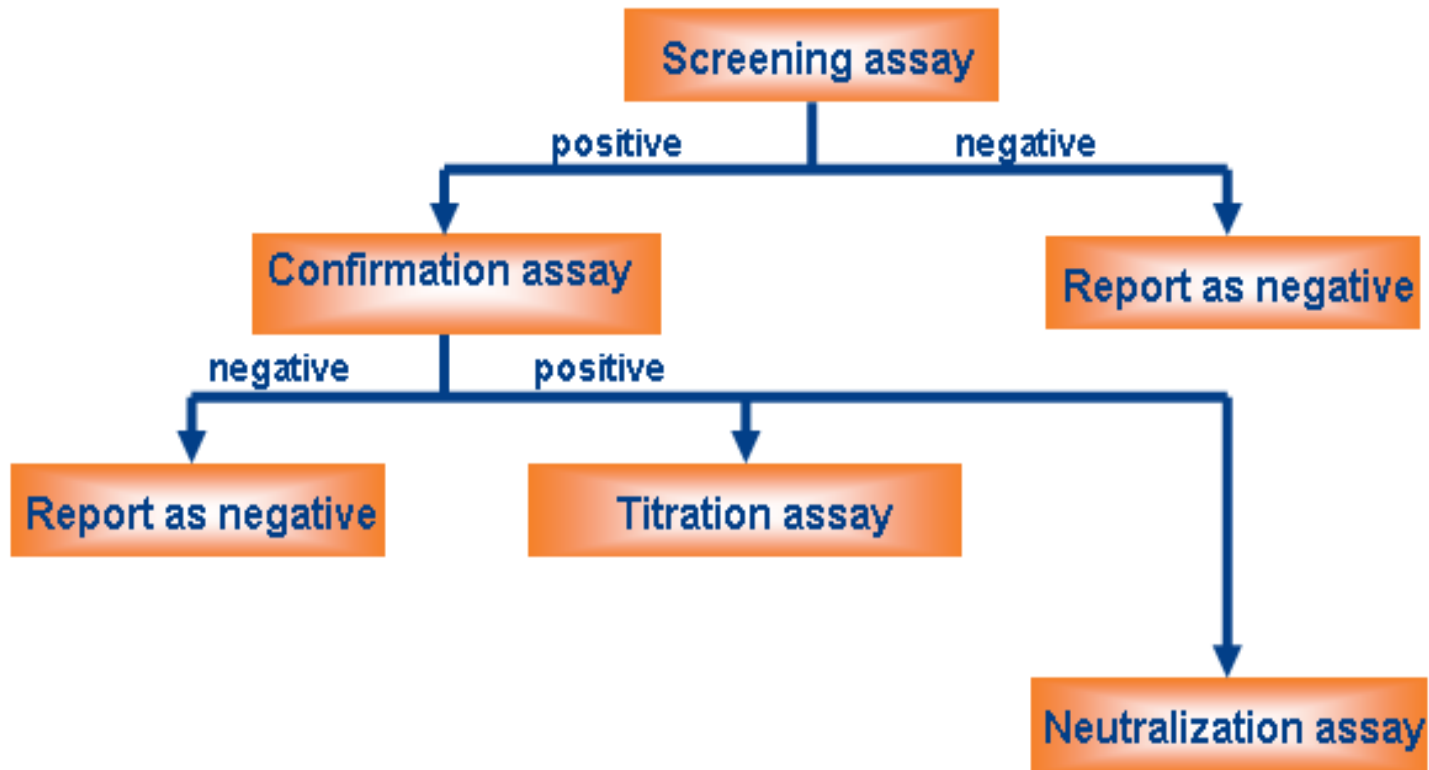
Phase: I

Dosing: Single.dose / Multi-dose

No of Subjects: 50+

Example of Sampling: day 1, day 14, day 35

Tiered Immunogenicity Testing



Presentation Topics

Assay Design

Assay Development and Optimization

Assay Qualification

Neutralization Assay Principle

Treatment of cells with a drug induces a functional response.

