Cellular High Throughput Screening assays using HTRF detection of cytokines

HTRF in Drug Discovery Symposium

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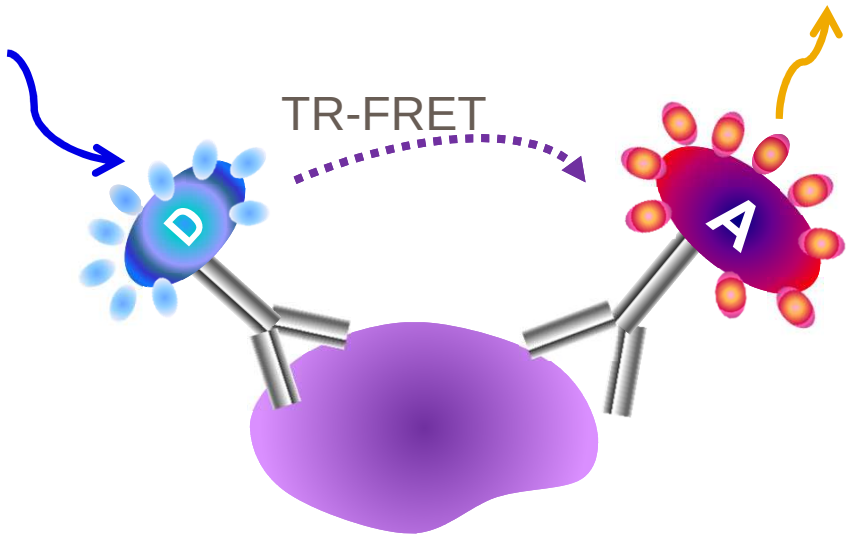
Lorena Kallal

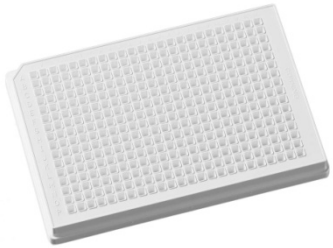
GlaxoSmithKline, Collegeville, PA USA



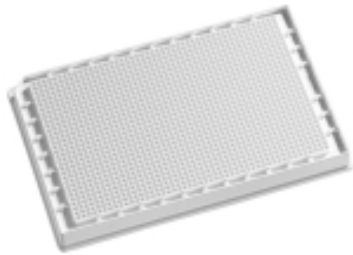
GlaxoSmithKline, Upper Providence (Collegeville), Pennsylvania, USA

- Assay development and screening environment
- 384 and 1536 well cell based assays

384 well



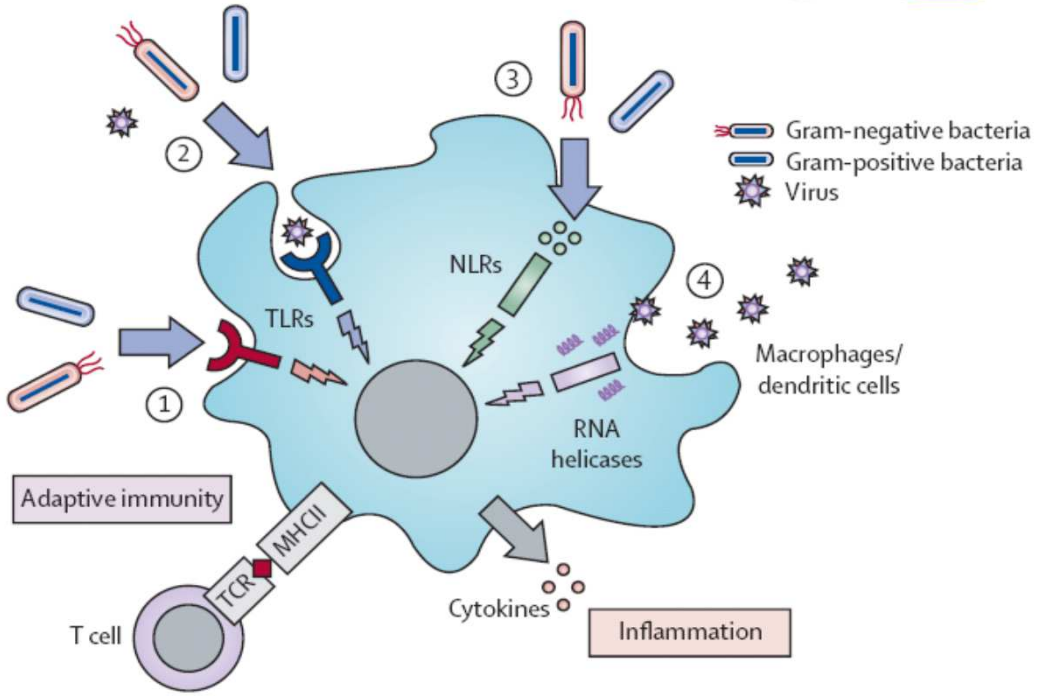
1536 well



Pattern Recognition Receptors: immunity

NOD, Toll, TLR, NLRPs, etc.

Activated by pathogenic & endogenous danger signals



Stimulate:

–Cytokine production

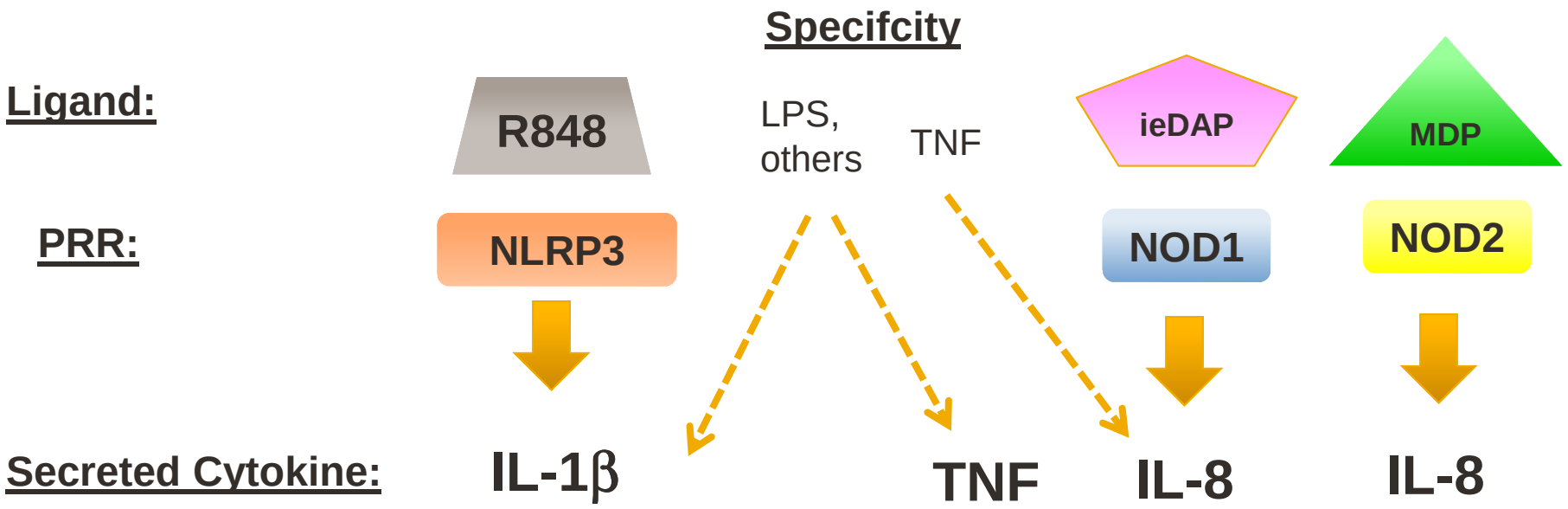
–inflammatory signal transduction cascades

Therapeutics:
inflammatory diseases

Bochud et al., 2007, Lancet Infect Dis 7:531

HTRF assays for the detection of cytokines

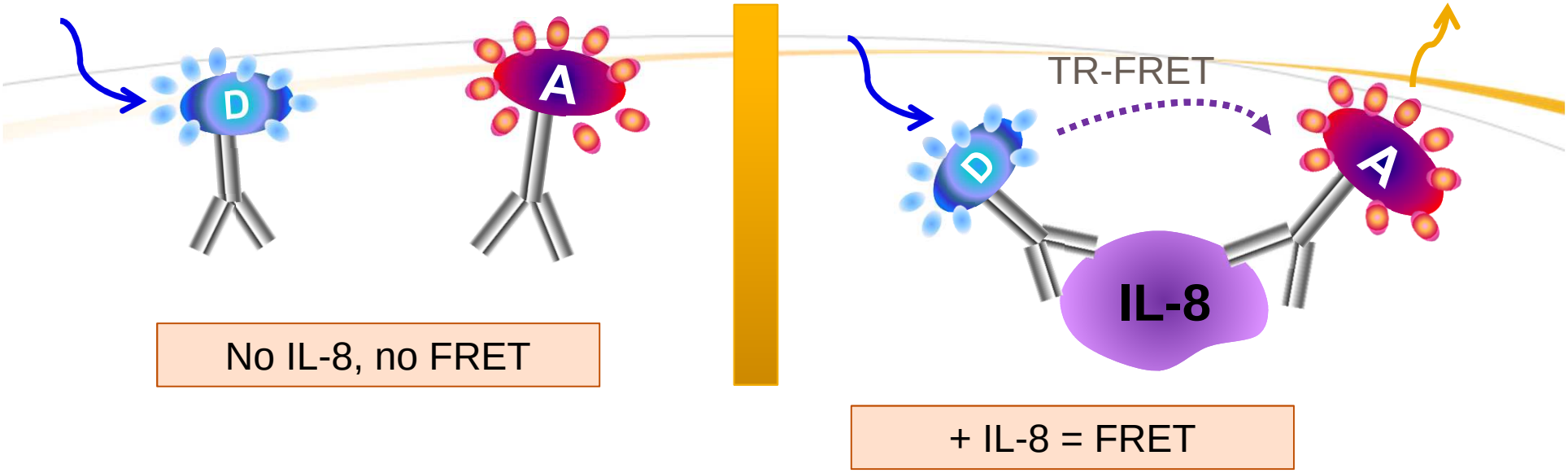
- Interested in NOD1, NOD2, and NLRP3 signaling
- Pathway specificity: different activators, different cytokines



Cell system: PMA differentiated THP1 cells:

Engineered HEK cells, stably expressing NOD1 or NOD2:

Assay development strategy: NOD1 and NOD2 HTSs:



- Goal: Establish 1536-well homogeneous cell based assays
- Also: Will assay-ready frozen cells work for the assay?

