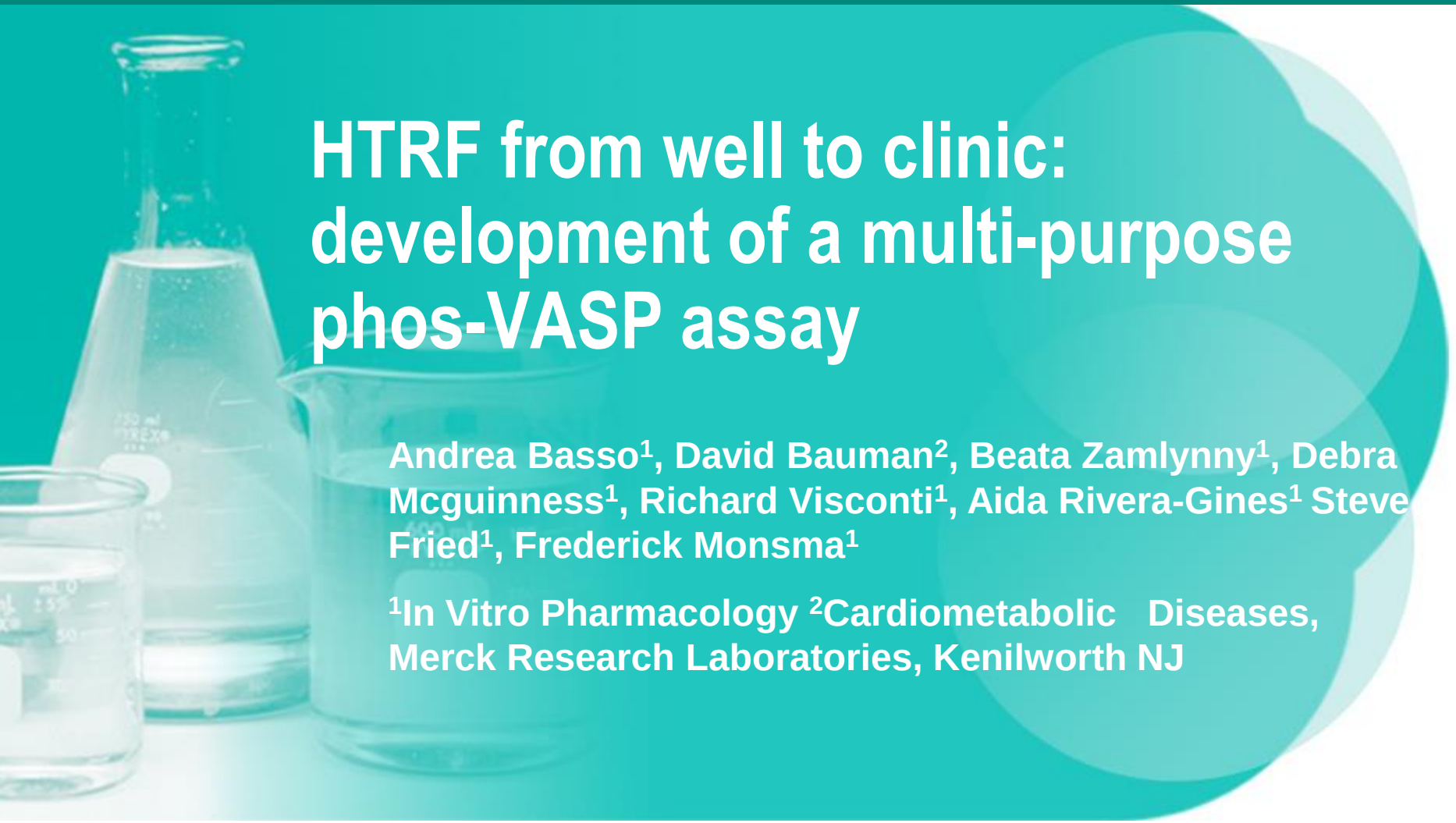


HTRF from well to clinic: development of a multi-purpose phos-VASP assay

Andrea Basso[1], David Bauman[2], Beata Zamlynny[1], Debra Mcguinness[1], Richard Visconti[1], Aida Rivera-Gines[1] Steve Fried[1], Frederick Monsma[1]

