HTRF®

A versatile approach for 7TM drug discovery

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Bioassays
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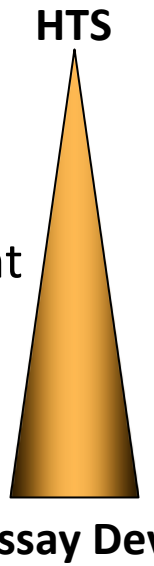
Outline

- Medical Research Council Technology, Centre for Therapeutics Discovery

1. Utilising HTRF® assays in an HTS environment
2. HTRF® Tag-lite® technology and secondary assay development
3. Investigational studies for receptor-protein interactions



- Conclusions and future perspectives

Applications of HTRF® to Assay Development/Screening/Profiling

- ***Establishing relevant HTRF assays at different points in an assay cascade***

- Historically have successfully applied HTRF to kinase programmes
- Increased number of 7TM and other receptor targets in our portfolio
- Receptor-relevant HTRF assays specifically for 7TM/GPCR (tyrosine kinase)
- Highlights of HTRF applications

MRC T

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- MRC Technology Centre for Therapeutics Discovery
- **Utilising HTRF® assays in an HTS environment**
- HTRF® Tag-lite® technology and secondary assay development
- Investigational studies for receptor-protein interactions
- Conclusions and future perspectives

Homogenous Time Resolved FRET (HTRF®): A versatile HTS tool

- Flexibility
- Sensitivity
- Throughput
- Low interference
- TR-FRET (lanthanide chemistry)
- Signal stability
- Ratiometric data transformation (correction)

- Stable XC50 (hours-days)
- Low volume assay
- Miniutarisation/Automation-friendly
- Fresh or frozen cells

HTRF® cAMP Assay - dynamic 2 Kit (Eu^{3+} Cryptate):

Melanocortin Receptor 3 (MC3) HTS: Positive Allosteric Modulators (PAM)

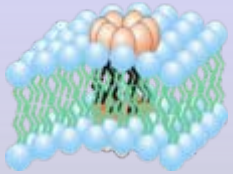
MC3

Gs-coupled

γ-MSH

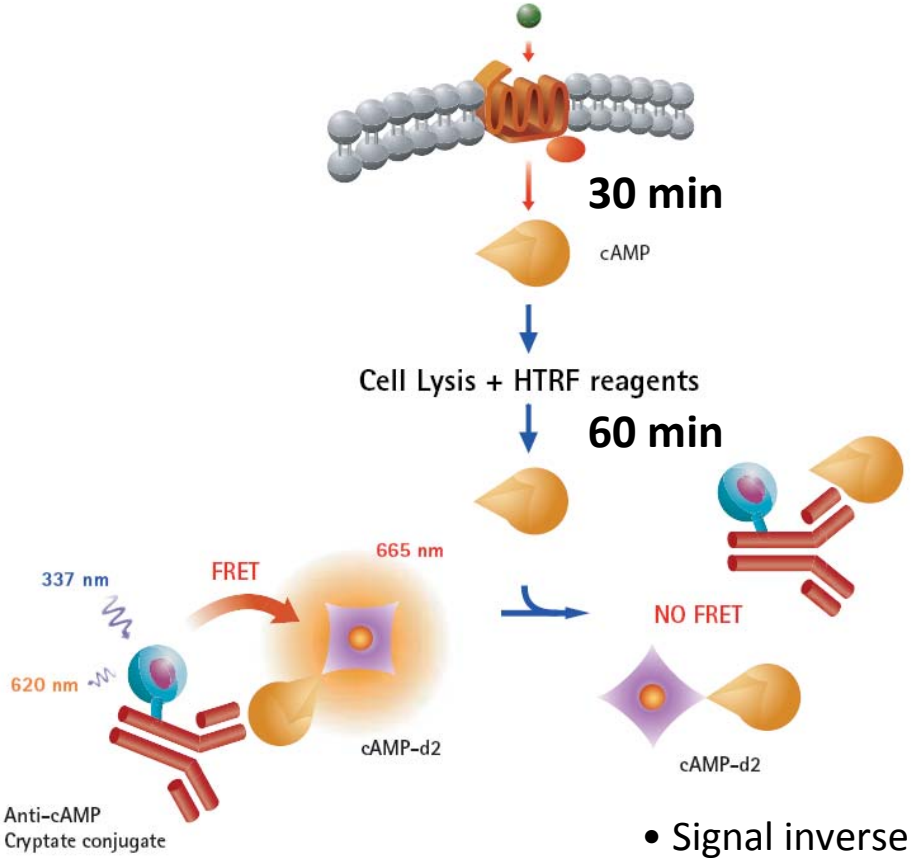
ACTH

α-MSH



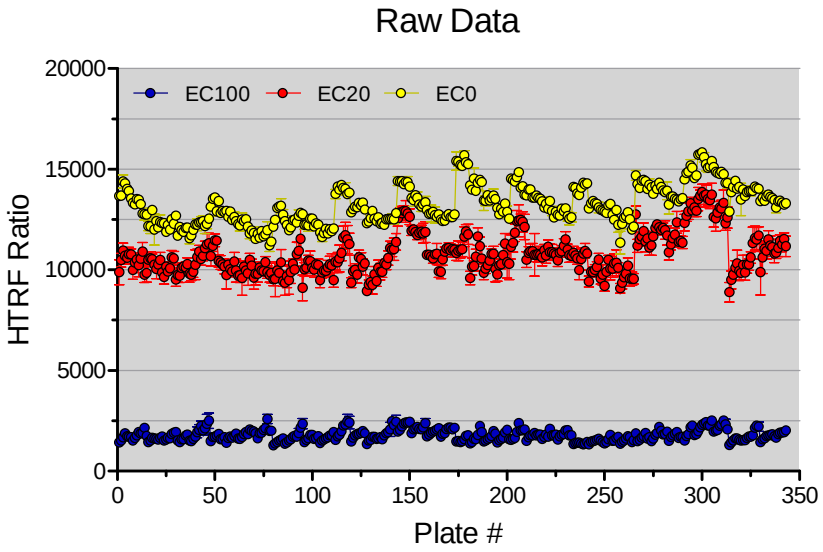
- Macrophages
- CNS
- Gut
- Placenta

Cardiovascular
Inflammation

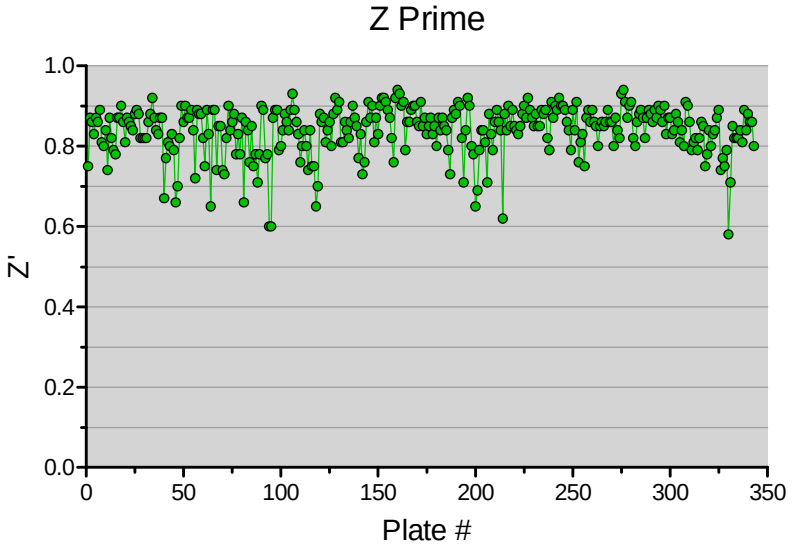


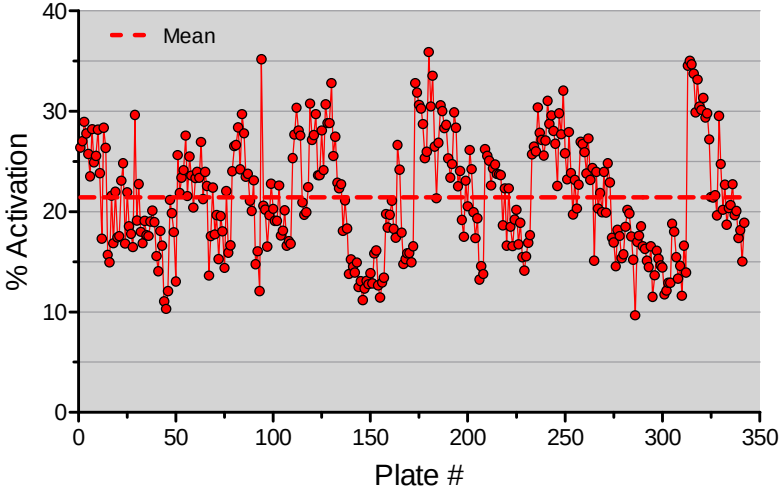
- Signal inversely proportional to cAMP
- Wide cAMP concentration range
- Gs or Gi coupled receptors
- Agonist/antagonist screening
- Phosphodiesterase
- Adenylate cyclase