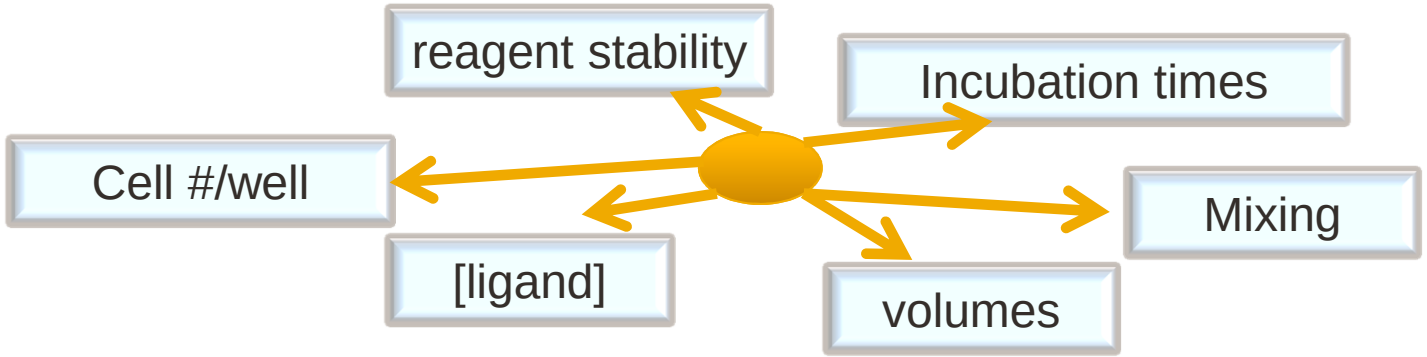
Assay development for high throughput screen (HTS):

HTS: ~2 M compounds; @10 µM — 1536 well plates

XC50 curves; primary assay + specificity assay — 384 well plates

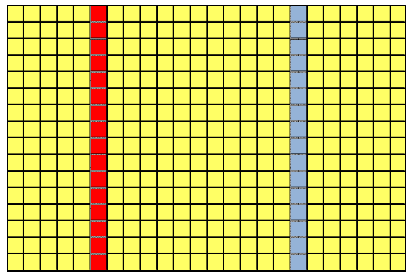
Other specificity assays and downstream assays → tox, off target…

- Test many conditions during assay development: will share only examples

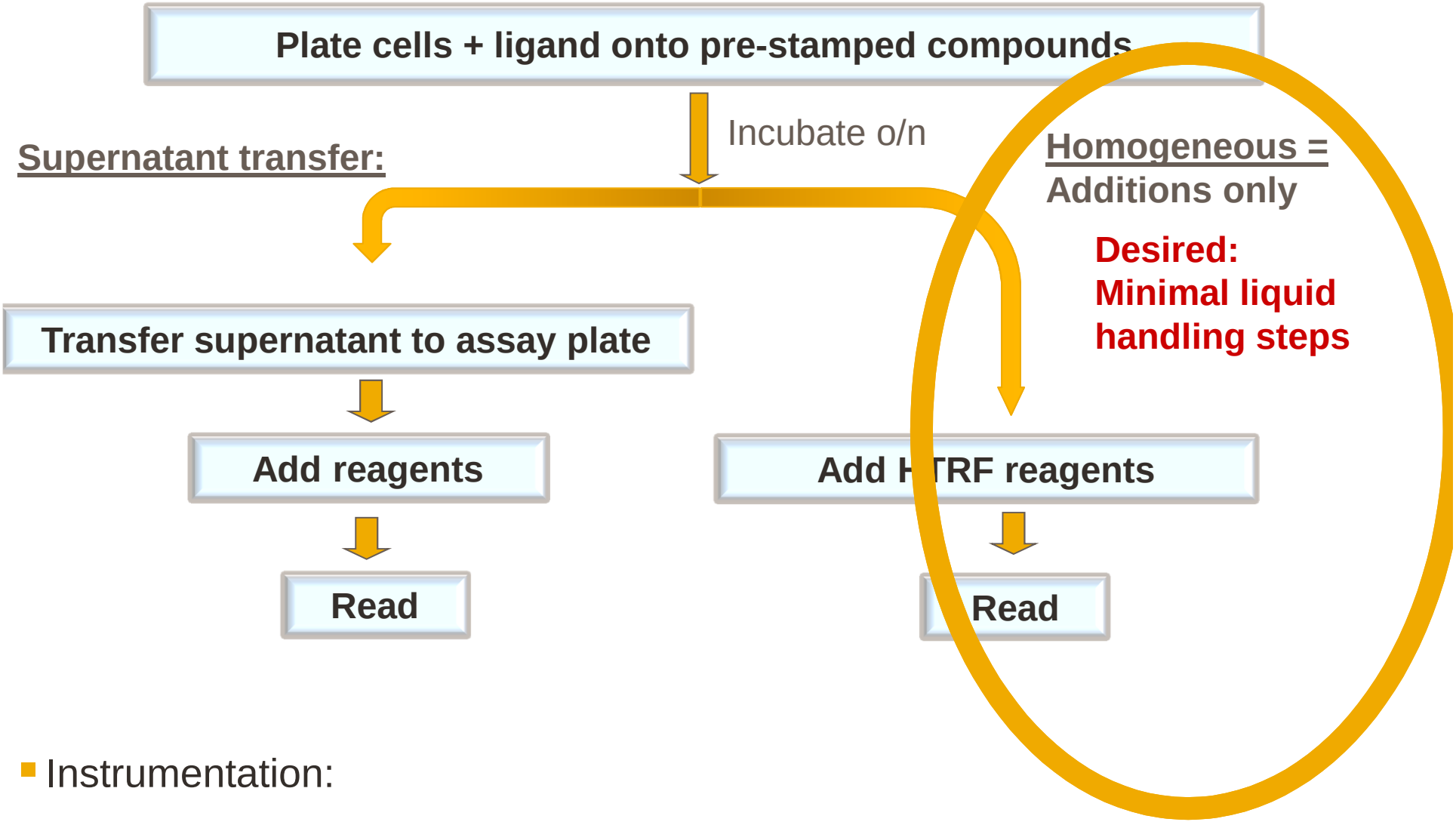


- **Statistics**
  - Signal / background (**S/B**)
  - **Z' gives "variability": want ≥ 0.4; 1 = perfect**

Control columns

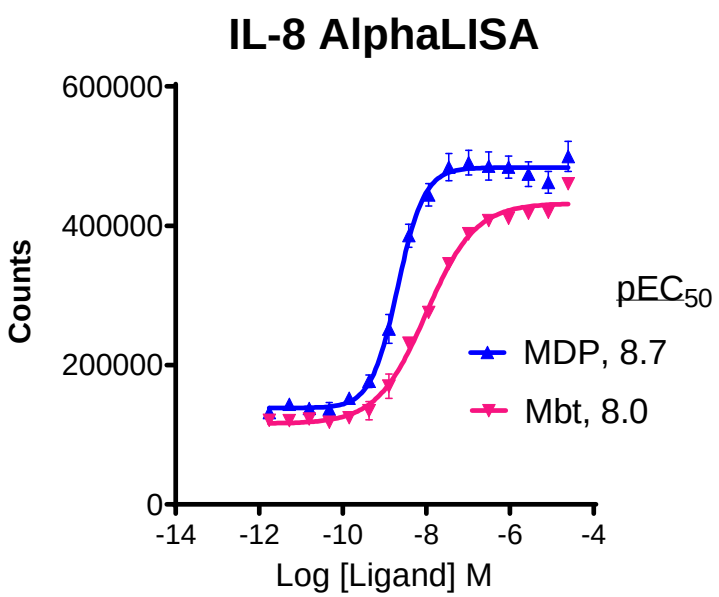


Flow chart: supernatant transfer vs homogeneous assays:

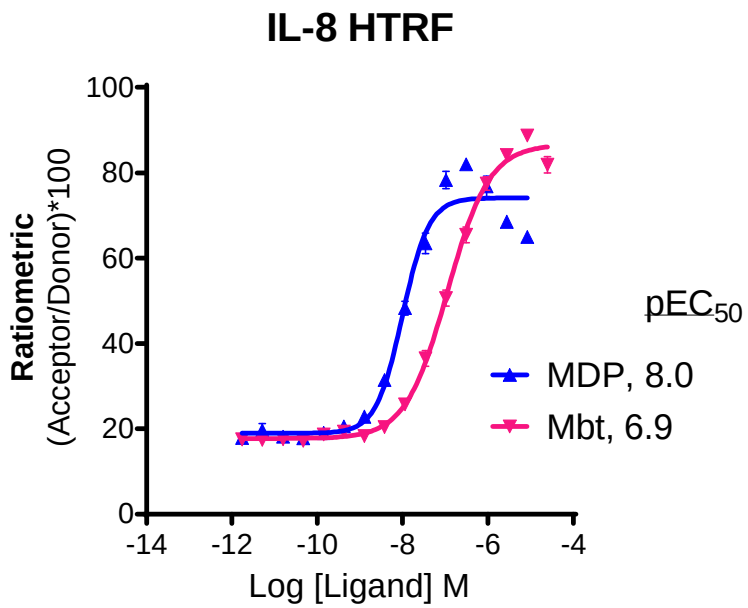


- Instrumentation:
  - Envision: PMT-based, sensitive, ~ 7 – 35 min per plate
  - Viewlux: CCD camera based, less sensitive, ~ 2 - 4 min per plate); ☺