If a sample containing NABs is introduced into this system it would reduce or abolish the response associated with the drug

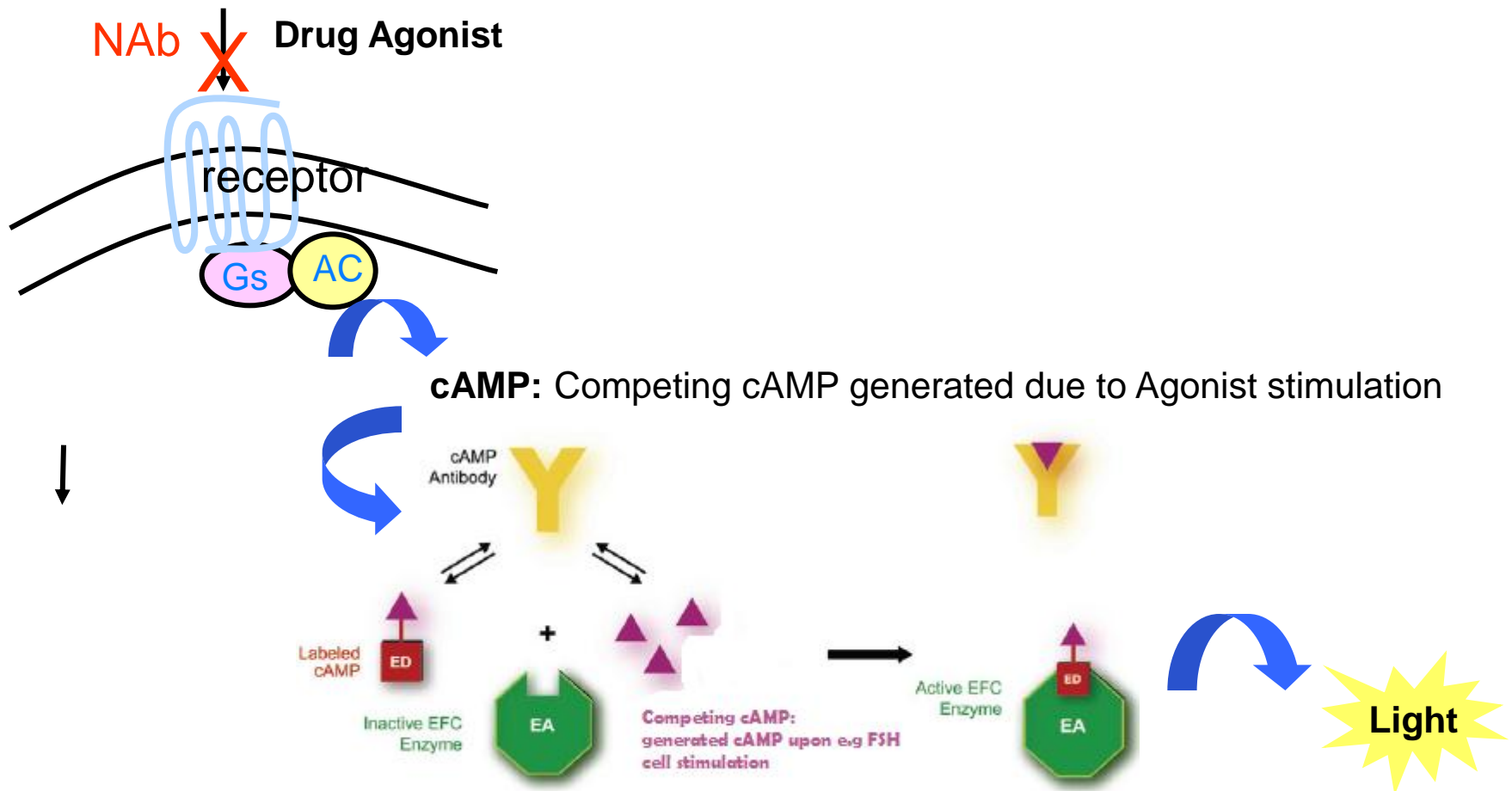
Examples of common neutralization assay end-points:

- Proliferation
- Cytokine secretion
- mRNA or target protein expression

The selected response should assess some aspect of the drug's biology

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cAMP Endpoint



Presentation Topics

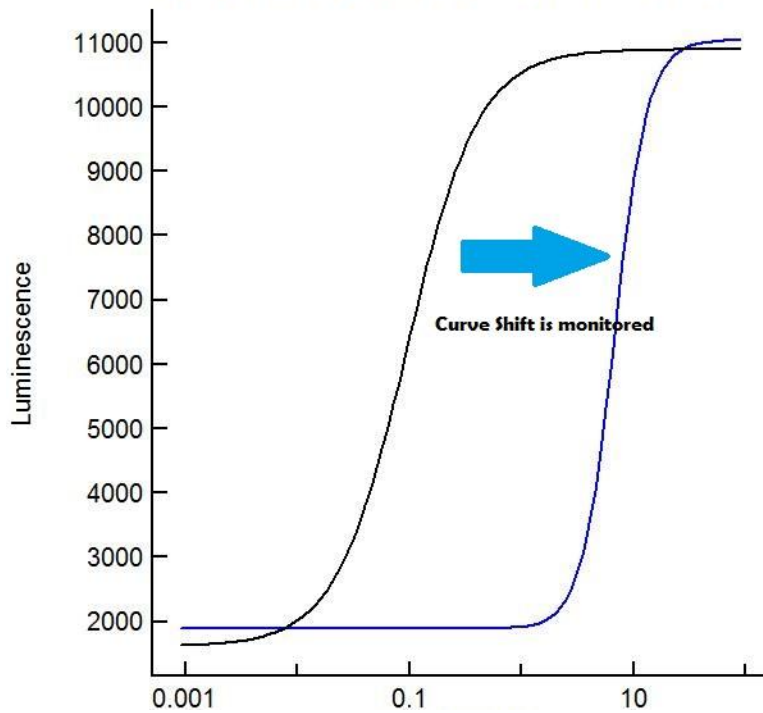
Assay Design