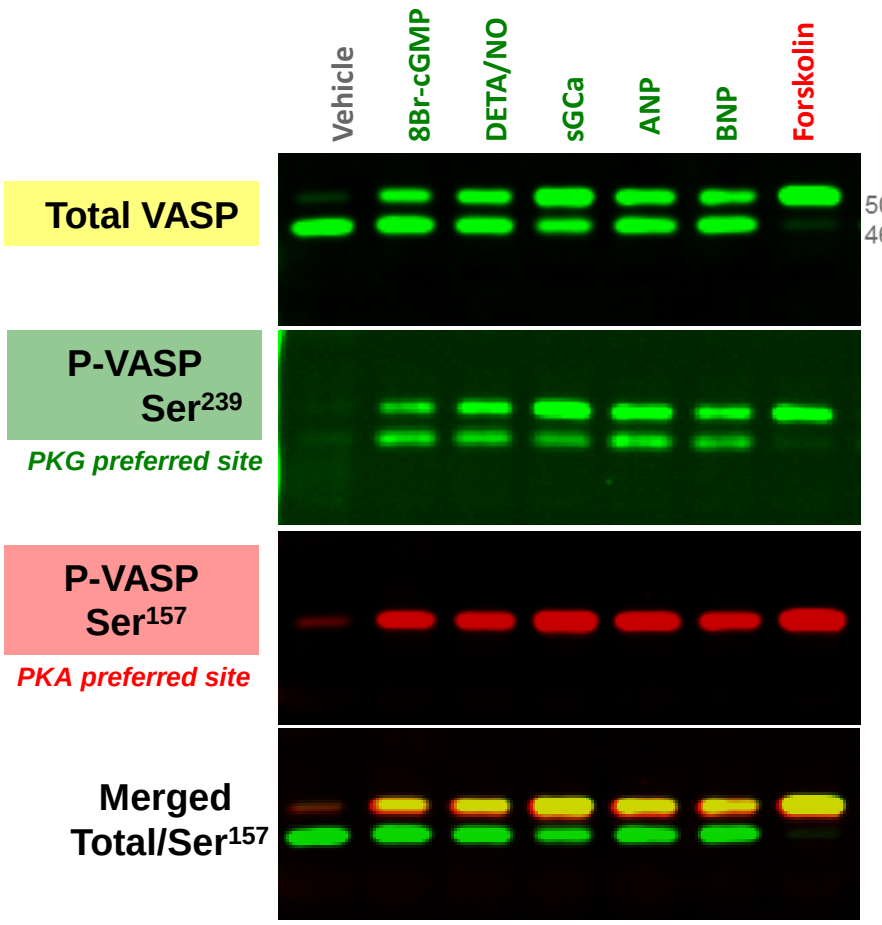
[1]In Vitro Pharmacology [2]Cardiometabolic Diseases, Merck Research Laboratories, Kenilworth NJ