NOD2 IL-8: AlphaLISA (Perkin Elmer) vs HTRF comparison



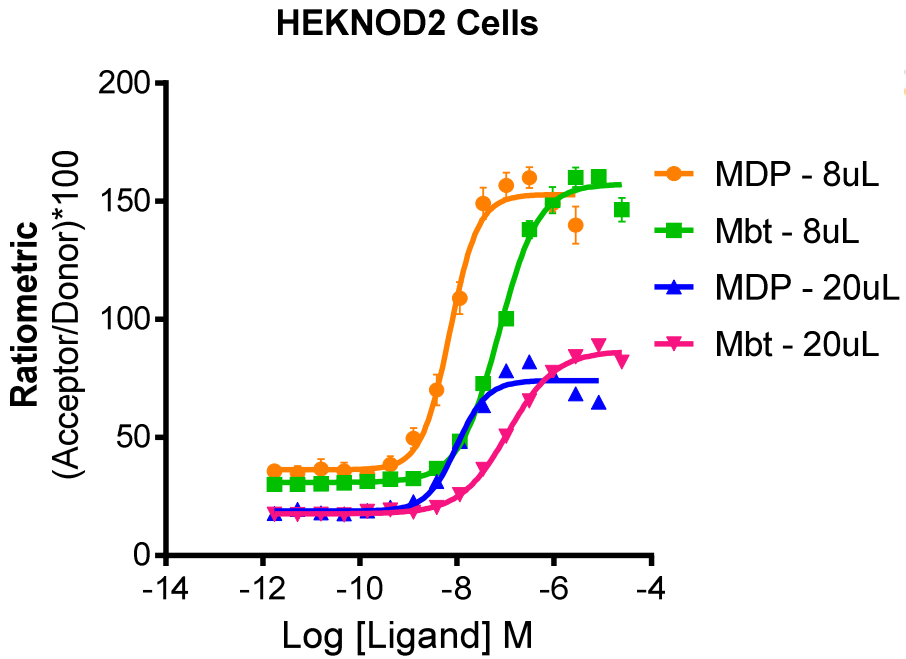
Ligand	S/B	Z'
MDP	2.3	0.45
MBt	2.2	0.33



Ligand	S/B	Z'
MDP	2.9	0.56
MBt	2.9	0.57

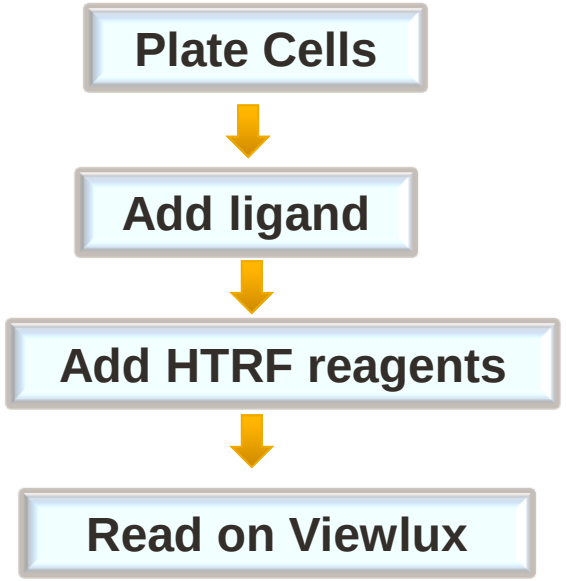
- AlphaLISA :higher pEC_{50}s, lower Z'
  - more steps, reagents less stable
    - Envision: longer read time (7 vs. 2 min on a Viewlux)
- **HTRF assay was chosen for screening**

NOD2 HTRF Assay: miniaturization: 8µL vs. 20µL (low vol 384):



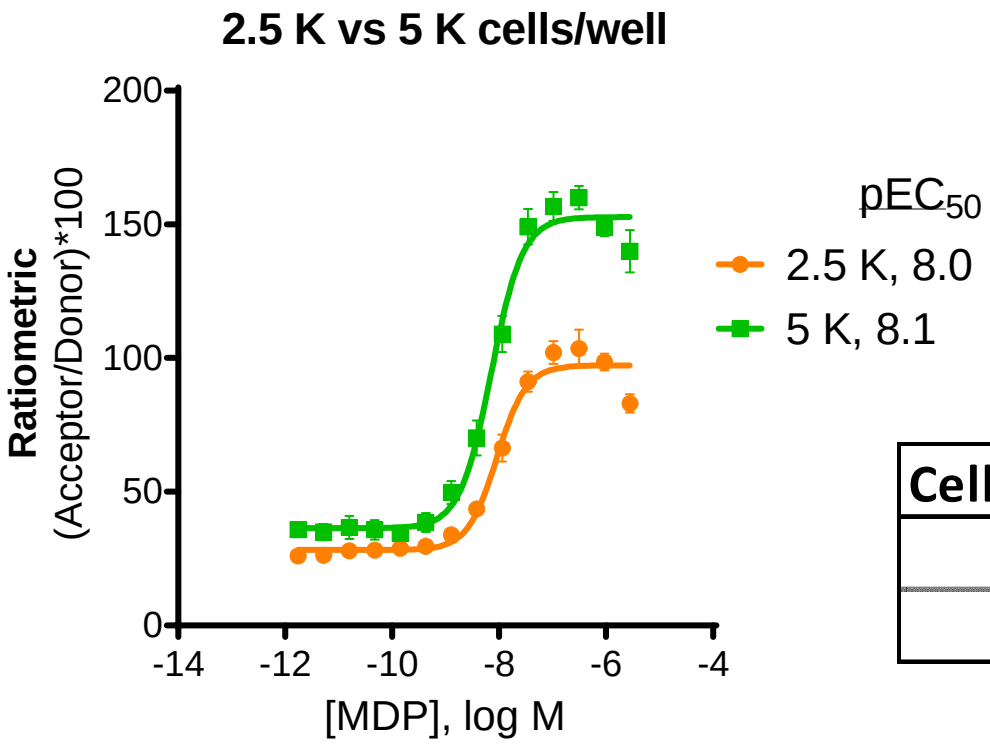
ligand	volume	S/B	Z' (16/16)
MDP	8	3.3	0.58
MDP	8	3.4	0.55
Mbt	20	2.9	0.56
Mbt	20	2.9	0.57

Assay flow chart:
"homogeneous"



- S/B higher in 8 µL than 20 uL assay
- EC_{50}s and Z' comparable → low vol assay OK → good for 1536

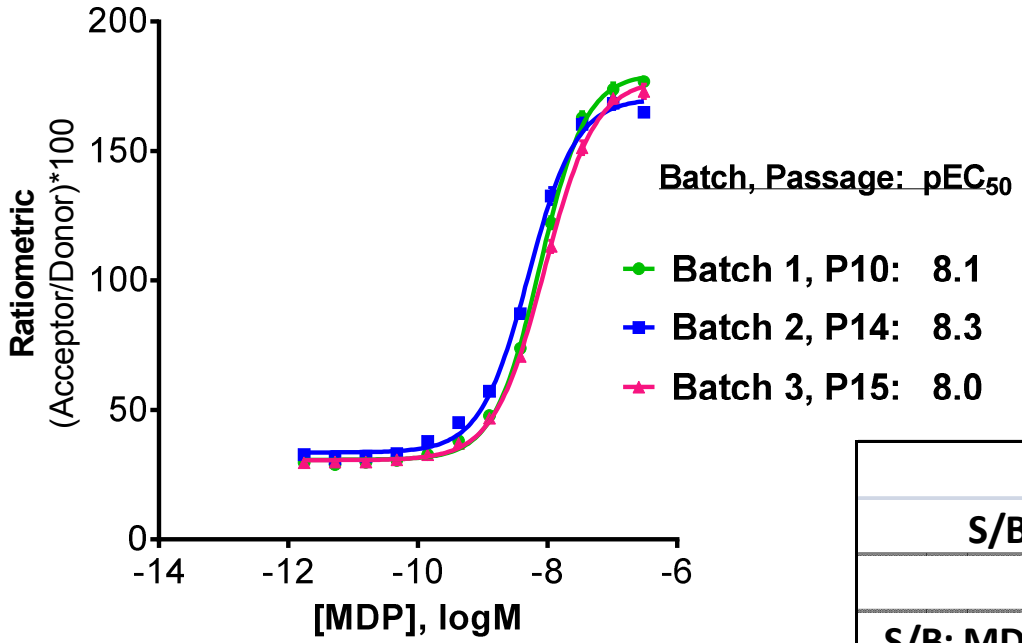
NOD2 Cell Density (#cells/well) optimization :



Cells/well	S/B	Z' (16/16)
2.5 K	2.6	0.45
5 K	3.3	0.58

- 5 K per well gave slightly higher S/B and Z' values than 2.5 K

NOD2: Assay-Ready Frozen cells were acceptable:

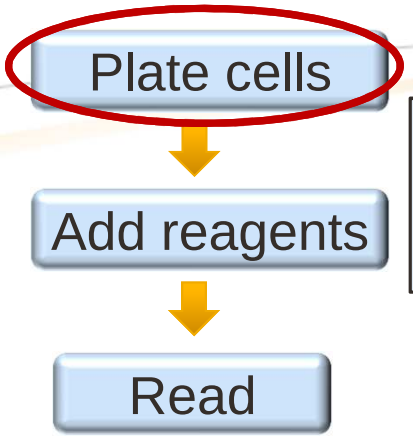


S/B and Z' statistics:

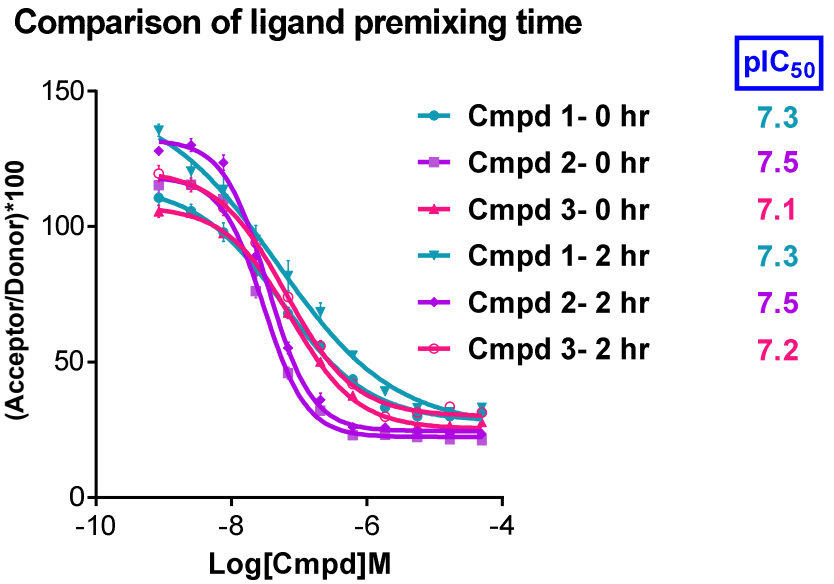
	Batch 1	Batch 2	Batch 3
S/B: MDP / DMSO	4.4	4.3	4.2
Z'	0.60	0.75	0.59
S/B: MDP / MDP + blocker 1	4.6	4.3	4.5
Z'	0.64	0.74	0.62
S/B: MDP / MDP + blocker 2	6.3	6	5.6
Z'	0.66	0.78	0.64

- Similar and consistent responses were obtained with assay-ready frozen cells

Addressing cell plating lag time:



Plating hundreds of plates takes time: is blocker potency affected?