Assay Development and Optimization

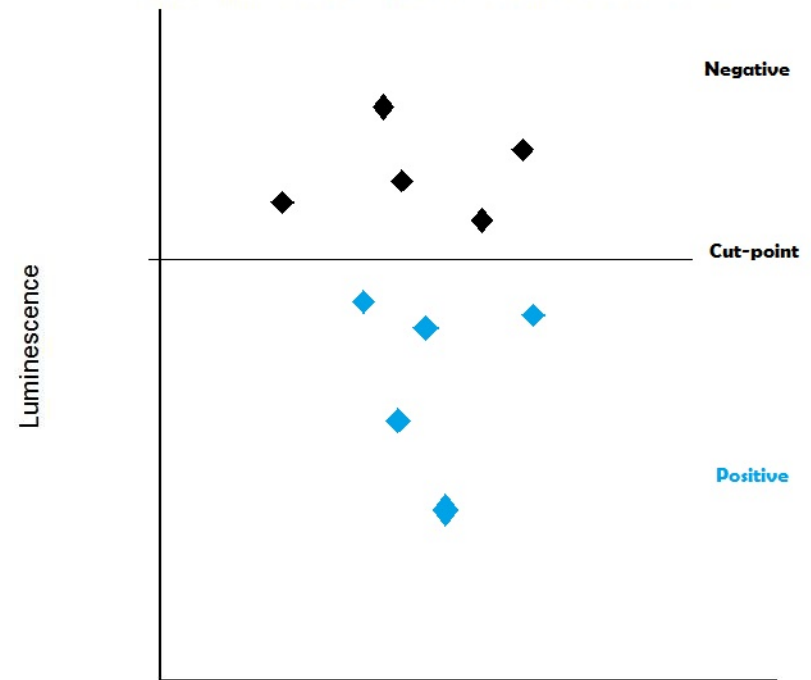
Assay Qualification

Assay Formats

Curve shift between the negative- and positive control



Signal inhibition by negative- and positive controls



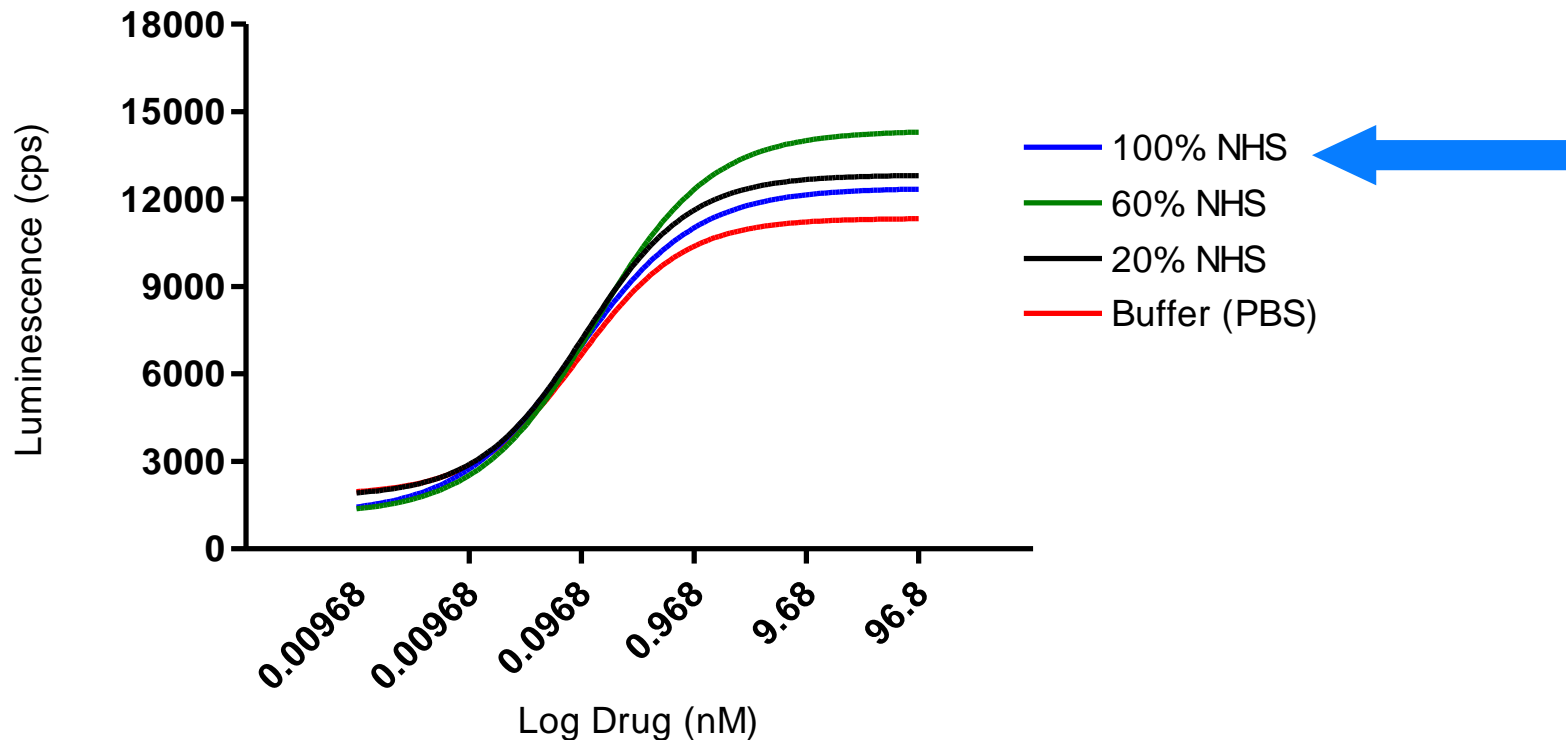
Seeding Density and Matrix Concentration