HTRF® cAMP dynamic 2: HTS Assay Performance

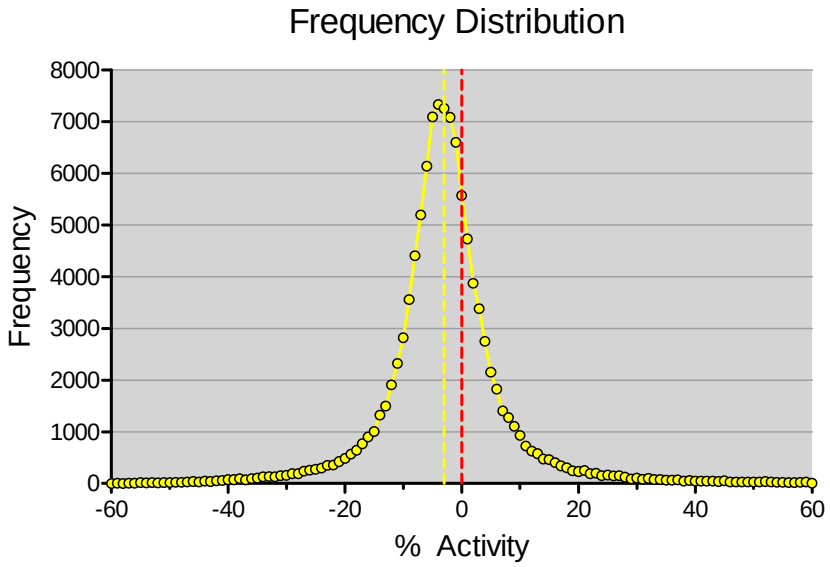


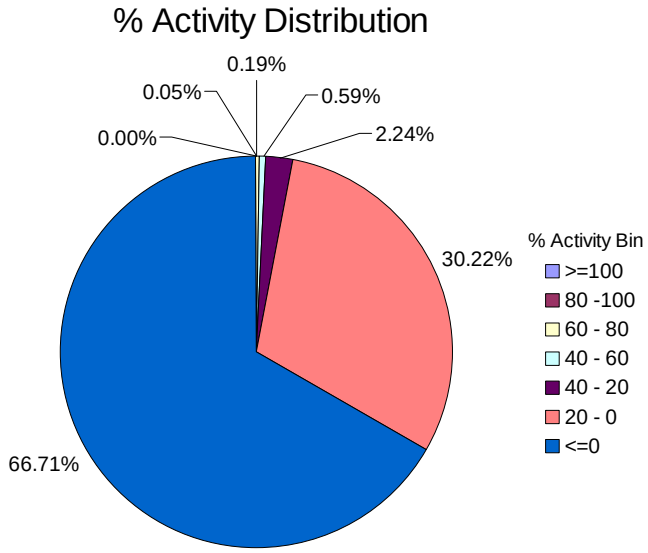
- GeneBLAzer® MC3R CRE-*bla* CHO-K1 Cells (Life Technologies)
- **Formatted as an MC3 potentiator/PAM HTS**
- γ–MSH native ligand (Lys-γ3-MSH)
- EC20 ± 10%
- Frozen cells
- Low volume 384
- Fully automated
- 32 plates/day
- >100K compounds





MC3 HTS Statistics



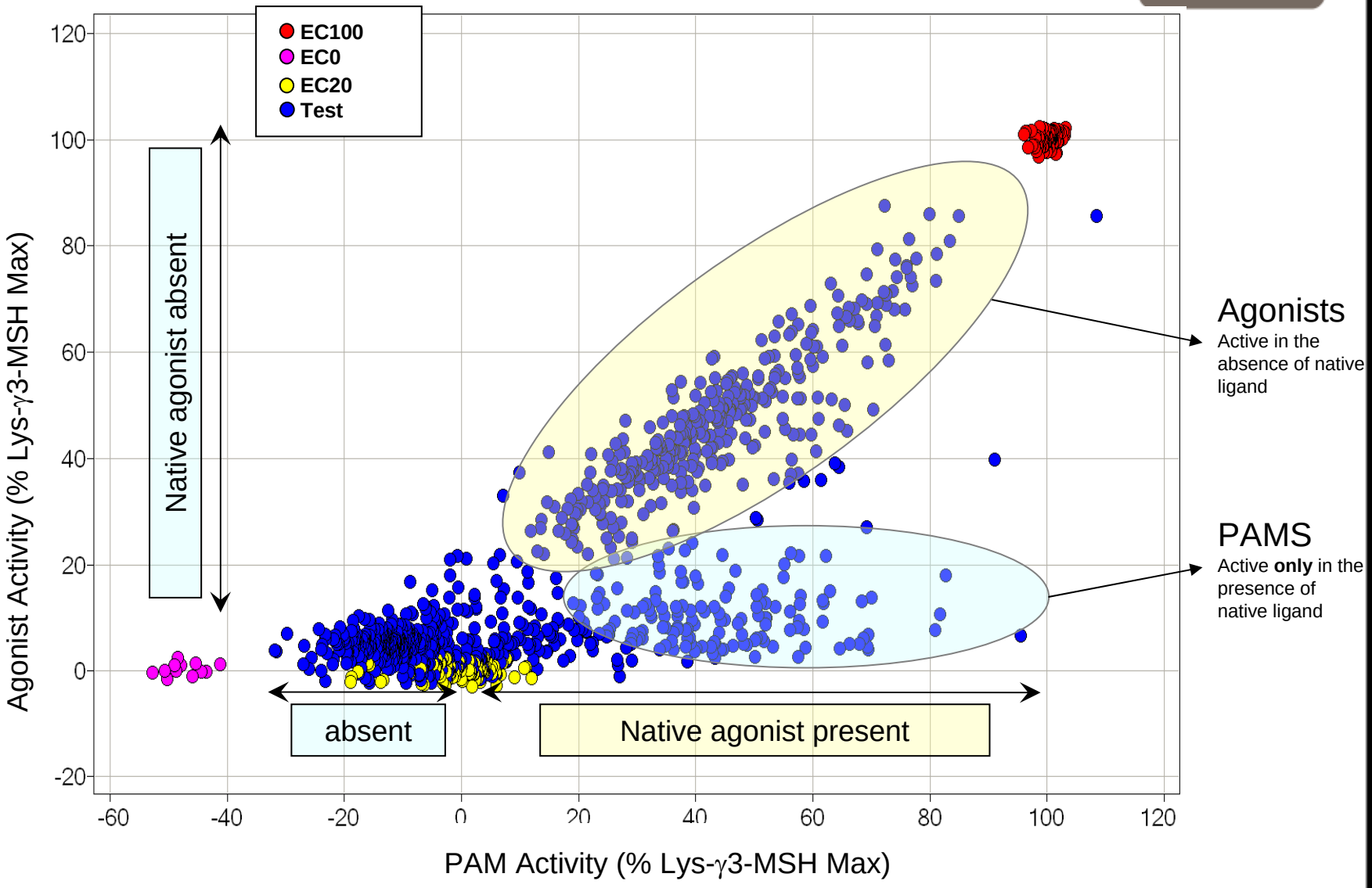


- 109760 compounds @ 10μM (1% DMSO)
- Mean Z' = 0.84 (± 0.06)
- Low Control %CV = 5.3 (± 2.7)
- High Control %CV = 3.6 (± 1.5)

Cutoff(%)	# Hits	% HR
40	912	0.83
50	520	0.47
60	269	0.25
70	147	0.13
80	62	0.06
90	12	0.01
100	2	0.00

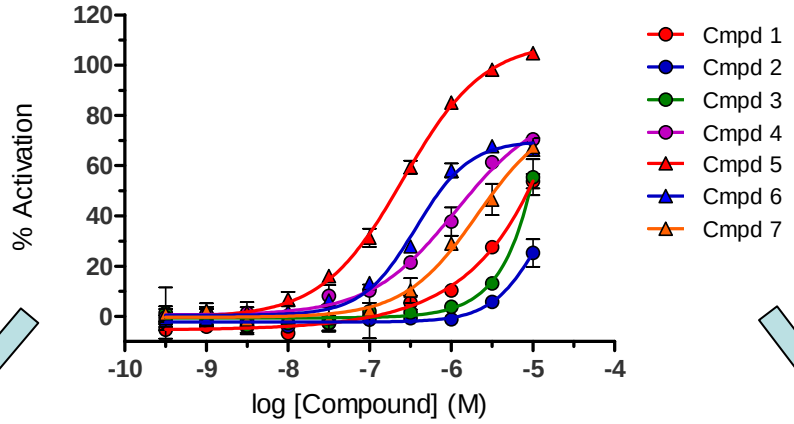
- Screened vs EC20 of native ligand (γMSH)
- Agonists
- Positive Allosteric Modulators (PAMs)
- Antagonists

MC3 HTS Hit Deconvolution: Agonist vs PAM



MC3 HTS Hit Profiling: Agonist vs PAM

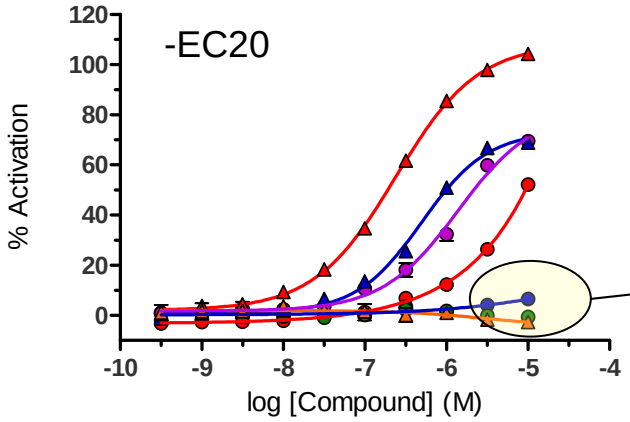
Increase in $[cAMP]_i$ in CHO cells stably expressing MC3 receptors



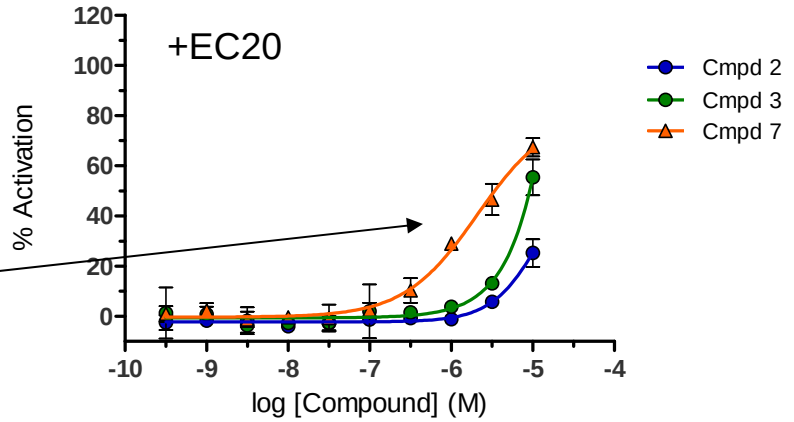
Agonists

PAMS

Agonist-induced increase in $[cAMP]_i$ in CHO cells stably expressing MC3 receptors