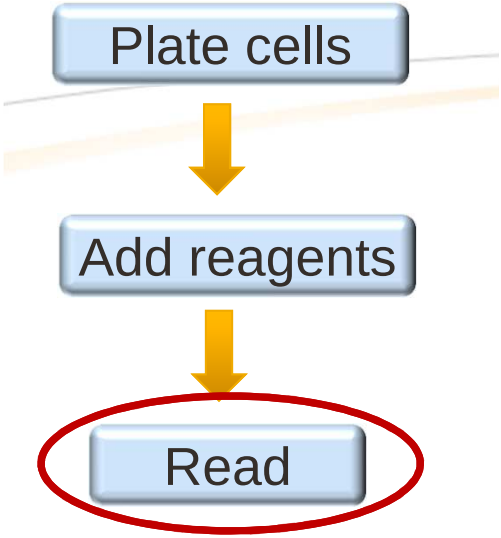


Plating time				
30nM EC80	0 hr		2hr	
	S/B	Z'	S/B	Z'
DMSO	4.0	0.72	4.5	0.73
Blocker 1	3.9	0.71	4.1	0.74
Blocker 2	4.8	0.74	4.8	0.76
Blocker 3	3.8	0.70	3.4	0.70

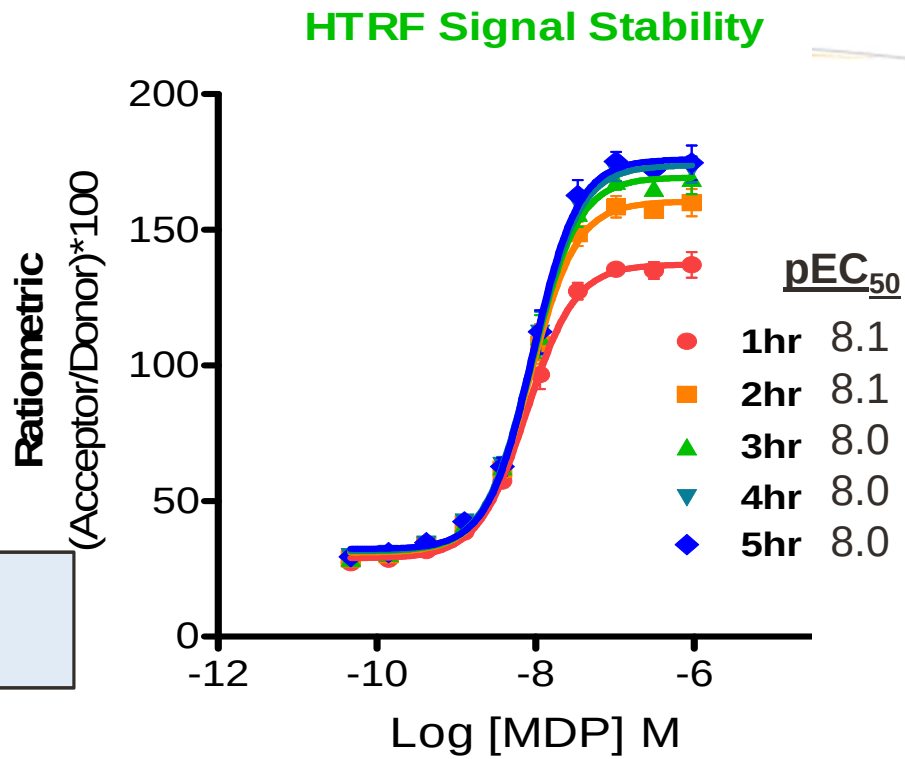
Low volume 384

- Blocker potencies did not change with cell pre-exposure to ligand for 2 hr

Addressing plate reading lag time:



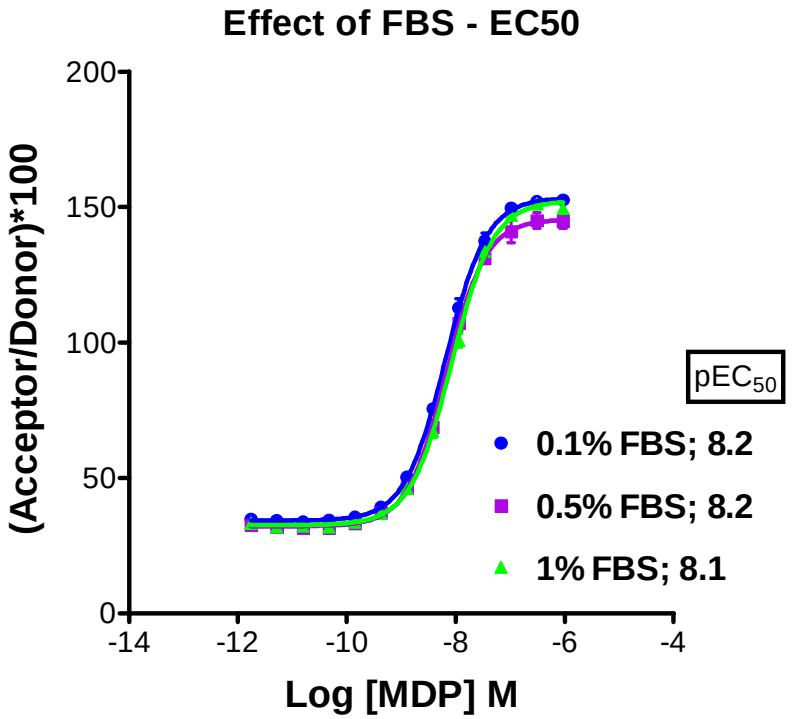
Reading hundreds of plates takes time

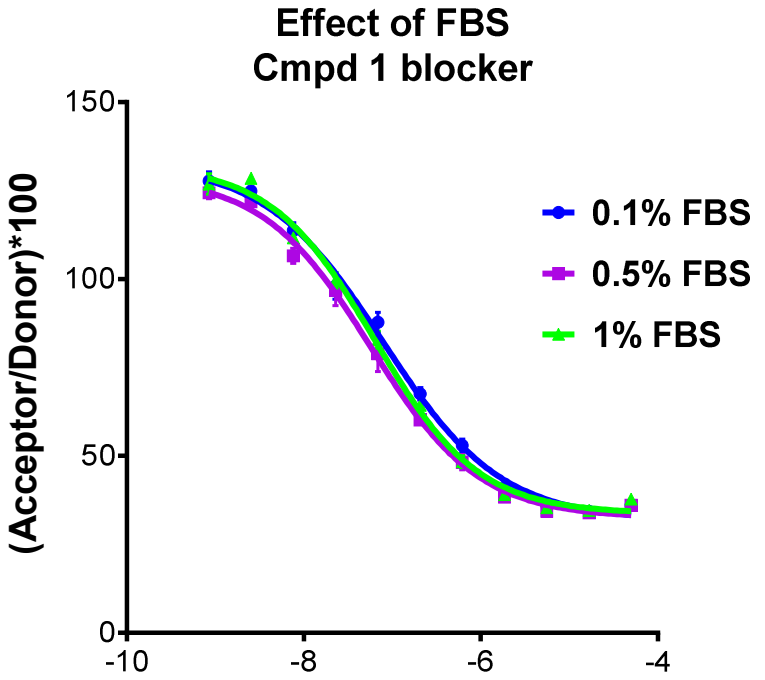


TIME (hr)	Z'
1	0.66
1	0.55
2	0.66
2	0.56
3	0.64
3	0.55
4	0.63
4	0.55
5	0.64
5	0.55

- The HTRF signal was stable 1-5 hrs after reagent addition

Low serum had no effect on the NOD2 IL-8 assay:



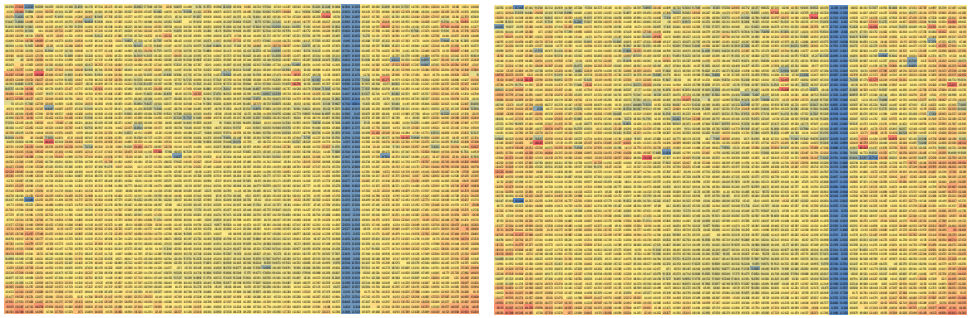


FBS:	0.1%	0.5%	1%
S/B	3.5	3.4	3.5
Z'	0.63	0.62	0.67

- Lowering the serum: no effect on MDP potency or compound blocker pIC_{50}

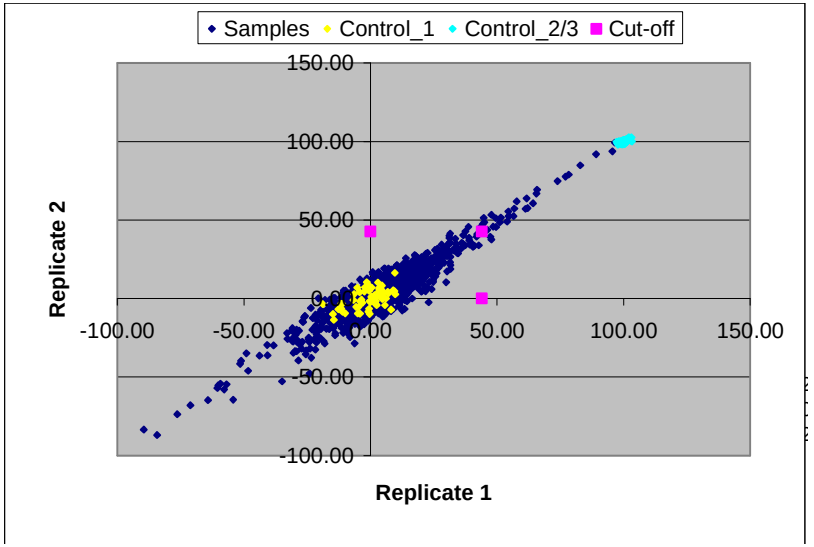
NOD2 assay statistics: small compound set testing: “robustness”:

2 replicates



	rep 1	DMSO 1	rep 2	DMSO 2
S/B	4.4	4.2	4.5	4.2
Z'	0.77	0.72	0.79	0.72

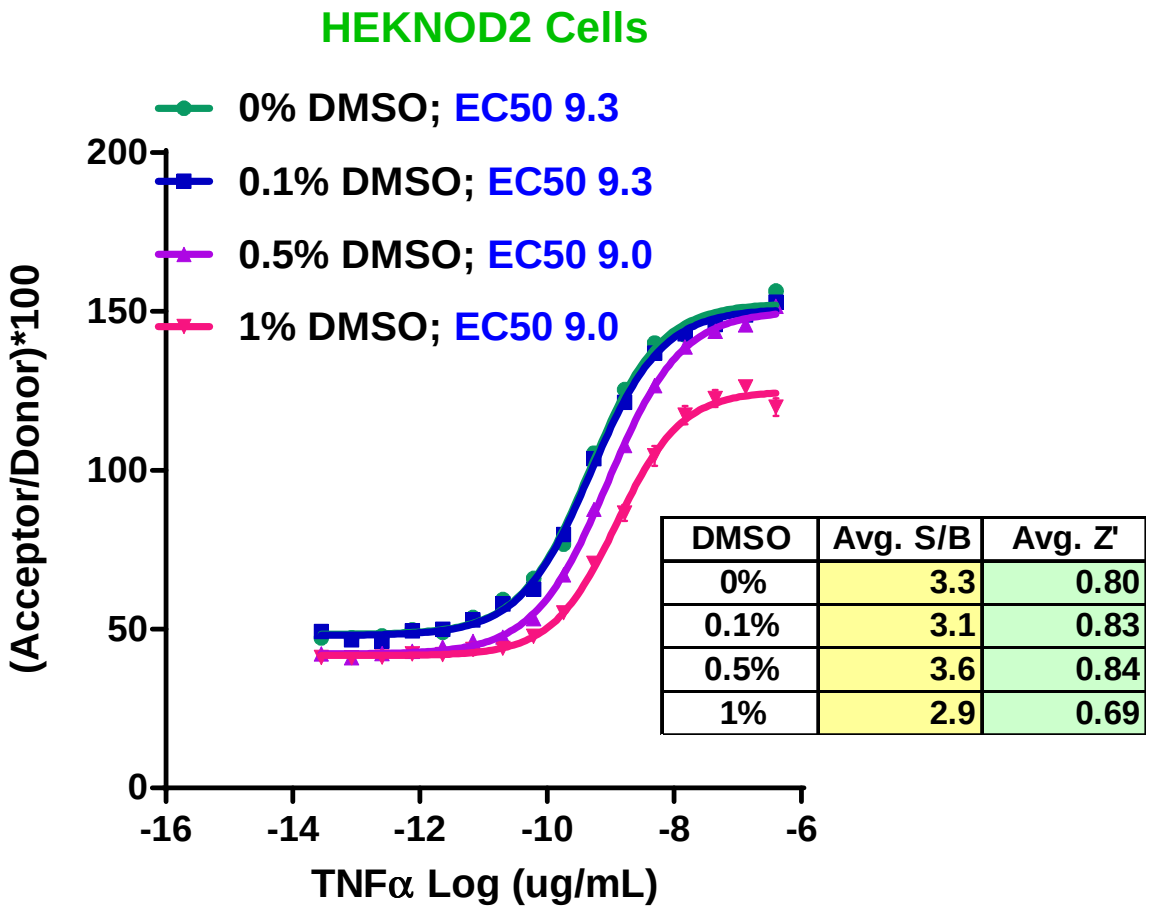
Figure 4. Correlation Plot



Replicate	Number of samples	% Inh Cut-off	N hits	HR(%)	Correlation Coeff.
1	1408	44	34	2.4	0.93
2		43	34	2.4	

- Random compound set of 1408 compounds used to assess statistics
  - Excellent for a cell based assay (Z’, hit rate, correlation)

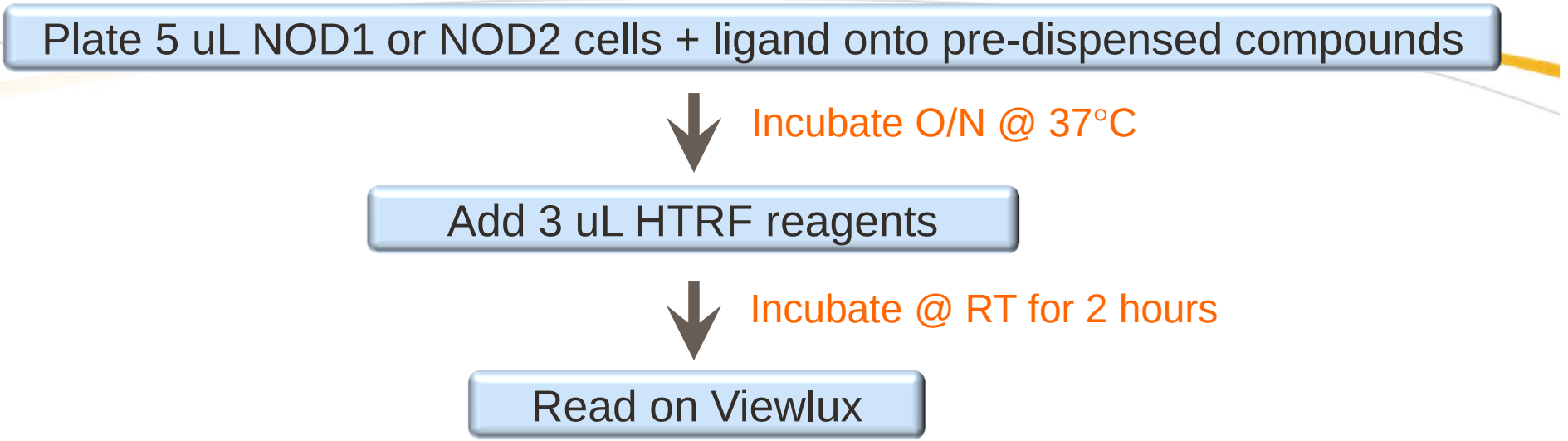
NOD2 Specificity Assay; TNFα Stimulation of IL-8:



DMSO	Avg. S/B	Avg. Z'
0%	3.3	0.80
0.1%	3.1	0.83
0.5%	3.6	0.84
1%	2.9	0.69

- TNFα-driven assay = similar responses/stats as the MDP NOD ligand

NOD1,2 IL-8 HTRF Homogenous Protocol (low vol. 384 or 1536):



- Simple protocol, amenable for HTS, screening 2M compounds

Summary of NOD1, NOD2 work:

- HTRF assays for IL-8 performed better than the AlphaLISA assay (Z')
- HTRF assays performed well in 1536 well format: excellent cellular assay Z'
- Assay-ready frozen cells for HTSs = no need for continuous culture