Cells/well	% Serum	EC50 (nM)	Basal CPS	S/B
20 000	100	0.113	1426	9
30 000	100	0.100	2282	5
40 000	100	0.114	2466	4
20 000	60	0.155	1664	9
30 000	60	0.427	1522	14
40 000	60	0.195	2678	7
20 000	20	0.131	1783	5
30 000	20	0.423	1314	4
40 000	20	0.232	3191	7

n = 3

Seeding Density and Matrix Concentration Cont.

Investigation of Optimal Matrix Concentration
20 000 cells/well



Further Parameters were Optimized

Sample Volume

Incubation Times

Plate types

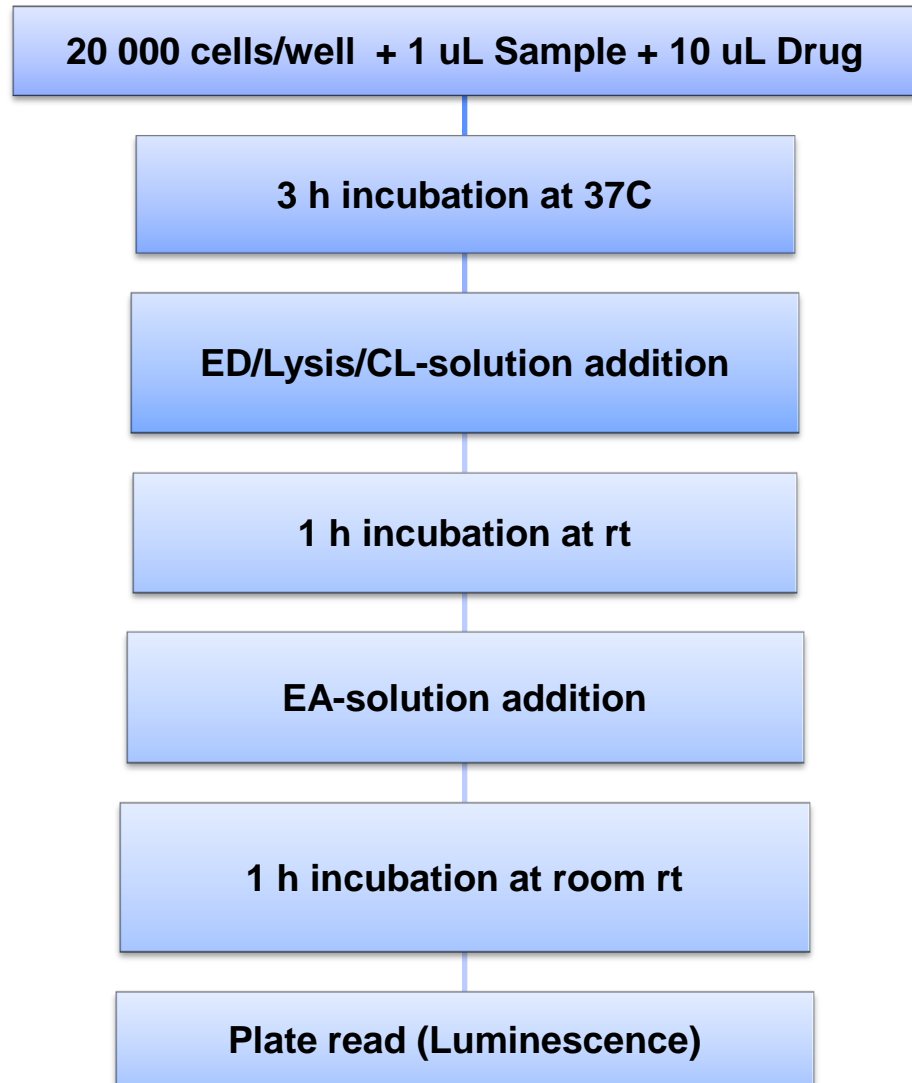
Pipetting

Plate Uniformity

Cell Maintenance

Final Assay

Total Assay Time: 7h



Acceptance Criterias

- ▶ Ratio of maximum to minimum counts per second must be ≥ 3 .
- ▶ Curves must have an slope value of at least ≥ 0.6
- ▶ Positive controls must always be positive i.e. above cut-point.
- ▶ The negative control must be below the upper limit of the negative control, as established in the qualification.

If any of the above criteria are not fulfilled for the positive- and negative control, the plate is not approved and has to be rerun. If any of the above criteria are not fulfilled for the sample, the sample curve is not approved and has to be excluded.

Presentation Topics

Assay Design

Assay Development and Optimization

Assay Qualification

Qualification Parameters

Replicate analysis of sample curves (data not shown)

Precision

Specificity of rare reagents

Sensitivity

Drug tolerance

Cut-point

Short-term stability

Freeze and thaw stability

Upper limit for negative control

Cut-point

Cut-point Determination (30 individual sera)									
	0.181	0.132	0.238	0.0943	0.113	0.173	0.0931	0.181	0.170
	0.121	0.145	0.158	0.101	0.102	0.252	0.0924	0.129	0.185
	0.126	0.182	0.168	0.115	0.223	0.146	0.106	0.164	0.220
	0.132	0.250	0.151	0.0986	0.181	0.219	0.107	0.220	0.160
	0.166	0.267	0.219	0.174	0.279	0.256	0.131	0.221	0.188
	0.0920	0.206	0.342	0.127	0.179	0.154	0.114	0.155	0.294
	0.0914	0.273	0.220	0.151	0.186	0.156	0.149	0.199	0.174
	0.149	0.158	0.164	0.137	0.156	0.171	0.308	0.150	0.193
	0.125	0.317	0.260	0.106	0.180	0.180	0.207	0.265	0.236
	0.118	0.166	0.149	0.107	0.217	0.392*	0.150	0.225	0.258
n	30			29			30		
Mean	0.176								
SD	0.0575								
Cut-point (Mean + 1.645 x SD)	0.270								

Through a parametric approach a floating cut-point was chosen.

The correction factor was determined to be: 0.134 (EC50)