PAM-induced increase in agonist-mediated $[cAMP]_i$ in CHO cells stably expressing MC3 receptors



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MC3 HTS *dynamic 2* Summary

• Target feasibility, assay development, automation/adaptation, HTS, hit follow-up and profiling – **single assay (cAMP dynamic 2)**

• Robustness, signal stability, low interference leading to a dual (tri) HTS format for simultaneous detection of both agonists and PAMs (antagonists)

• The capacity to control an EC20 value throughout a HTS campaign (*potent native peptide ligands*)

• Deconvolution and hit profiling clearly distinguishes specific PAMs and agonists by quantitative pharmacology

• Assay employed as a counterscreen using other non-MC3 cell lines

***MP63*: Jerman et al. Identification and pharmacological characterisation of novel positive allosteric modulators (PAM) of Melanocortin 3 Receptors**

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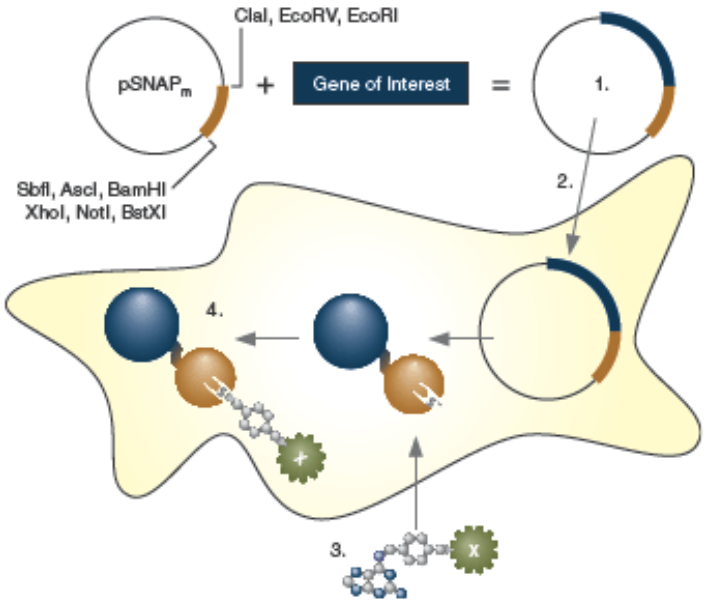
SNAP-Tag Technology and Tag-lite®

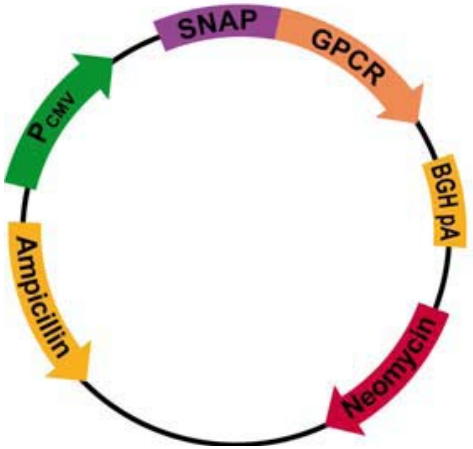


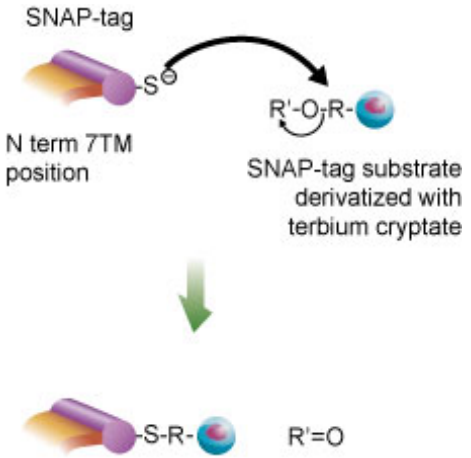
SNAP-tag

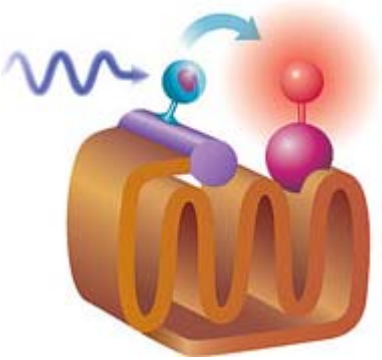
- SNAP-tag (New England BioLabs)
- O^6-alkylguanine-DNA alkyltransferase
- Benzylguanine derivatives
- Irreversible covalent labelling of SNAP-tag

Tag-lite®

- Terbium cryptate-labelled SNAP-tag substrate
- Acts as donor in HTRF
- Receptor ligand labelled with d2 - acceptor







Formyl Peptide Receptor (FPR) Receptor Family

- Class A receptors
- G_i/G_o and G_q/G_{11} coupled
- Chemotaxis, superoxide production, pro/anti-inflammatory functions

FPR1

Formyl Peptides
WKYMVm
Annexin 1 Peptides
Cyclosporins

- Neutrophils
- Immature DC
- Epithelial
- CNS

Chemotaxis
Inflammation

FPR2/ALX

Lipoxin A4
Formyl Peptides
WKYMVm
SAA
Annexin 1

- Neutrophils
- Monocytes
- Macrophages
- Epithelial
- Immature DC

Chemotaxis
Inflammation

FPR3

F2L
Humanin
Annexin 1 Peptides
WKYMVm

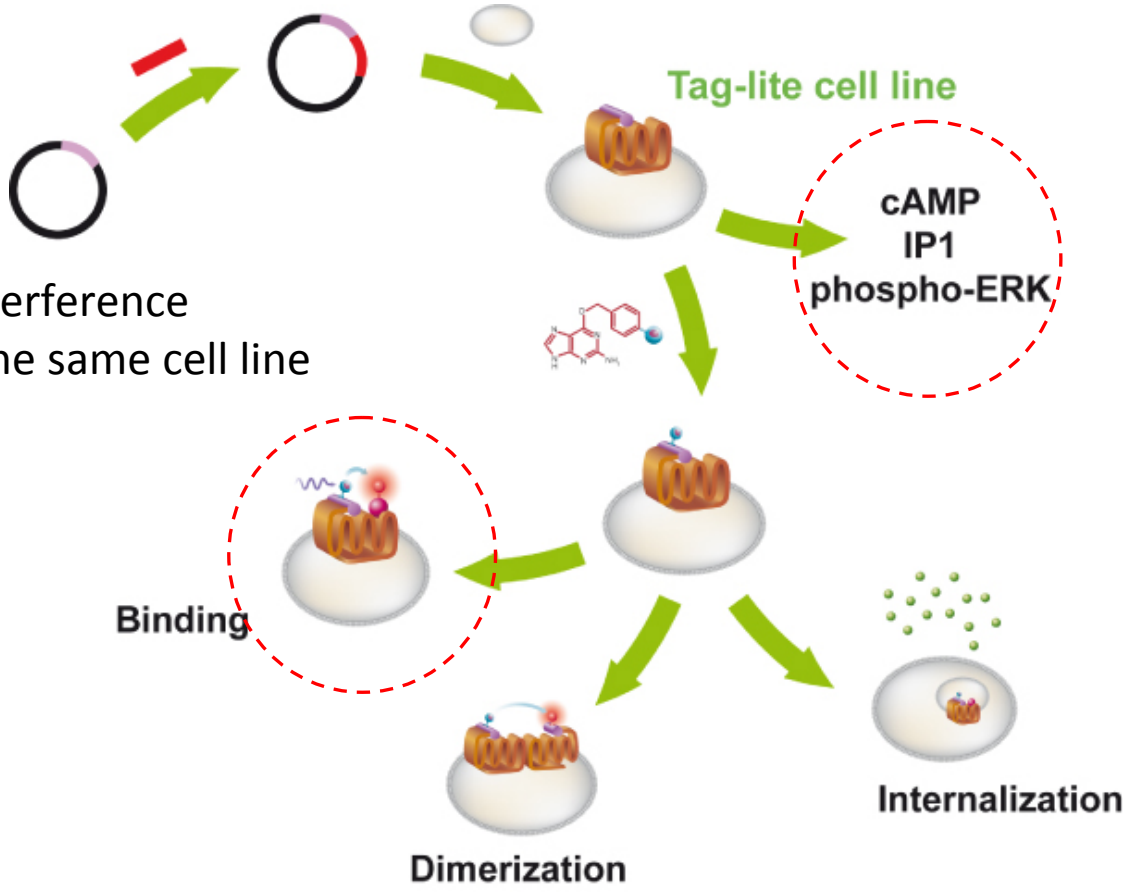
- Monocytes
- Mature DC

Chemotaxis
Function (s) ?