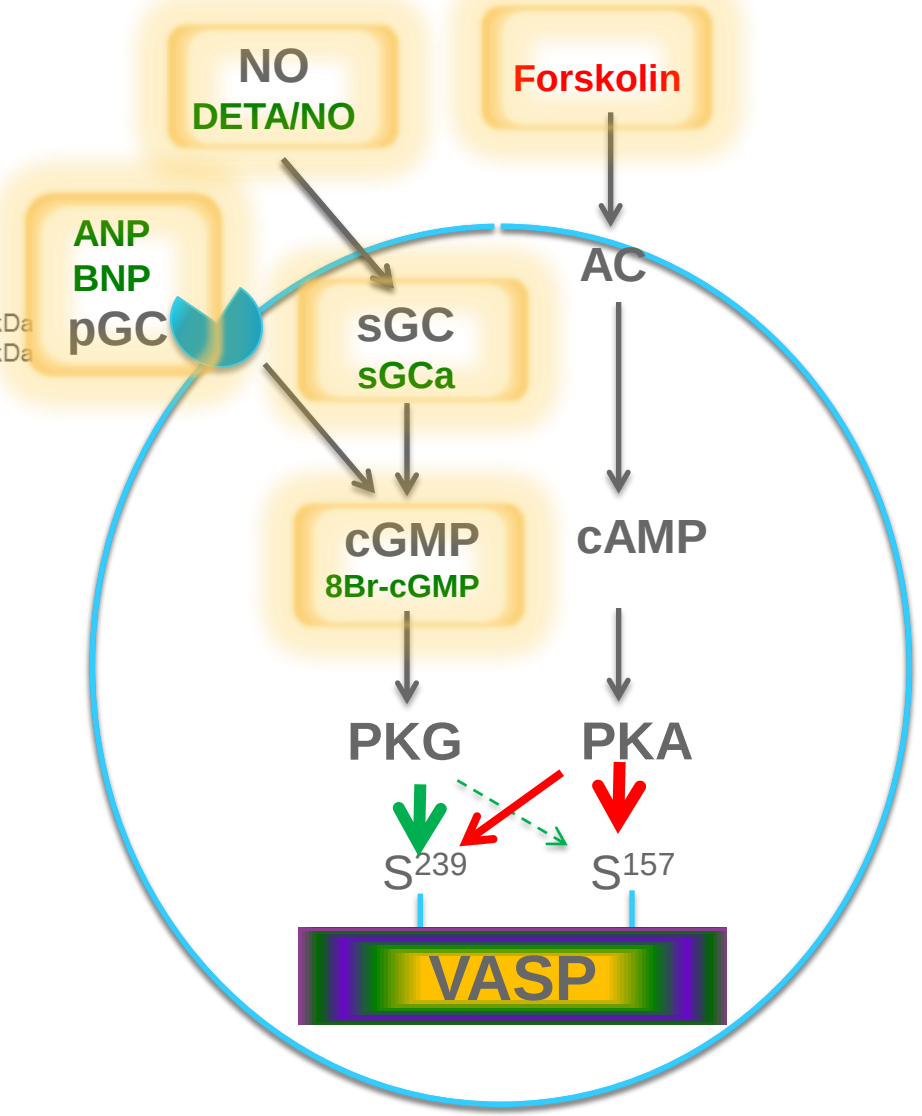
MERCK | In Vitro Pharmacology

VASP

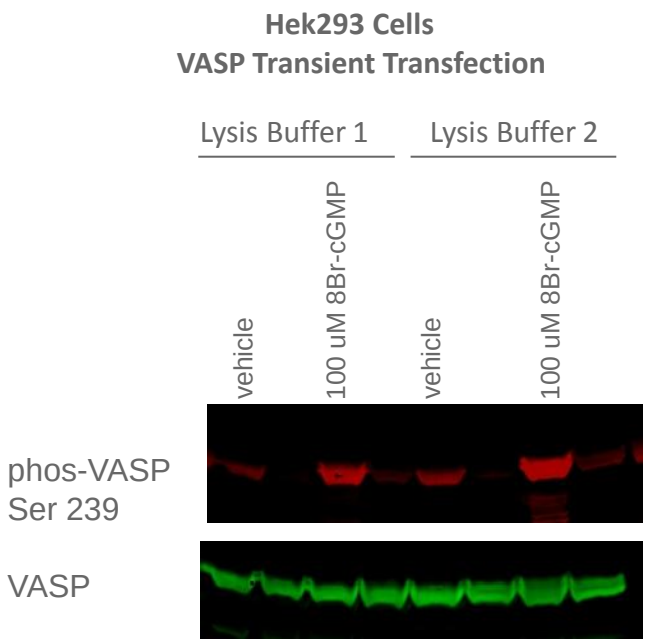
- VASP= vasodilator-stimulated phosphoprotein
- Regulates actin cytoskeleton dynamics and plays a role in smooth muscle contraction.
- Modulated by cAMP and cGMP pathways
- We sought to develop a phos-VASP HTRF assay for both Ser157 and Ser239 phosphorylation in order to evaluate signaling downstream of cAMP and cGMP pathways.
- Goals:
  - Develop an assay to be utilized for routine screening
    - Miniaturize
    - Minimize plate handling
    - Robust signal
    - High Z-prime
  - Assay platform translatable to physiologically relevant cells
  - Assay platform translatable to in vivo studies
  - Potential for human clinical biomarker studies