Inter- and intra-assay precision

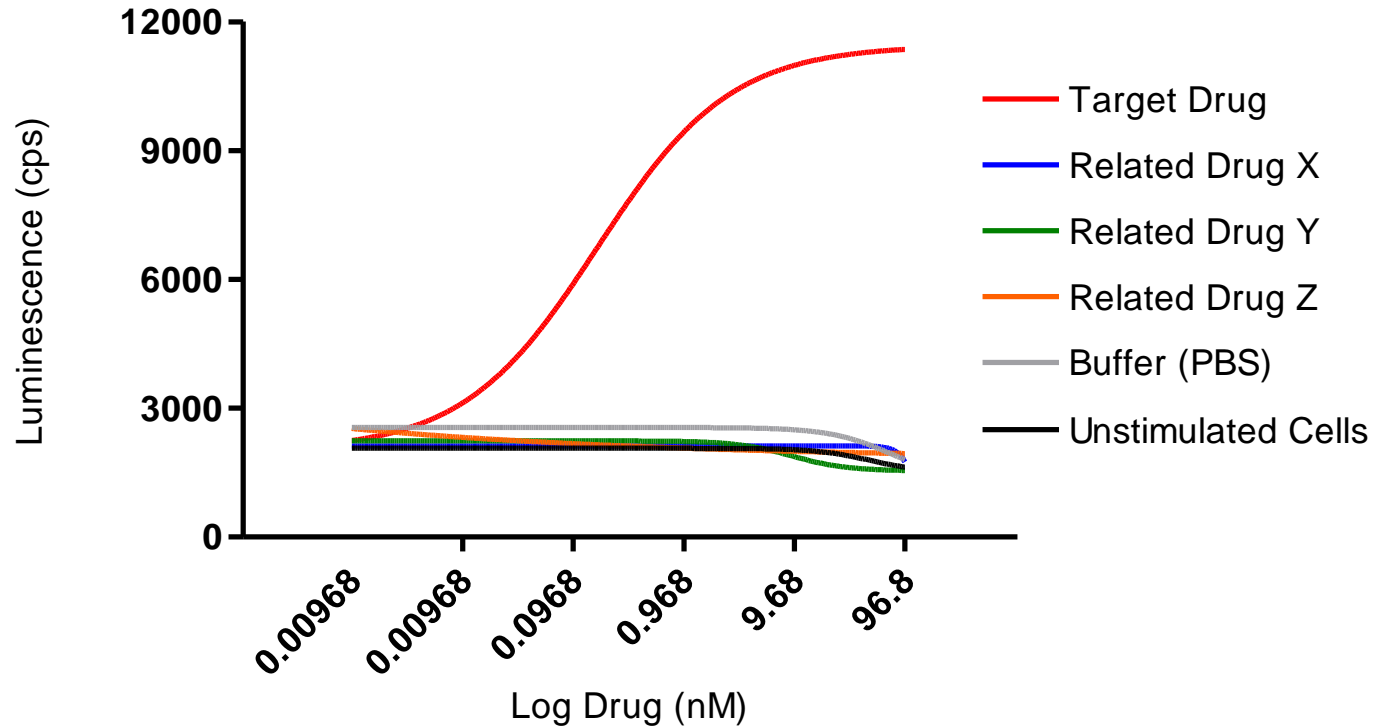
	Inter-assay Precision			Intra-assay Precision		
	LOW	MID	HIGH	LOW	MID	HIGH
EC50 (nM)	0.472	1.70	6.54	0.472	1.70	6.54
	0.610	1.86	7.25	0.610	1.86	7.25
	0.573	1.90	7.39	0.573	1.90	7.39
	0.423	1.82	6.21	0.423	1.82	6.21
	0.554	2.04	8.45	0.554	2.04	8.45
	0.478	1.49	7.94	0.478	1.49	7.94
	0.318	1.21	9.71			
	0.311	1.40	11.2			
	0.319	1.21	9.66			
	0.267	1.23	7.07			
	0.314	1.45	8.76			
	0.336	1.32	8.22			
	0.503	1.08	6.57			
	0.504	1.30	7.58			
	0.542	1.16	6.37			
	0.673	1.27	10.2			
	0.608	1.77	6.06			
	0.519	1.33	12.5			
Mean	0.462	1.48	8.21	0.518	1.80	7.30
SD	0.124	0.295	1.83	0.071	0.187	0.836
CV (%)	27	20	22	14	10	11
n	18	18	18	6	6	6