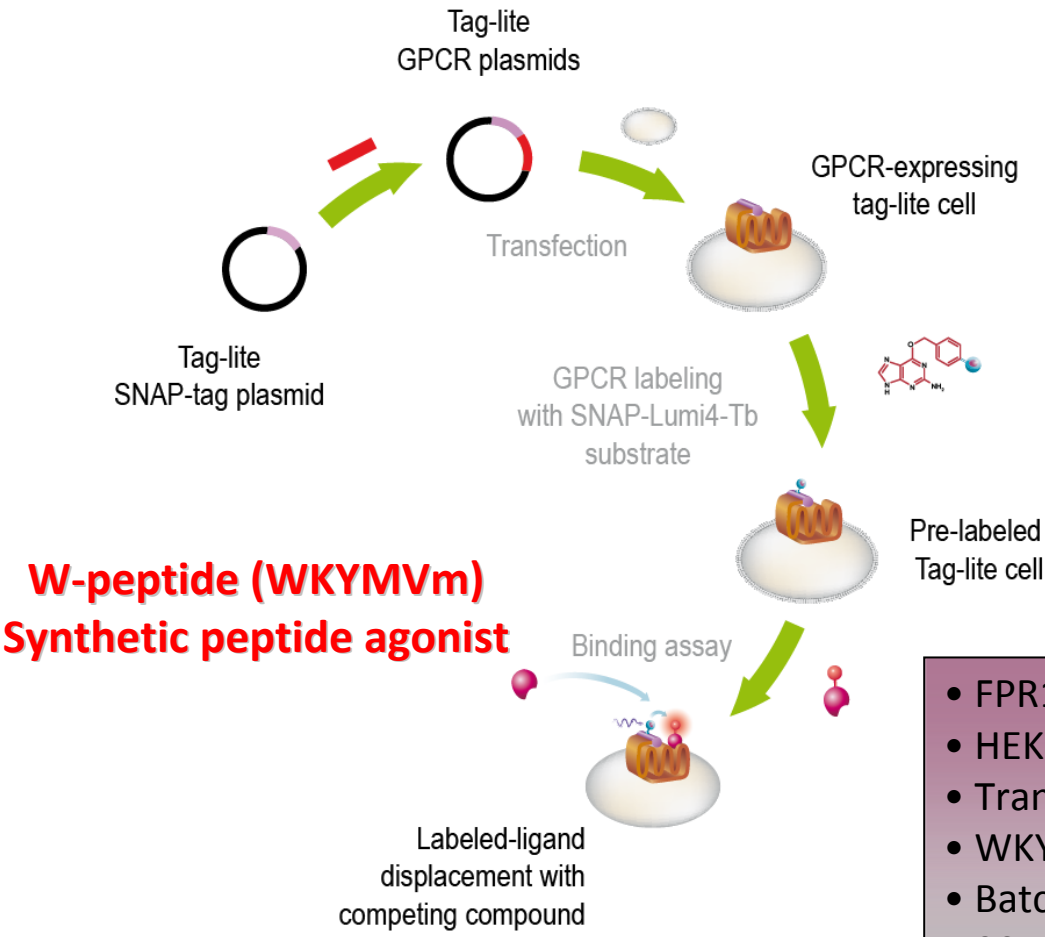
FPR Receptor Binding: Tag-lite®

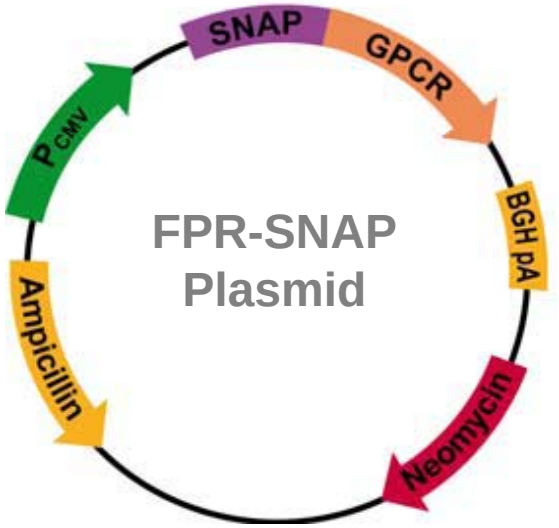
Why Tag-lite?
Why Not!

- Non-radioactive
- Homogenous
- High-throughput
- HTRF sensitivity
- Low compound and matrix interference
- Different assay endpoints in the same cell line



Generating Tag-lite® FPR Cell Lines:
Transient Expression

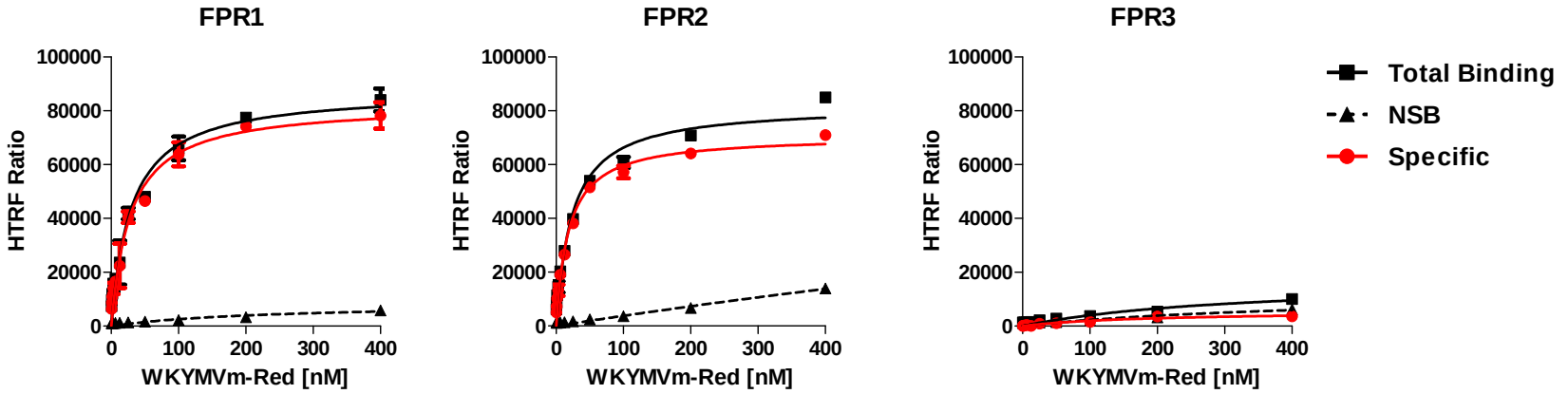


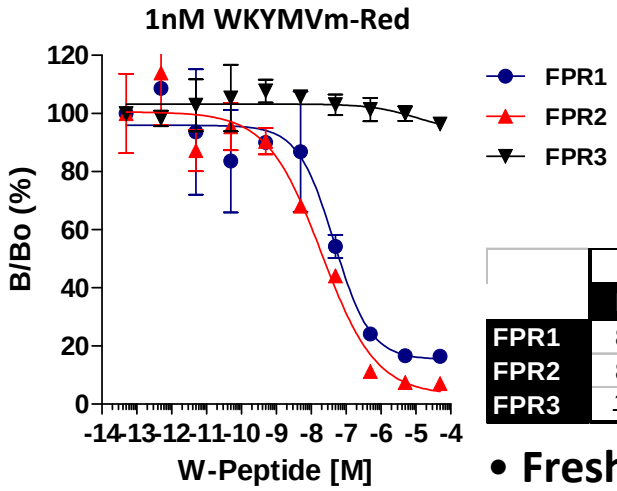


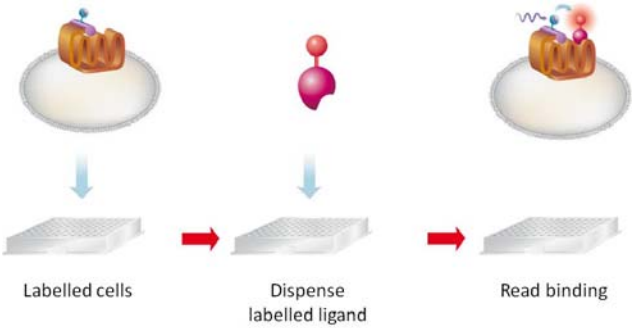
W-peptide (WKYMVm)
Synthetic peptide agonist

- FPR1-3 SNAP-tag constructs (Cisbio)
- HEK293
- Transient transfection
- WKYMVm red HTRF acceptor
- Batch transfection and labelling (Lumi4-Tb)
- 90 minute incubation

Tag-lite® Saturation and Competition Binding:
W-peptide (WKYMVm) Transient Expression





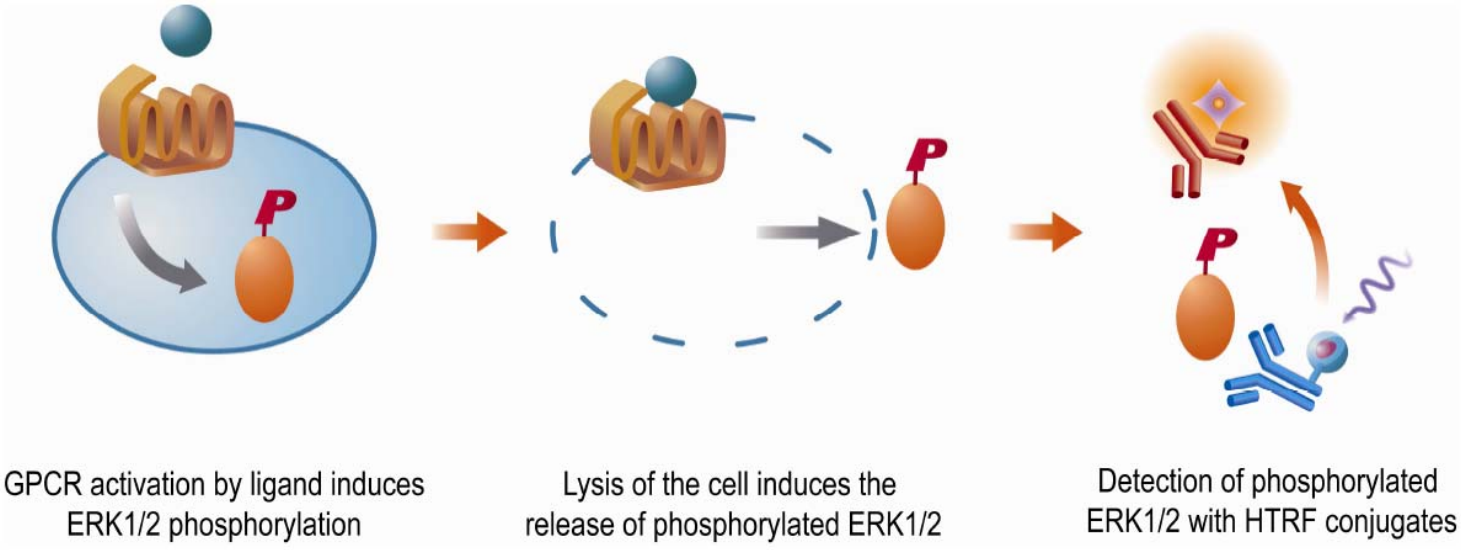


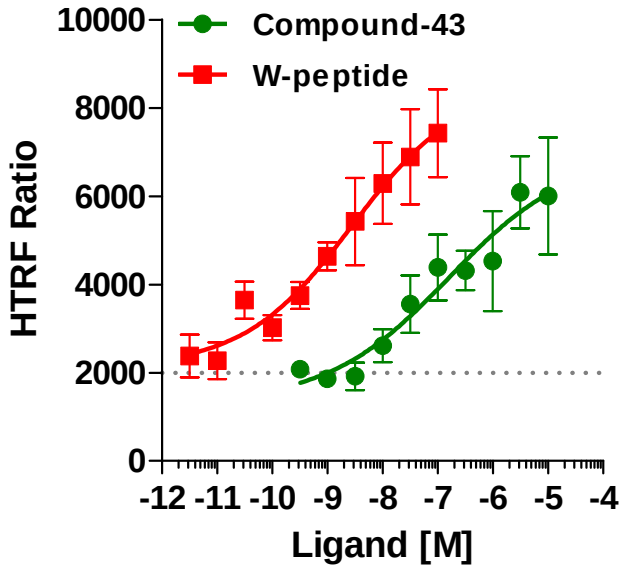
	Total		Specific		Competition		IUPHAR	MRCT
	Bmax	Kd (nM)	Bmax	Kd (nM)	EC50 (nM)	pEC50	pKd	
FPR1	87547	30	82618	29	46	7.3	-	7.5
FPR2	82016	24	70775	19	20	7.7	8.7-10.13	7.7
FPR3	*17366*	*331*	*5678*	*191*	*9600*	*5.0*	-	*6.5*