Differentiating cGMP and cAMP Pathways

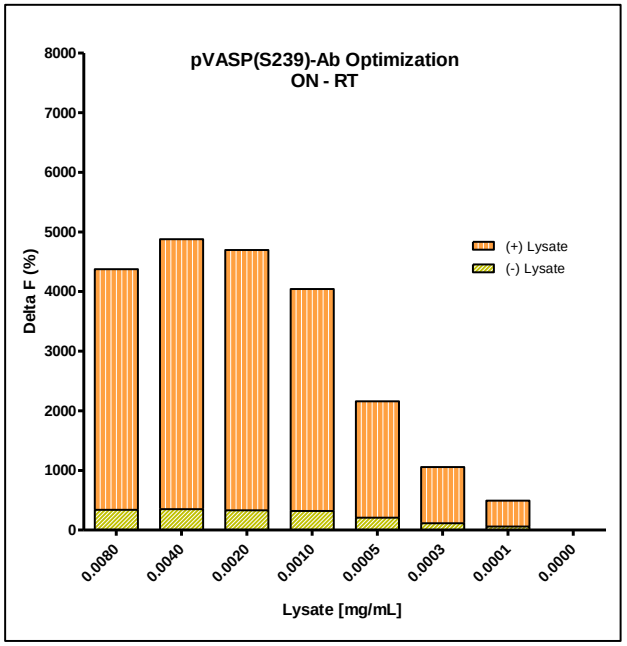




Custom Assay Development for VASP Phos-Ser239



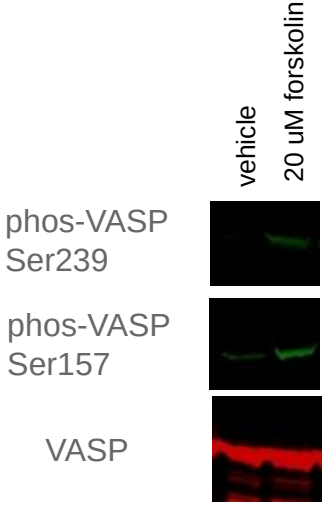
ANTIBODIES		
ID#	Name	Clonality
1	VASP Antibody-K	Poly
2	VASP Antibody-d2	Poly
3	VASP-K	Mono
4	VASP-d2	Mono
5	Anti-pVASP (Ser239)-K	Mono
6	Anti-pVASP (Ser239)-d2	Mono
7	VASP Antibody (Ab-239)-K	Poly
8	VASP Antibody (Ab-239)-d2	Poly
9	VASP (phospho-Ser239)-K	Mono
10	VASP (phospho-Ser239)-d2	Mono



- Tested 2 total VASP and 3 phos-VASP antibodies; each labeled with donor and acceptor using 3 concentrations of lysate samples
- Optimized concentration of each antibody using 3 concentrations each antibody in matrix fashion on 7 concentrations of lysate samples
- Final selection tested on titration of lysate samples (graphed)
  - Monoclonal phospho-VASP (Ser239) labeled d2
  - Monoclonal VASP labeled K
- Significant signal-to-noise between treated and untreated samples

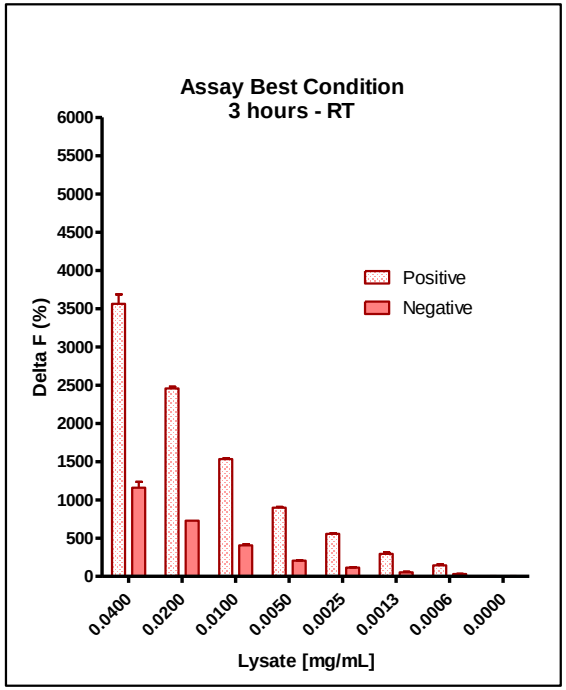
Custom Assay Development for VASP Phos-Ser157

Hek293 Cells
VASP Transient Transfection



HTRF Assay Development (Cisbio)

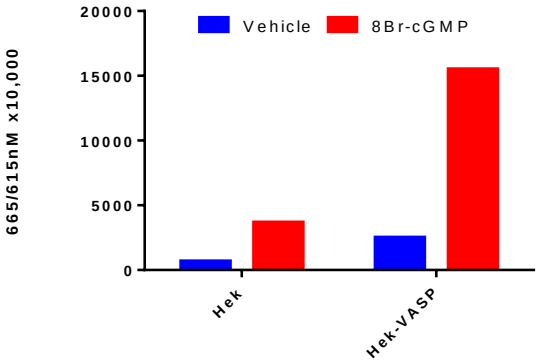
	ANTIBODIES	
ID#	Name	Clonality
1	VASP Antibody-K	Poly
2	VASP Antibody-d2	Poly
3	VASP-K	Mono
4	VASP-d2	Mono
5	Phospho-VASP (Ser157)-K	Poly
6	Phospho-VASP (Ser157)-d2	Poly
7	Anti-VASP (phospho S157)-K	Poly
8	Anti-VASP (phospho S157)-d2	Poly
9	Anti-VASP (phospho S157)-K	Mono
10	Anti-VASP (phospho S157)-d2	Mono