HTS 1.9 M

↓

4000 XC_{50}s

↓

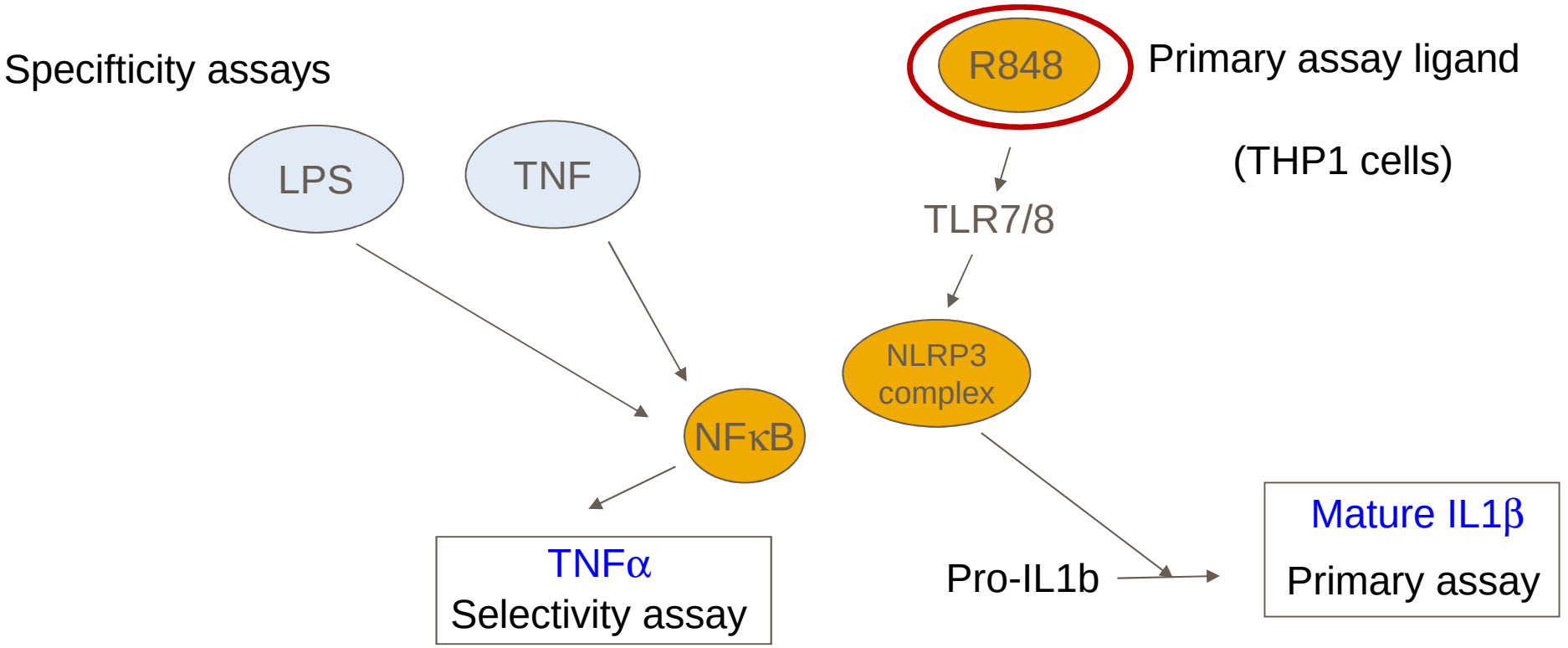
Other specificity assays

↓

= 1 NOD2 specific cmpd

GSK Paper submitted

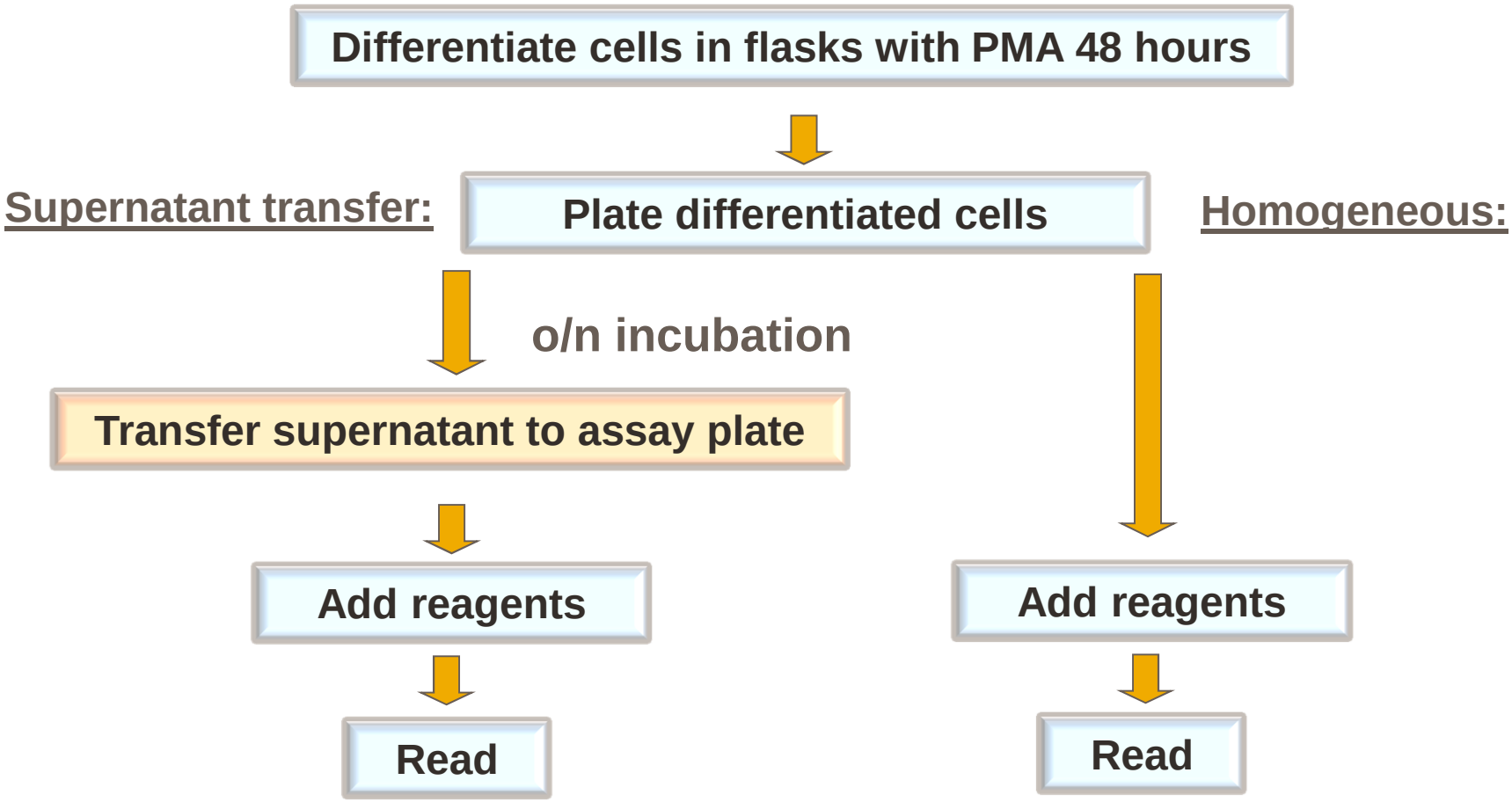
NLRP3 primary and specificity cellular assays:



IL-1β and TNFα assays:

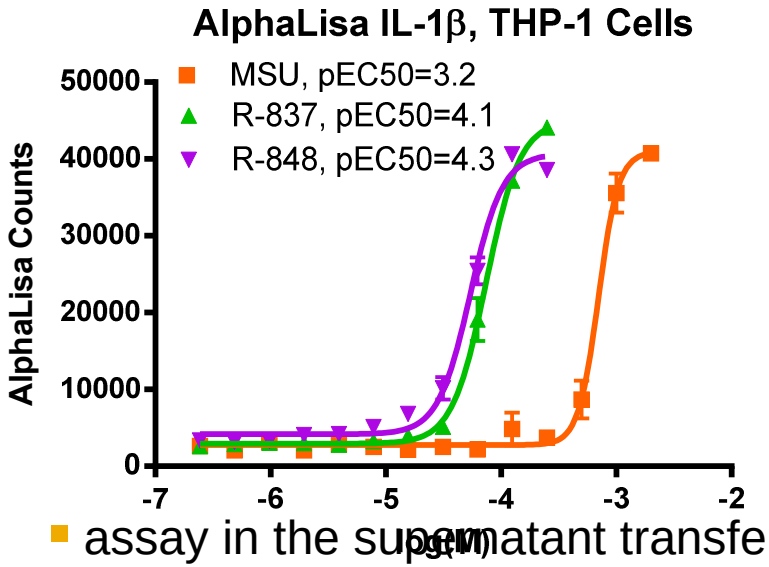
- HTRF (Cisbio) and AlphaLISA (Perkin Elmer) kits were available for TNFα
- HTRF for IL-1β, yes; **AlphaLISA: no. Only pre-market test reagents (PE)**

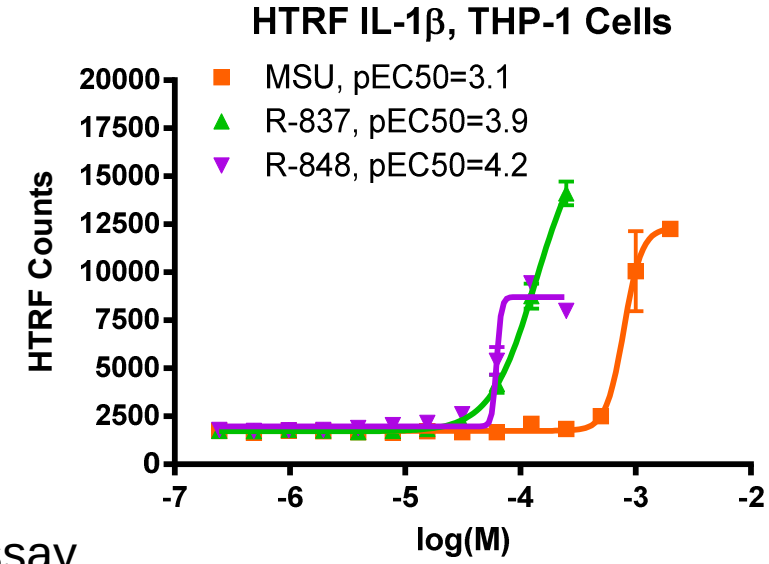
Flow chart for NLRP3 assays: supernatant vs homogeneous assays:



- Time on Envision instrument to read a plate: ~7 – 35 min
- Time on Viewlux instrument to read a plate: ~2 – 4 min

IL-1β : supernatant assay: both AlphaLISA and HTRF OK:





- assay in the supernatant transfer assay

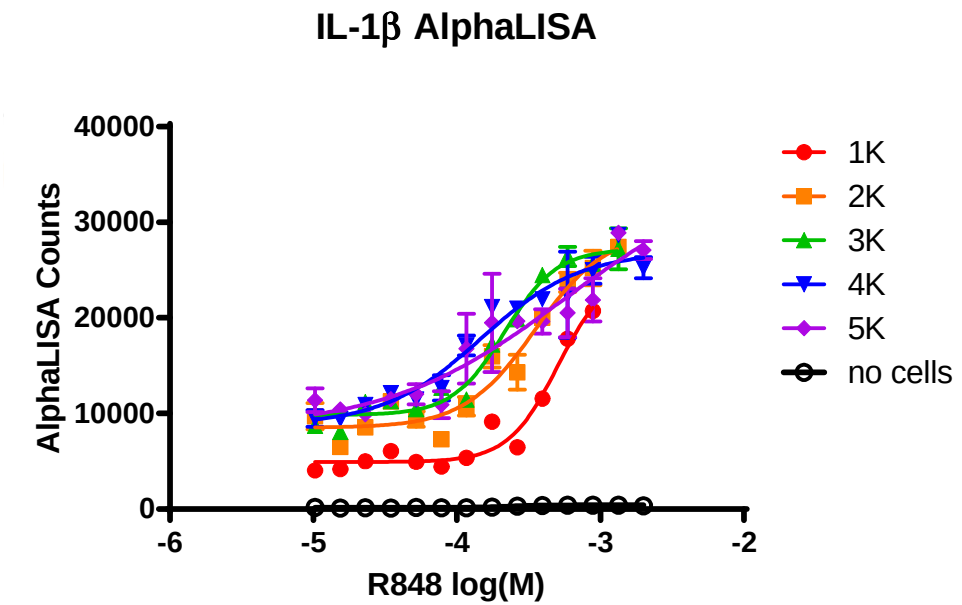
Single concentration stats:

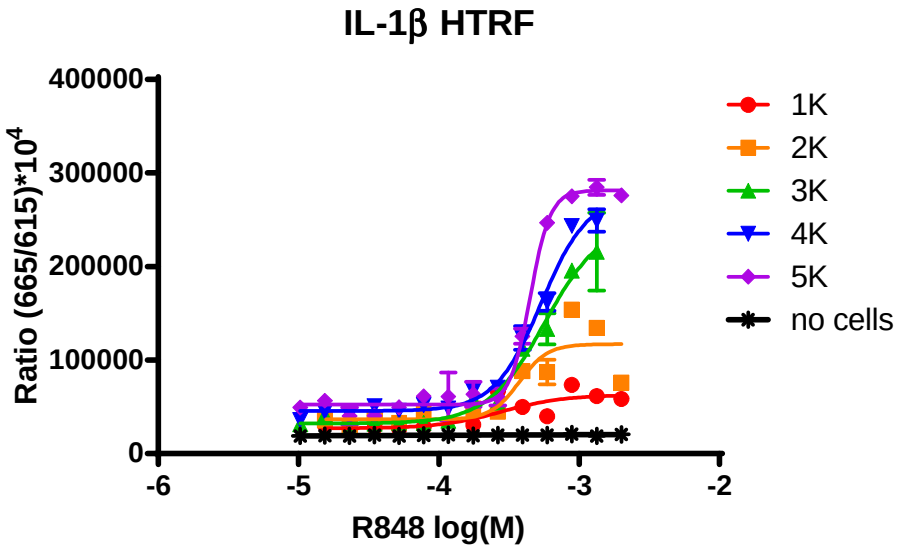
AlphaLISA (n=2)				
Cells	Ligand	pEC50	Z'	S/B
THP-1	MSU	3.4	0.7	14
	R837	3.7	0.6	16
	R848	4.2	0.7	14

HTRF (n=2)				
Cells	Ligand	pEC50	Z'	S/B
THP-1	MSU	3.2	0.8	10
	R837	3.5	0.7	10
	R848	4.0	0.7	7

- Both kits OK for IL-1β in a supernatant transfer assay

IL-1β homogeneuous: HTRF performed better:





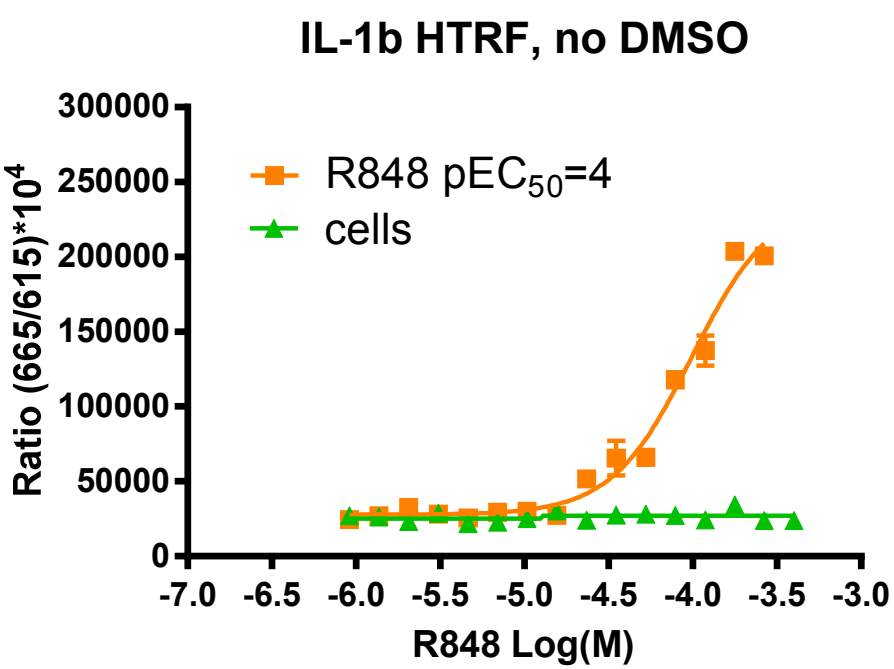
A-LISA	
Cells/well	**S/B**
1K	5
2K	5
3K	4
4K	3
5K	3

HTRF	
Cells/well	**S/B**
1K	2
2K	3
3K	8
4K	6
5K	5

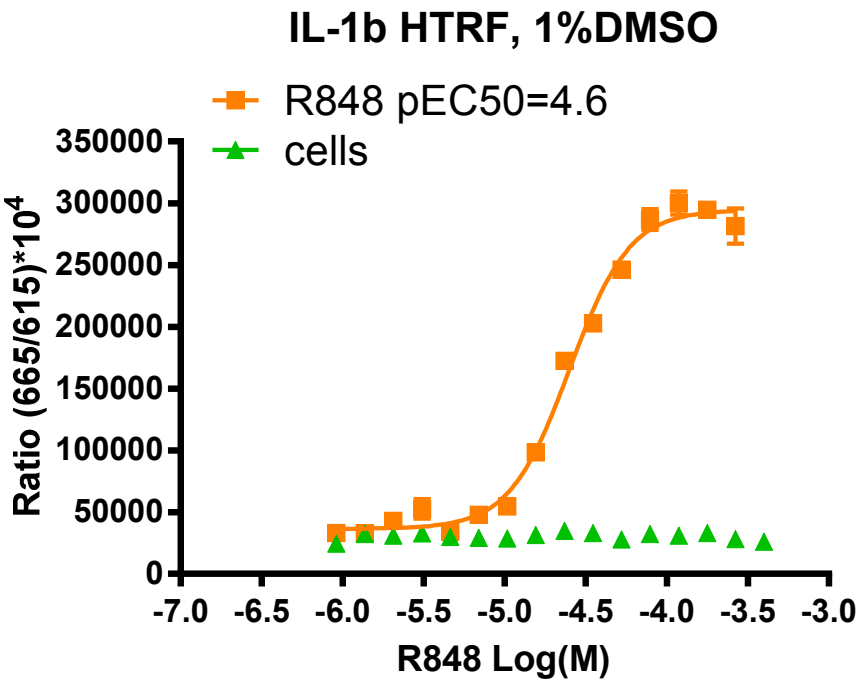
- IL-1β AlphaLISA did not perform as well as HTRF in homogeneous format

IL-1β: DMSO helped the R848 ligand:

No additional DMSO

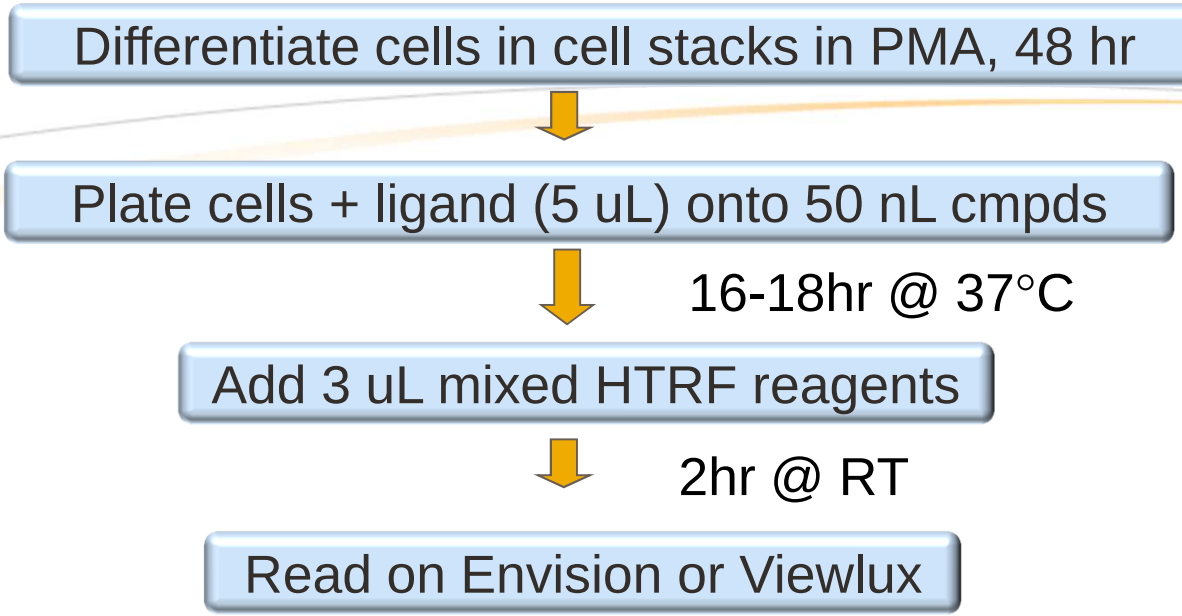


1% DMSO



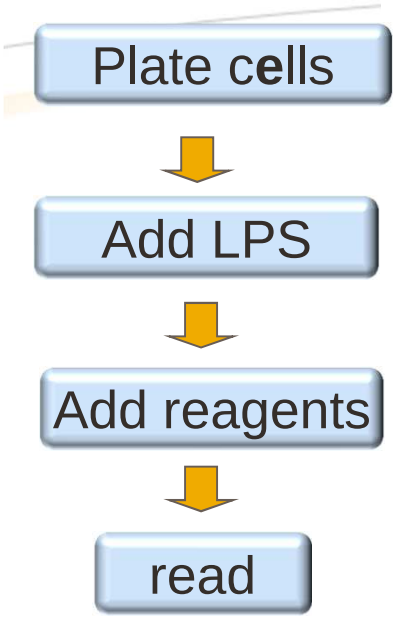
- DMSO increased the R848 potency by approximately 0.5 log