- **Freshly cultured or frozen cells**
- **Batch transfection/labelling**

Cellul'erk (phospho-Erk) Assay:

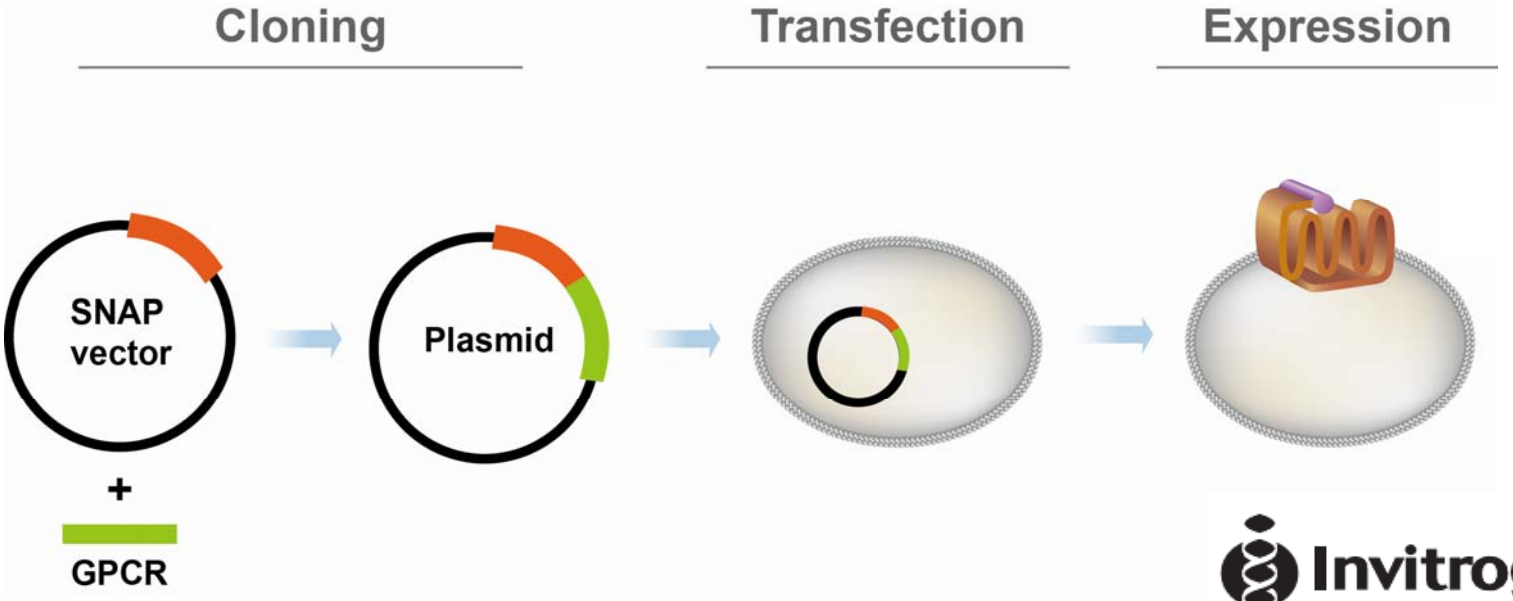
Transiently transfected FPR2 Cells





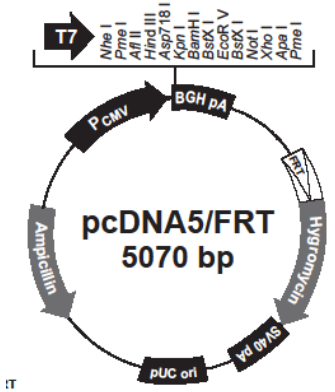
- Measures intracellular phosphorylated Erk1/2
- 96-well to 384-well format
- Signal proportional to phosphorylation
- 384-well format being developed
- Success with AlphaScreen/LISA

Generating Tag-lite® FPR Flp-In™ Cell Lines:
Stable Expression



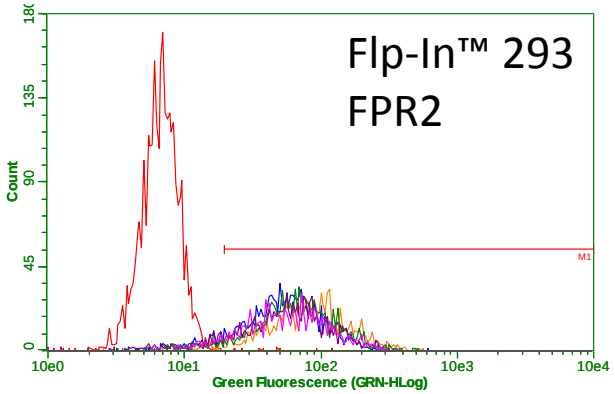
Invitrogen™ life technologies

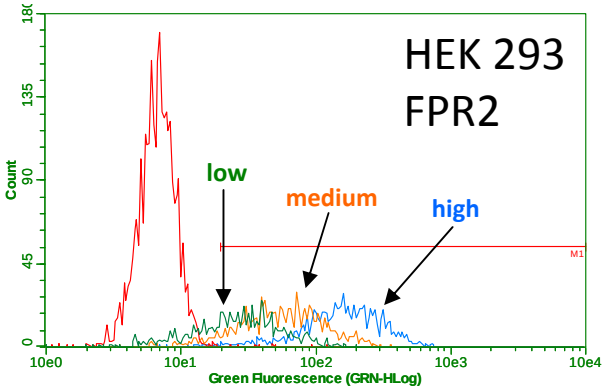
- FPR1 and FPR2 SNAP-tag constructs (Cisbio)
- Sub-cloned in pcDNA5/FRT Flp-In vector (Life Technologies)
- Flp-In™ 293 and HEK293 (Cisbio constructs)
- Transient transfection
- Selection in hygromycin/neomycin
- Isogenic clones selected
- **Screened for receptor expression, binding and function**



Tag-lite® FPR2 Flp-In™ Cell Lines:

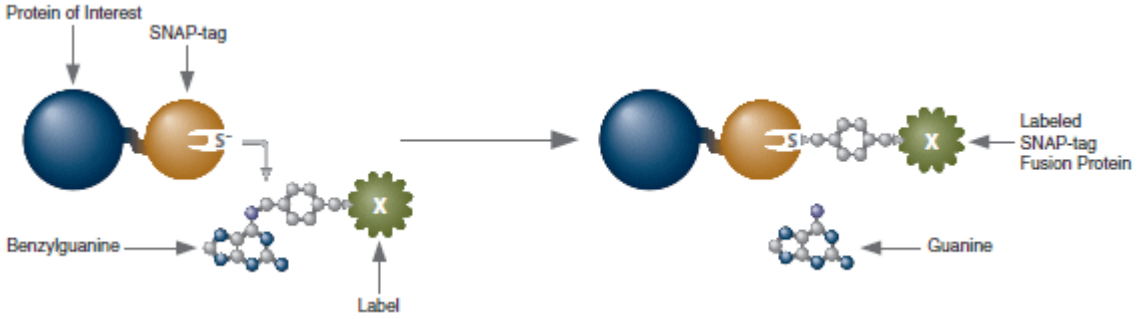
Receptor Expression – SNAP Fluorescence





Flp-In isogenic clones
Homogenous FPR2 expression

HEK293 clones
Differential FPR2 expression

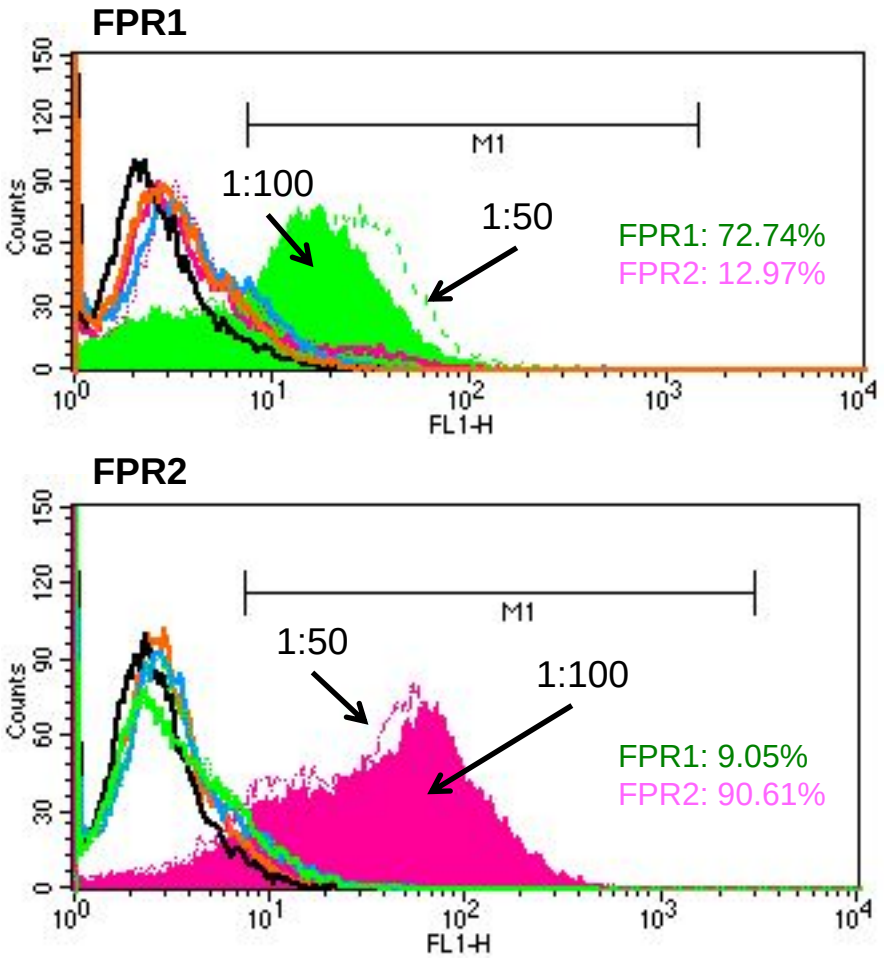


SNAP-Surface®488: non-cell-permeable fluorescent SNAP-tag substrate

(New England BioLabs)