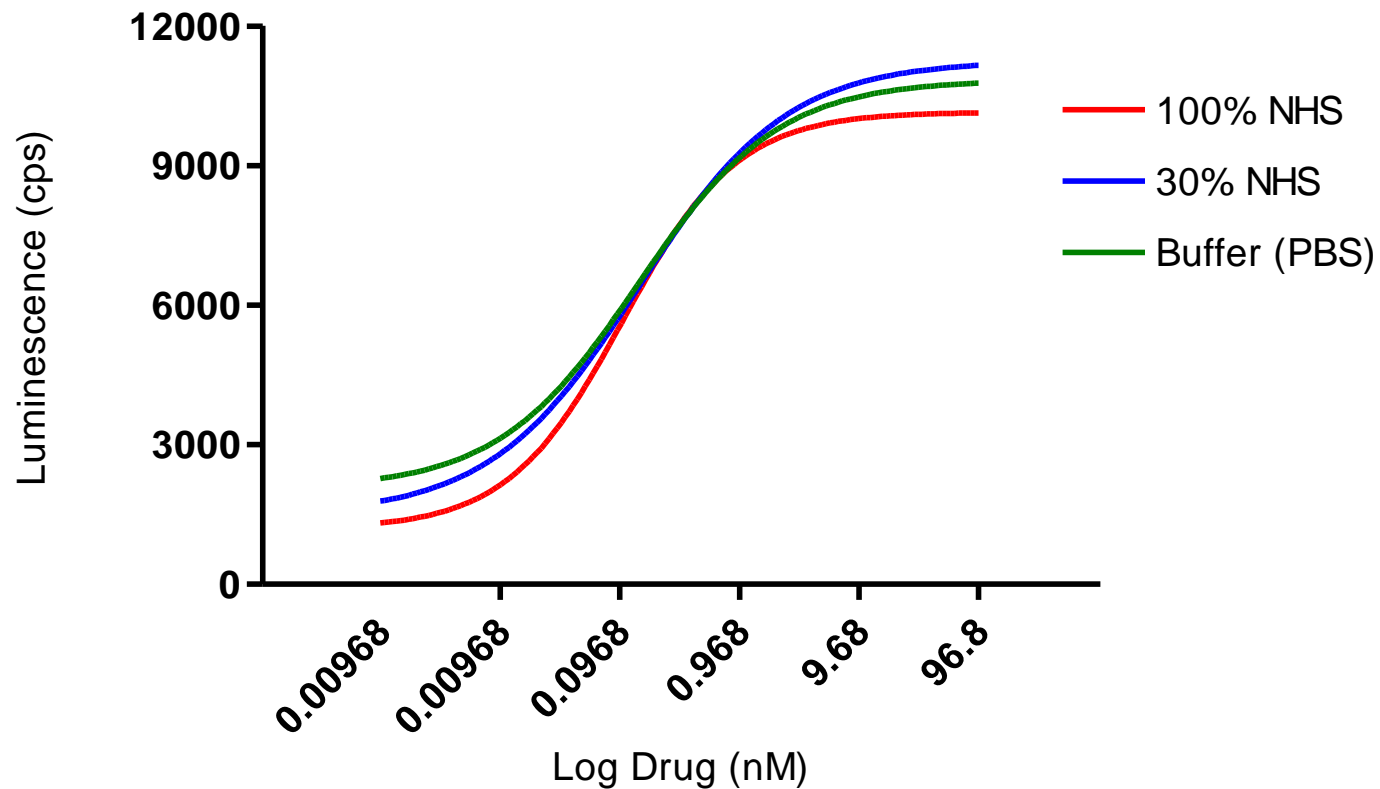
Specificity of Rare Reagents: Reference Ab

Specificity of the Analytical Reference					
Sample ID	EC50 (nM)	Pos/Neg	Sample ID	EC50 (nM)	Pos/Neg
LOW Unspiked	0.507	Pos	LOW Spiked with Relevant Substance X	0.381	Pos
LOW Unspiked	0.776	Pos	LOW Spiked with Relevant Substance X	0.388	Pos
LOW Unspiked	0.428	Pos	LOW Spiked with Relevant Substance X	0.387	Pos
LOW Unspiked	0.509	Pos	LOW Spiked with Relevant Substance X	0.415	Pos
LOW Unspiked	0.403	Pos	LOW Spiked with Relevant Substance Y	0.499	Pos
LOW Unspiked	0.440	Pos	LOW Spiked with Relevant Substance Y	0.393	Pos
			LOW Spiked with Relevant Substance Y	0.306	Pos
			LOW Spiked with Relevant Substance Y	0.432	Pos
			LOW Spiked with Relevant Substance Z	0.367	Pos
			LOW Spiked with Relevant Substance Z	0.421	Pos
			LOW Spiked with Relevant Substance Z	0.385	Pos
			LOW Spiked with Relevant Substance Z	0.447	Pos

Specificity of Rare Reagents: Cell Line



Specificity of Rare Reagents: Matrix



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Sensitivity: In Pooled and Individual Serum

Sample	EC50	Pos/Neg	Sample	EC50	Pos/Neg	Sample	EC50	Pos/Neg
Dilution: 1 to 1000	nM		Dilution: 1 to 1000	nM		Dilution: 1 to 2000	nM	
SENS/500	0.472	Pos	SENS/1000	0.256	Pos	SENS/2000	0.304	Pos
SENS/500	0.610	Pos	SENS/1000	0.263	Pos	SENS/2000	0.308	Pos
SENS/500	0.573	Pos	SENS/1000	0.292	Pos	SENS/2000	0.370	Pos
SENS/500	0.423	Pos	SENS/1000	0.251	Pos	SENS/2000	0.257	Neg
SENS/500	0.554	Pos	SENS/1000	0.283	Pos	SENS/2000	0.379	Pos
SENS/500	0.478	Pos	SENS/1000	0.261	Pos	SENS/2000	0.205	Neg
SENS/500	0.318	Pos	SENS/1000	0.181	Neg	SENS/2000	0.217	Neg
SENS/500	0.311	Pos	SENS/1000	0.204	Neg	SENS/2000	0.195	Neg
SENS/500	0.319	Pos	SENS/1000	0.178	Pos	SENS/2000	0.275	Pos
SENS/500	0.267	Pos	SENS/1000	0.188	Neg	SENS/2000	0.186	Neg
SENS/500	0.314	Pos	SENS/1000	0.197	Pos	SENS/2000	0.228	Neg
SENS/500	0.336	Pos	SENS/1000	0.216	Pos	SENS/2000	0.215	Neg
SENS/500	0.503	Pos	SENS/1000	0.188	Neg	SENS/2000	0.176	Neg
SENS/500	0.504	Pos	SENS/1000	0.180	Neg	SENS/2000	0.188	Neg
SENS/500	0.542	Pos	SENS/1000	0.200	Pos	SENS/2000	0.257	Pos
SENS/500	0.673	Pos	SENS/1000	0.242	Pos	SENS/2000	0.164	Neg
SENS/500	0.608	Pos	SENS/1000	0.208	Pos	SENS/2000	0.256	Pos
SENS/500	0.519	Pos	SENS/1000	0.208	Pos	SENS/2000	0.154	Neg
Positives (%)		100 (18/18)			72 (13/18)			39 (7/18)