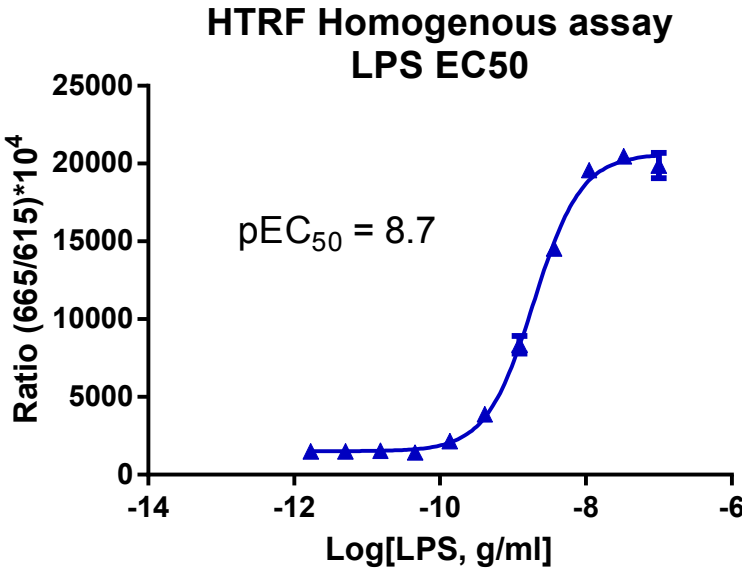
Homogeneous HTRF assay protocol for Profiling and Screening:

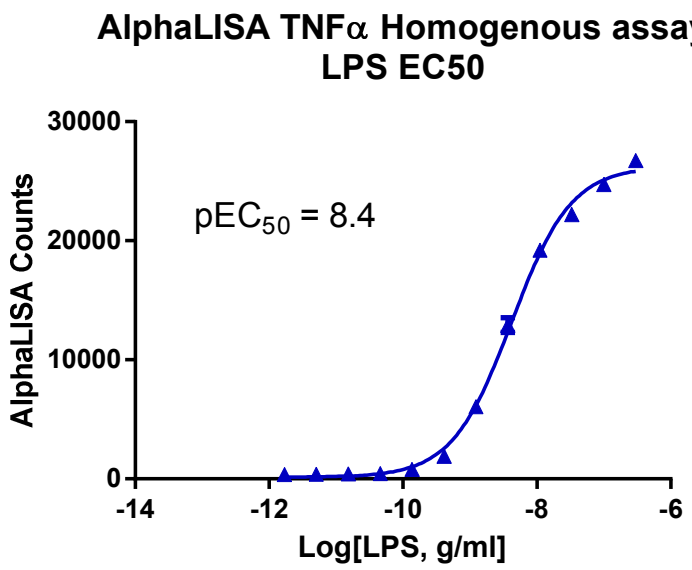


- Cellular model: PMA differentiated THP1 cells
- With much effort, frozen cells did not respond well: fresh cells for HTS

NLRP3 Specificity assay; TNFα AlphaLISA vs. HTRF





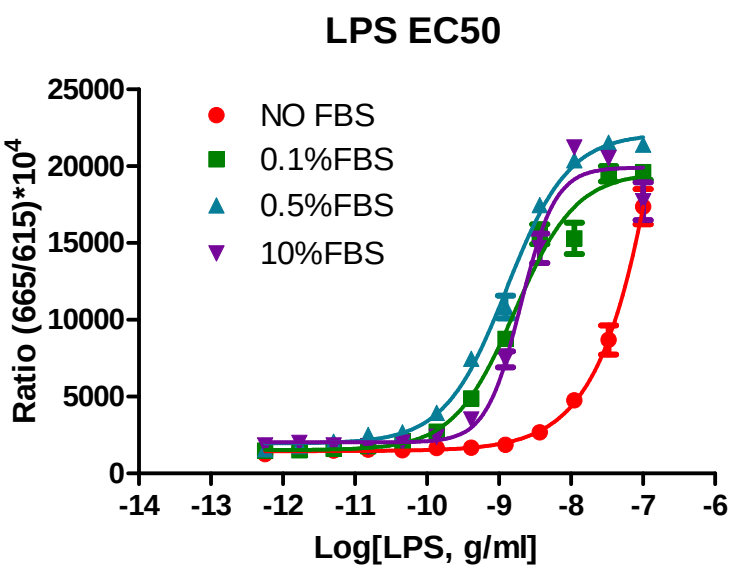


	Z'	S/B
AlphaLISA	**0.88**	**56**
HTRF	**0.69**	**9**

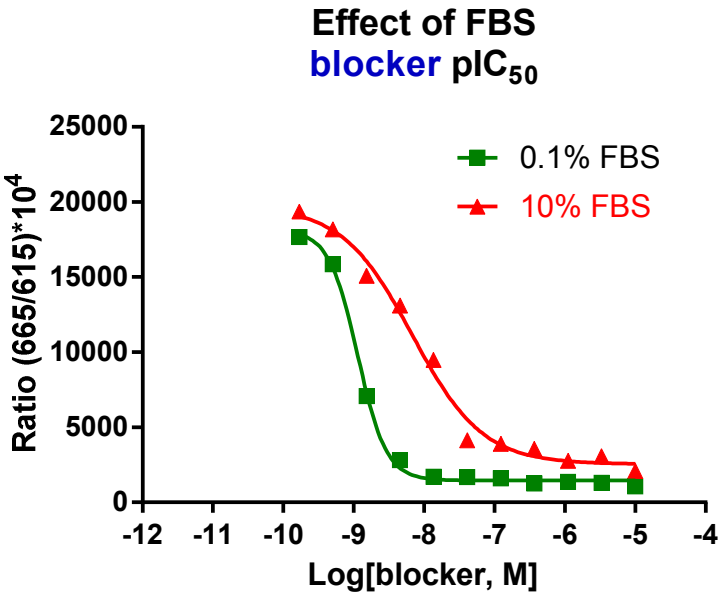
- For TNFα, the AlphaLISA performed better than HTRF
  - Higher Z', S/B
- HTRF statistics were acceptable
  - Used HTRF to match the primary screening assay

FBS effect on blocker potency:TNF example:

Ligand:



Inhibitor:



- LPS ligand response: required at least 0.1% FBS
- Compound blocker lost potency in 10% FBS
  - **common observation: reason to use reduced FBS in cellular assays**

Summary:

- HTRF assays for cytokines performed well in homogeneous format
  - 384 or 1536 well formats, excellent statistics
  - Have run numerous full HTS campaigns using HTRF for cellular assays

- Comparisons to AlphaLISA; in some cases performed better
  - In the assays shown, HTRF was better than AlphaLISA for IL-1β
  - AlphaLISA better than HTRF for TNFα

- Ease of use was better for HTRF than for AlphaLisa in our hands
  - HTRF reagents less light sensitive than AlphaLISA
  - Envision read times were longer than Viewlux
    - AlphaLISA cannot be read on a Viewlux

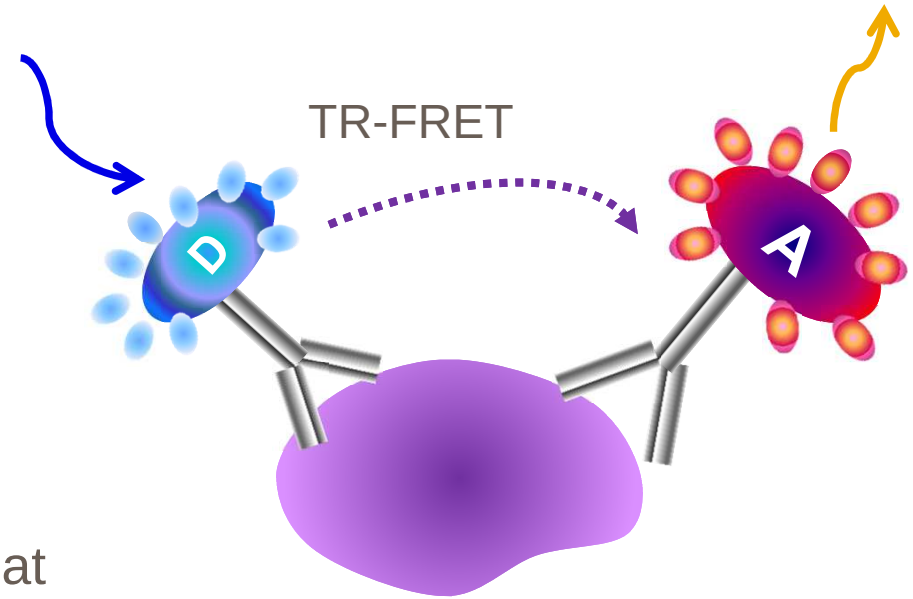
- Not all cells could be adapted to an “assay-ready” frozen cell format

Currently:

- Using HTRF for the detection of an intracellular nuclear protein

- Using dual acceptor approach (one donor fluor, 2 acceptor fluors)

About **HTRF** ……..



Has **T**he **R**ight homogeneous **F**ormat

Helps you **T**o **R**un assays **F**ast

Cisbio **H**elps **T**est new **R**eagents to **F**ind new assays with custom labeling

Scientists are **H**appy **T**o **R**un these **F**RET assays…… (OK maybe not….)

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