Tag-lite® FPR Flp-In™ Cell Lines:

Receptor Expression – Anti-receptor antibodies

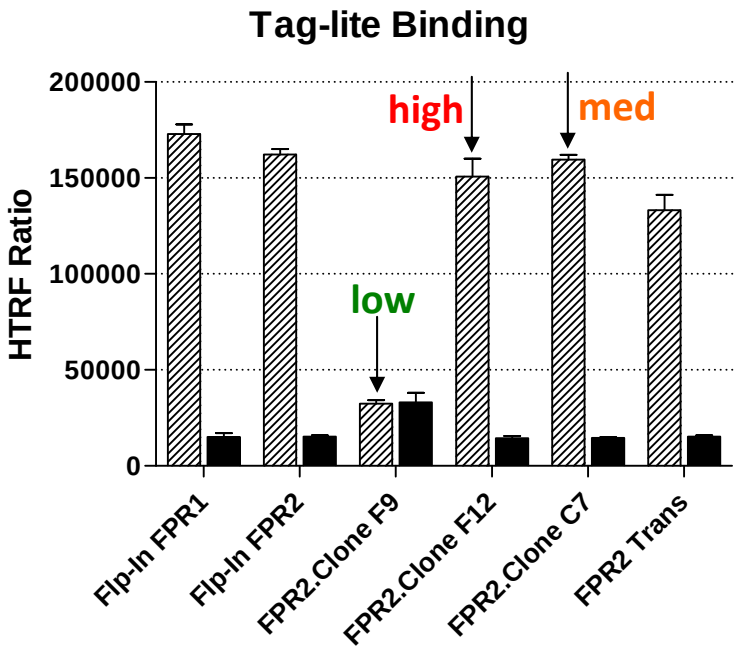


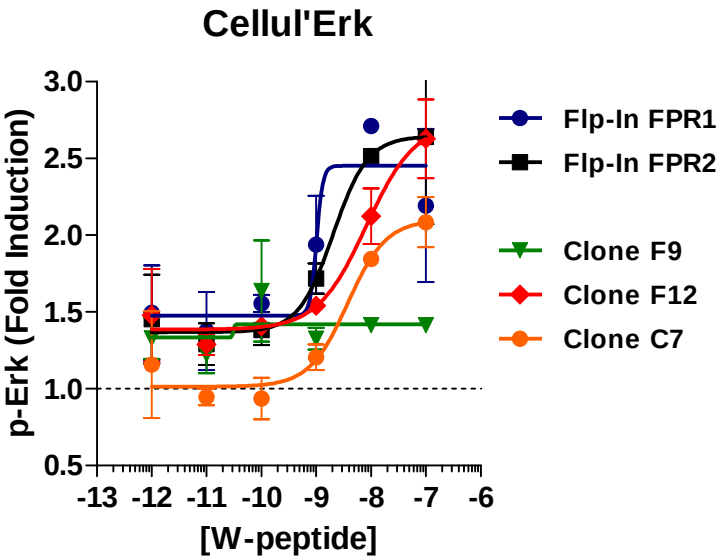
Unstained
Isotype
Secondary
FPR1
FPR2

- Commercially available anti-FPR1 and FPR2 monoclonal antibodies
- Further confirmation of surface receptor expression

Tag-lite® FPR Flp-In™ Cell Line Profiling:

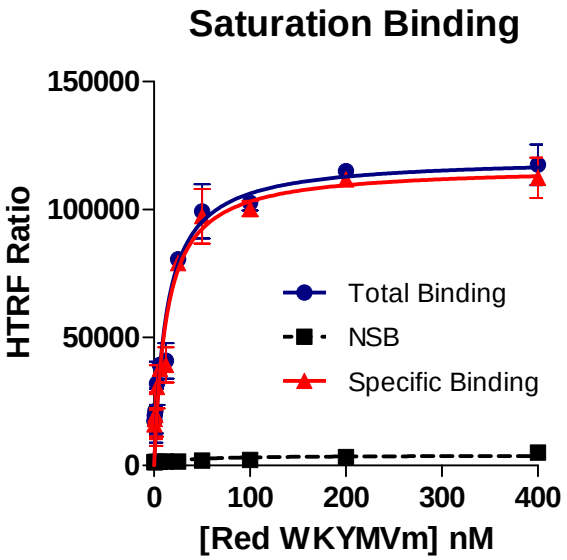
Flp-In™ vs HEK293 (high, medium , low MC3) Stable Expression

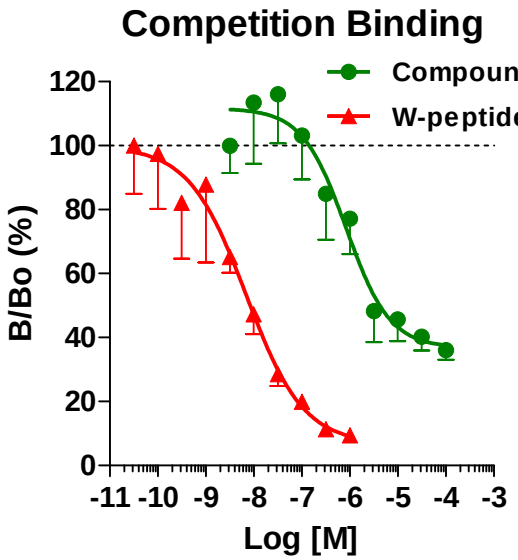


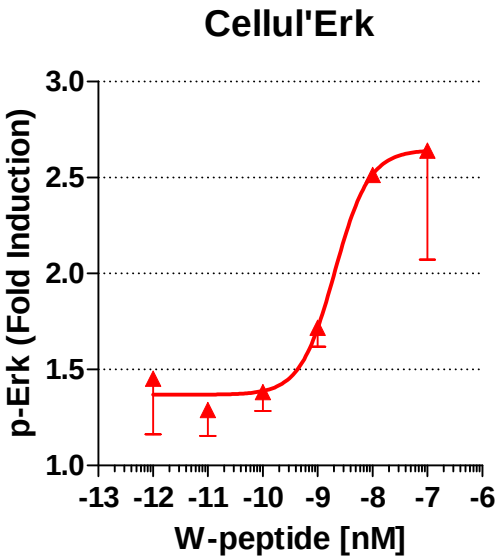


- Isogenic Flp-In clones – good specific binding
- HEK293 – good specific binding in high/med expression, but not low
- Cellul'Erk data correlates well with Tag-lite binding

HTRF-Based Profiling of FPR2 Tag-lite® Flp-In™ Cell Line







W-peptide (WKYMVm)

Total		Specific		Competition		Erk Phosphorylation		IUPHAR	MRCT
Bmax	Kd (nM)	Bmax	Kd (nM)	EC50 (nM)	pEC50	EC50 (nM)	pEC50	pKd	
120363	13.27	116819	13.25	6.9	8.16	2.04	8.69	8.7-10.13	7.8

- Multiple read-outs from a stable Tag-lite cell line
- Ligand affinity under-estimated (?)
- Amplification of binding affinity with phospho-Erk response

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FPR Receptor Summary

- Tag-lite binding assays successfully formatted for FPR receptors
- Reproducible pharmacology in transient and stable format
- Comparable pharmacology to published values*
- Receptor G_i coupling measured with Cellul'erk assay and correlates with binding
- Stable cell lines conveniently generated in Flp-In™ cell background