Established Sensitivity: 2.0 ug/mL

Upper Limit for Negative Control

The mean response and standard deviation determined for the negative control analyzed in all accepted qualification runs were used to establish an upper limit for the NegC as follows:

$$= \text{meanNegC} + t_{0.01,df} \times \text{SDNegC}$$

Run ID	Sample	EC50 (nM)	Run ID	Sample	EC50 (nM)
1	NegC	0.182	7	NegC	0.123
1	NegC	0.153	7	NegC	0.152
2	NegC	0.107	7	NegC	0.166
2	NegC	0.128	7	NegC	0.152
2	NegC	0.105	8	NegC	0.112
2	NegC	0.140	8	NegC	0.155
2	NegC	0.105	8	NegC	0.165
2	NegC	0.121	8	NegC	0.211
2	NegC	0.172	8	NegC	0.165
3	NegC	0.0777	9	NegC	0.0596
3	NegC	0.104	9	NegC	0.0939
3	NegC	0.132	9	NegC	0.143
3	NegC	0.0832	9	NegC	0.130
3	NegC	0.0913	9	NegC	0.143
3	NegC	0.169	10	NegC	0.0596
3	NegC	0.258	10	NegC	0.0939
3	NegC	0.169	10	NegC	0.143
4	NegC	0.107	10	NegC	0.130
4	NegC	0.164	10	NegC	0.143
4	NegC	0.267	11	NegC	0.0822
4	NegC	0.236	11	NegC	0.0432
5	NegC	0.0851	12	NegC	0.0775
6	NegC	0.0571	12	NegC	0.0969
7	NegC	0.136	12	NegC	0.0908
7	NegC	0.184	13	NegC	0.107
7	NegC	0.0918			
Mean					0.131
SD					0.0486
Upper Limit for NegC					0.247

Drug Tolerance

Drug Tolerance								
Sample ID	EC50 (nM)	% Change from LOW/unspiked	Sample ID	EC50 (nM)	% Change from MID/unspiked	Sample ID	EC50 (nM)	% Change from HIGH/unspiked
LOW/unspiked	0.457	0	MID/unspiked	2.00	0	HIGH/unspiked	9.47	0
LOW/0.864 ng/mL	0.449	-2	MID/0.864 ng/mL	2.80	29	HIGH/0.864 ng/mL	11.0	14
LOW/1.464 ng/mL	0.506	10	MID/1.464 ng/mL	2.91	31	HIGH/1.464 ng/mL	10.5	9
LOW/2.064 ng/mL	0.476	4	MID/2.064 ng/mL	1.57	-27	HIGH/2.064 ng/mL	9.26	-2
LOW/2.664 ng/mL	0.373	-22	MID/2.664 ng/mL	2.23	10	HIGH/2.664 ng/mL	8.09	-17
LOW/3.264 ng/mL	0.495	8	MID/3.264 ng/mL	1.83	-9	HIGH/3.264ng/mL	11.5	18
AVG	0.459		AVG	2.22		AVG	9.97	
STDEV	0.047		STDEV	0.538		STDEV	1.27	
CV (%)	10		CV (%)	24		CV (%)	13	

Assay tolerance of drug level: approximately 3.26 ng/mL

Short Term- and Freeze/Thaw Stability

Requirement for stability: difference from reference is $\leq -20\%$.

Treatment	% Difference from Reference Samples
3.5 h at Room Temperature	-5%
2 Freeze/Thaw Cycles	Cycle 1: 14% Cycle 2: 17%

Conclusion

A functional method for determination of neutralizing antibodies against a target drug in human serum, using stably transfected HEK 293 cells was successfully qualified

Development and Optimization Time 1.5 months

Qualification Time: 1 month

Number of Qualification Runs: 14 runs (51 plates)

Analysts: 1

Acknowledgements

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