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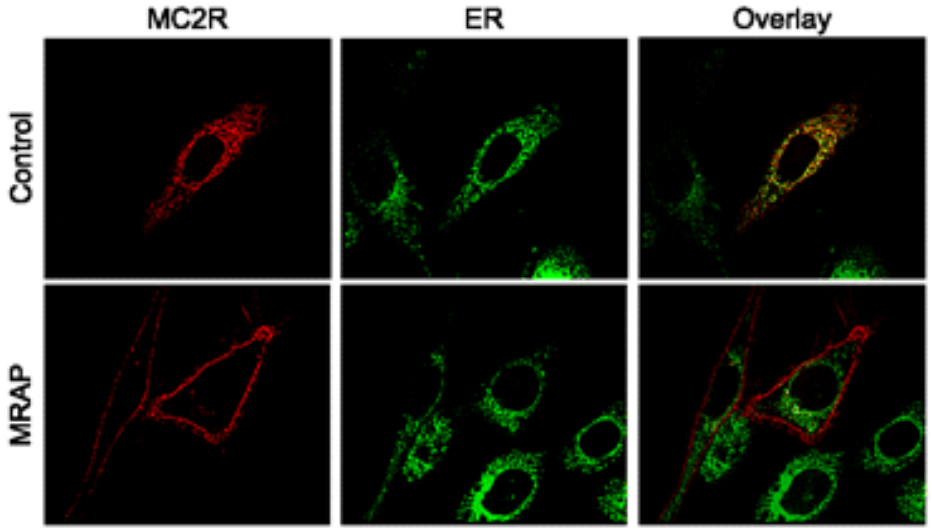
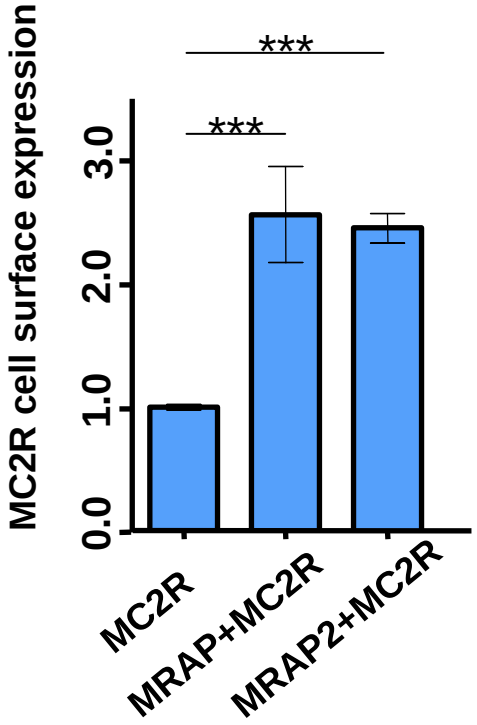
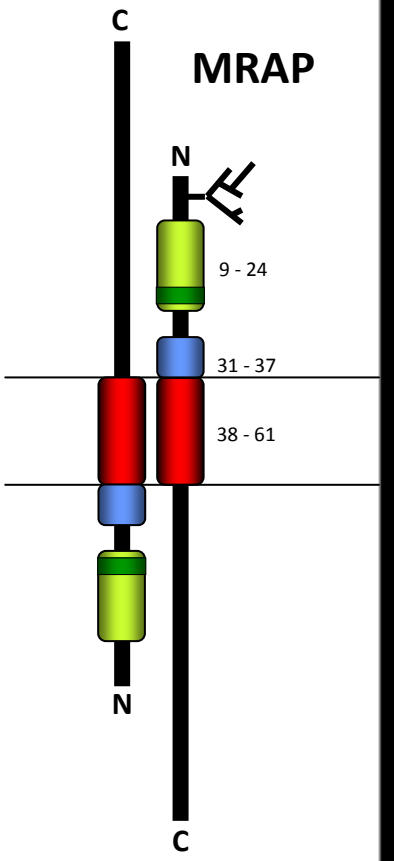
Melanocortin Receptor 2-MRAP Interaction

MC2: ACTH receptor responsible for steroidogenesis
MRAP: melanocortin receptor accessory protein





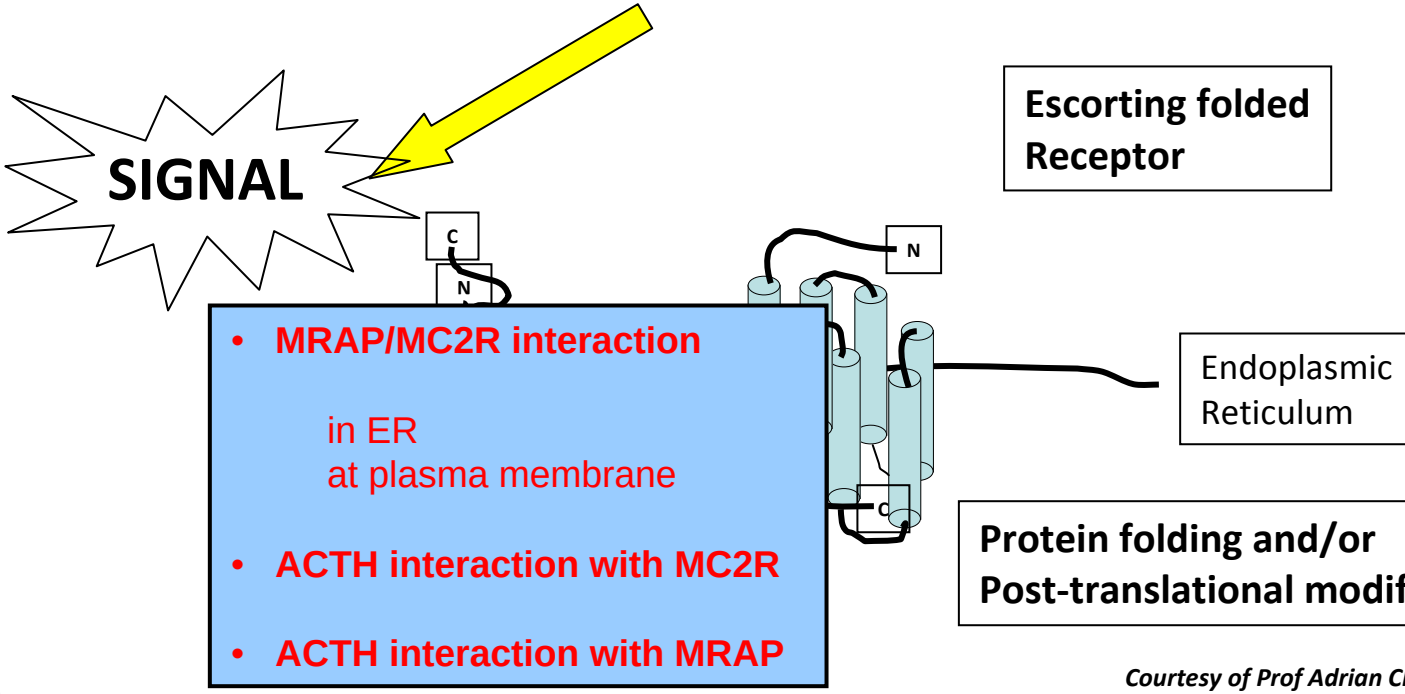


1. *J Biol Chem. (2009). 284:22641*
2. *Prof Adrian Clark, St Georges Hospital, London*

Mechanisms of MRAP-MC2 action

As dimer creating functional receptor complex

ACTH

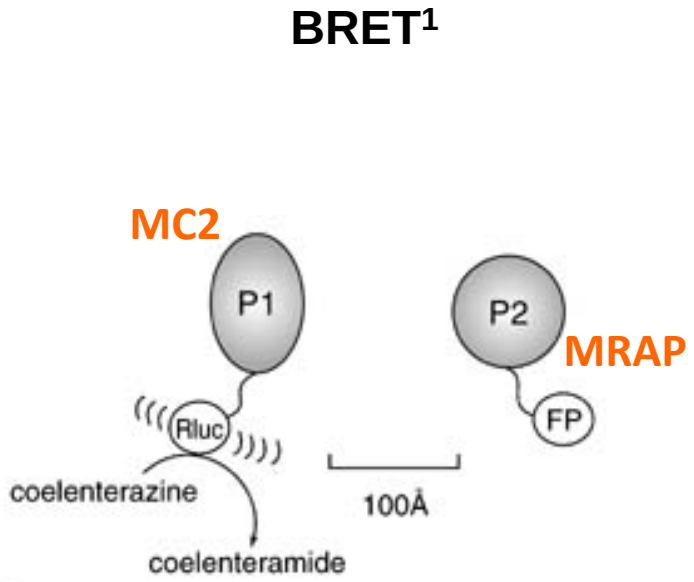


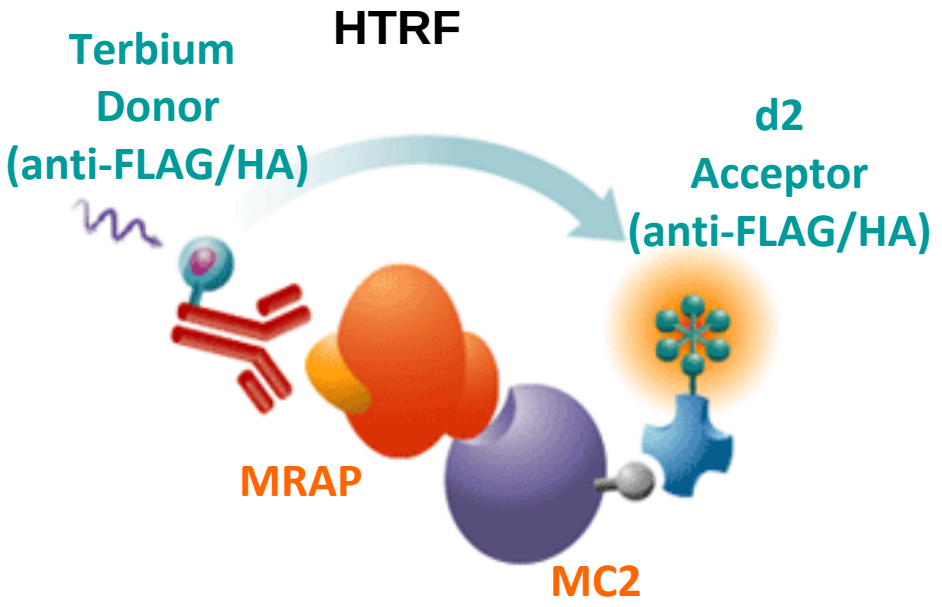
- **MRAP/MC2R interaction**
  - in ER
  - at plasma membrane
- **ACTH interaction with MC2R**
- **ACTH interaction with MRAP**

Courtesy of Prof Adrian Clark, St Georges Hospital, London

MC2-MRAP Proximity Assays: HTRF® Anti-tag Reagents

Anti-HA/FLAG-conjugated Tb/d2 Antibodies





- MC2R-Rluc, c-terminal (pRLuc-N1; PerkinElmer)
- MRAP-EYFP, n-terminal (pEYFP-C1; Clontech)
- HEK-293 cells
- Lipofectamine 2000
- Coelenterazine h
- Fluostar Optima (BMG Labtech)
- Published protocol

- MC2R-HA, n-terminal (cDNA.org)
- MRAP-FLAG, c-terminal (AC/LC)
- HEK-293 cells
- Lipofectamine 2000
- Anti-FLAG/Anti-HA labelled antibodies
- Pherastar Plus (BMG Labtech)
- Pilot evaluation

MC2-MRAP Proximity Assays: HTRF® Anti-tag Reagents

Anti-HA/FLAG-conjugated Tb/d2 Antibodies

- HEK293 transient transfection
- MC2-HA or MRAP-FLAG
- Suspension vs adherent
- Anti-tag reagents:

Combination 1

anti-HA-Lumi4-Tb (MC2 donor)

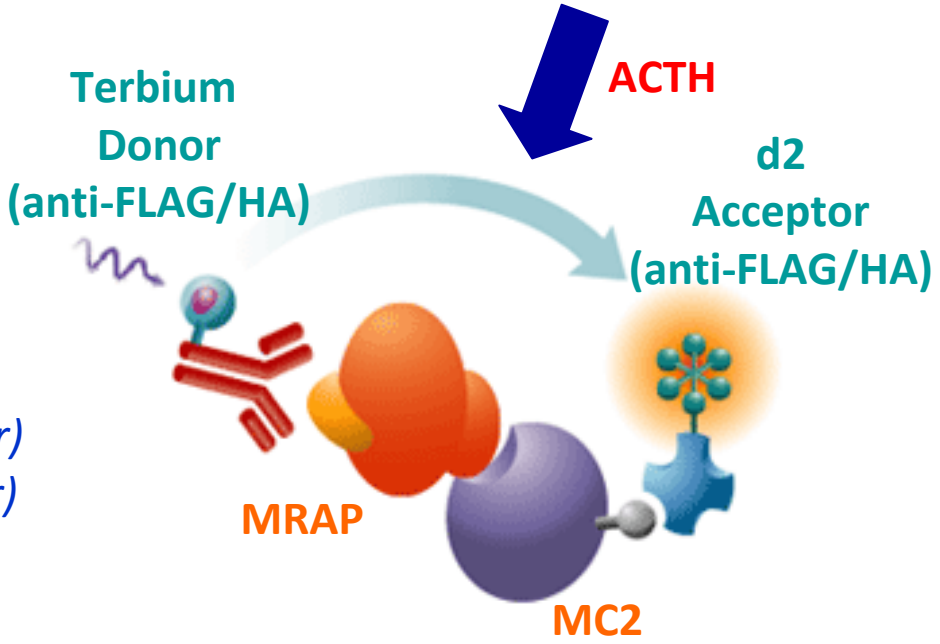
anti-FLAG-d2 (MRAP acceptor)

Combination 2

anti-FLAG-Lumi4-Tb (MRAP donor)

anti-HA-d2 (MC2 acceptor)

- Expression (WB), function (cAMP)
- HTRF-based proximity assay



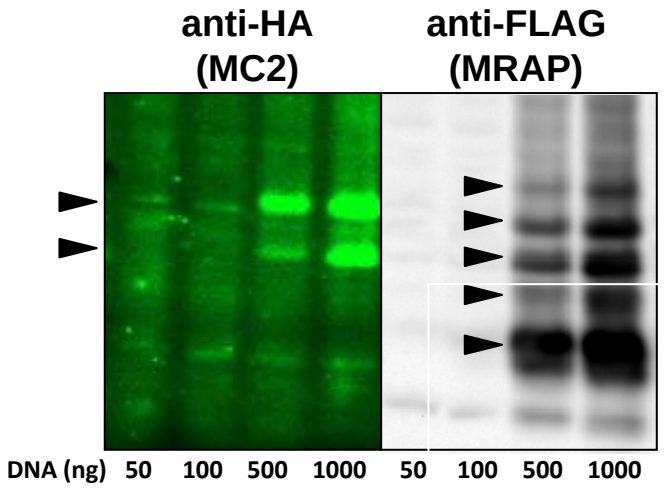
Delta F Calculation: Specific HTRF Signal

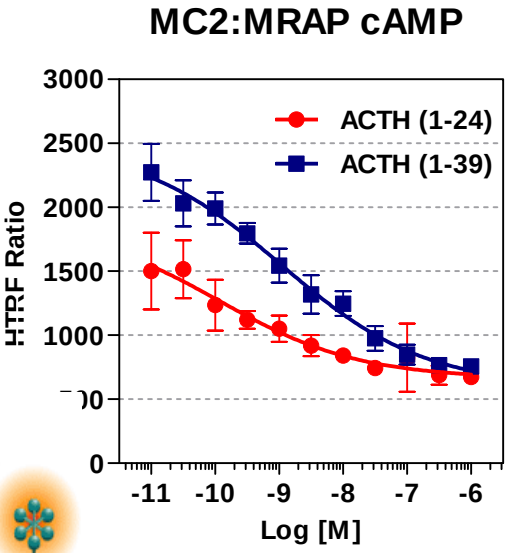
HTRF Ratio = (665nm/620nm) x 10000

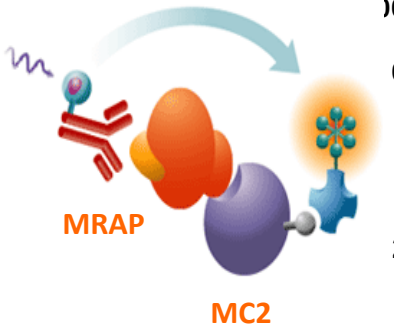
$$\text{Delta F } (\Delta F) = \frac{\text{Ratio Positive (ACTH)} - \text{Ratio Negative (unstimulated)}}{\text{Ratio Negative (unstimulated)}} \%$$

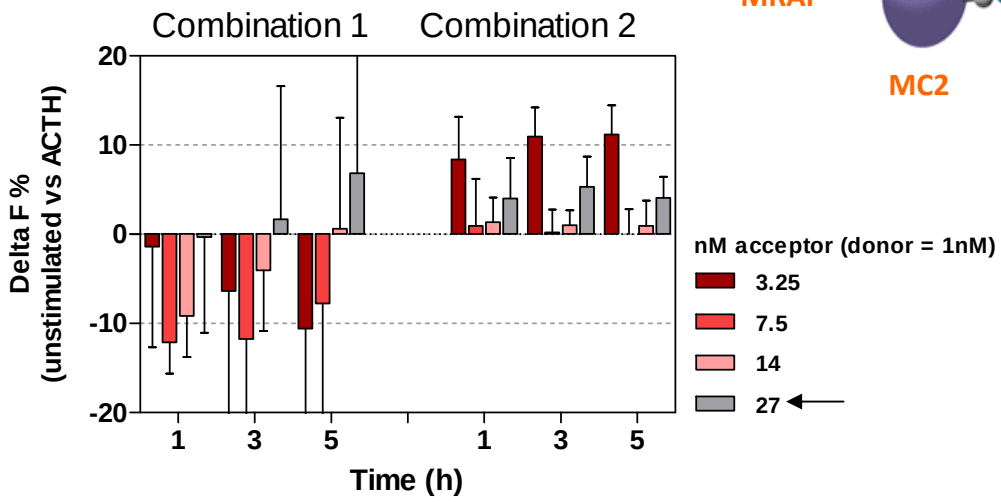
MC2-MRAP Proximity Assays: HTRF® Anti-tag Reagents:

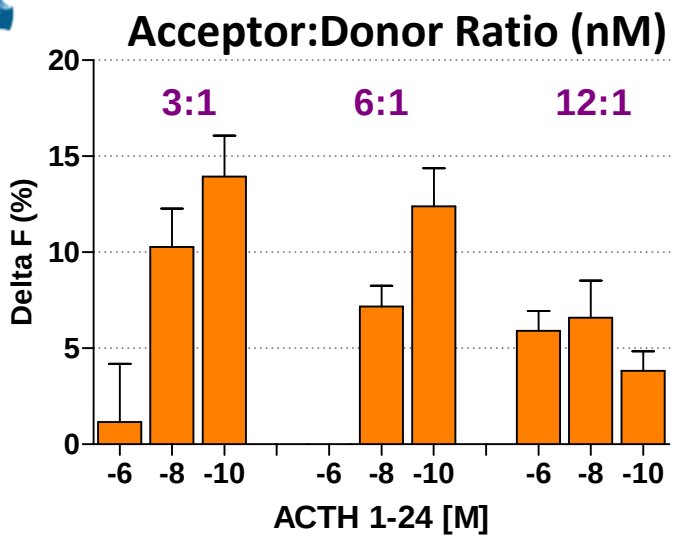
Anti-HA/FLAG-Tb/d2 Antibodies – Suspension Format 4⁰C











MRC T

MC2-MRAP Protein Interaction Summary

- BRET assay unsuccessful
- small signal, large signal contamination – donor/acceptor (RLuc/EYFP)

- HTRF anti-tag reagents – small, reproducible signal (ΔF ~ 10-15%)
- Ligand (ACTH) concentration-dependent effects
- Further optimisations:

  - ✓ Incubation temperature (4^0C/22^0C)
  - ✓ Suspension vs adherent
  - ✓ Buffers/matrix
  - ✓ Receptor internalisation (sodium azide)
  - ✓ Antibody concentrations and ratios
  - ✓ Cell number

- Challenging target!

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Outline

- MRC Technology Centre for Therapeutics Discovery
- Utilising HTRF® assays in an HTS environment
- HTRF® Tag-lite® technology and secondary assay development
- Investigational studies for receptor-protein interactions
- **Conclusions and future perspectives**

MRC T

Conclusions and Future Perspectives

- HTRF is a powerful and versatile tool for interrogating functional 7TM biology
- Robust and reproducible data
- HTRF is applicable to all stages of a project cascade
- Advantages of commonality of assay formats for automation and screening
- Tag-lite a convenient and sensitive format for receptor binding
- Tag-lite cell lines: flexibility, assay multiplexing
- Open assay platform for bespoke assay development

MRC T

HTRF® 7TM/GPCRs and . . . ?

- HTRF technology is not limited to 7TM biology!
- Kinease™ HTRF assays
- HTRF Transcreener® ADP
- Cortisol (steroid hormone)
- Cytokines (TNFα, IL-1β)

Extracellular targets

- IP1 (IP-One)

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MRC Technology

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MC2-MRAP BRET

Sadani Cooray (QMUL)

Group Leader

Debbie Taylor

***MP63*: Jerman et al. Identification and pharmacological characterisation of novel positive allosteric modulators (PAM) of Melanocortin 3 Receptors**