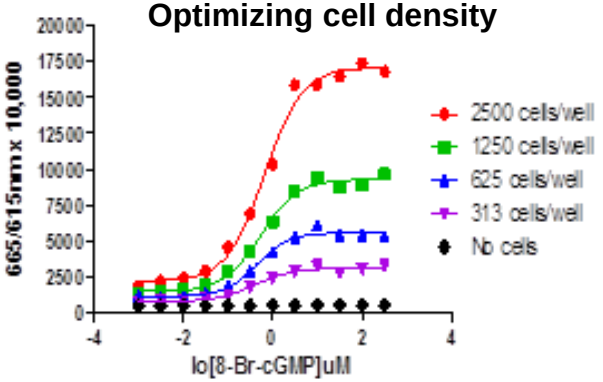
- Tested 2 total VASP and 3 phos-VASP antibodies; each labeled with donor and acceptor using 3 concentrations of lysate samples
- Optimized concentration of each antibody using 3 concentrations each antibody in matrix fashion on 7 concentrations of lysate samples
- Final selection tested on titration of lysate samples (graphed)
  - Monoclonal phospho-VASP labeled K
  - Monoclonal VASP labeled d2
- Significant signal-to-noise between treated and untreated samples

Optimizing Cell Type, Cell Density and Time Course

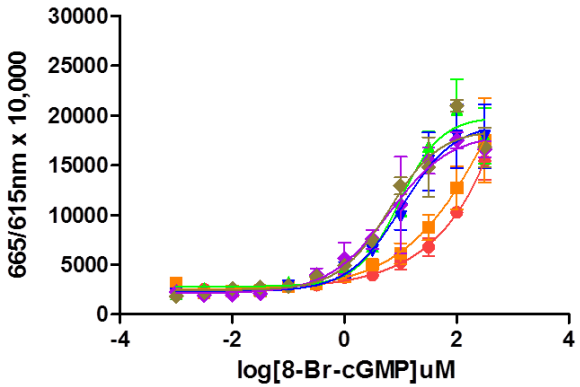
Phos-VASP Ser239
Hek vs. Hek VASP Stable cell line



Phos-VASP Ser239
Optimizing cell density



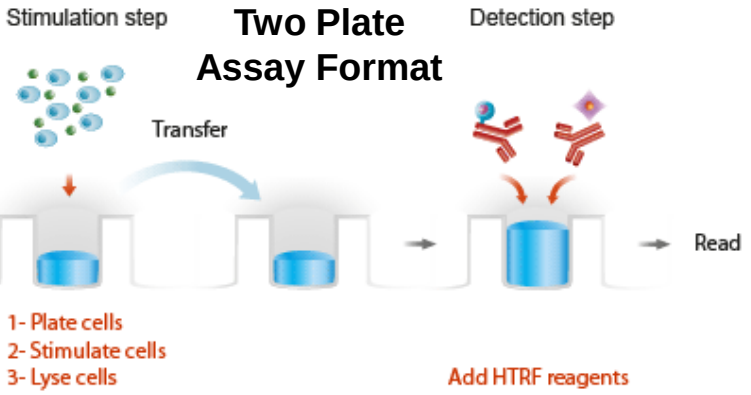
Phos-VASP Ser239
Optimizing Cell Treatment Time



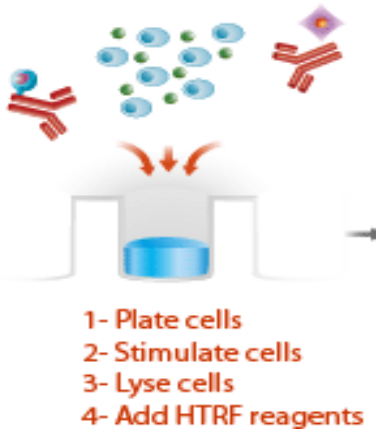
	EC50
10mins	~ 5.772e+007
20mins	671.6
30mins	9.596
40mins	10.44
50mins	6.202
60mins	6.215

Preferred Method: Hek-VASP cell line, 1250 cells/well, 30 min treatment

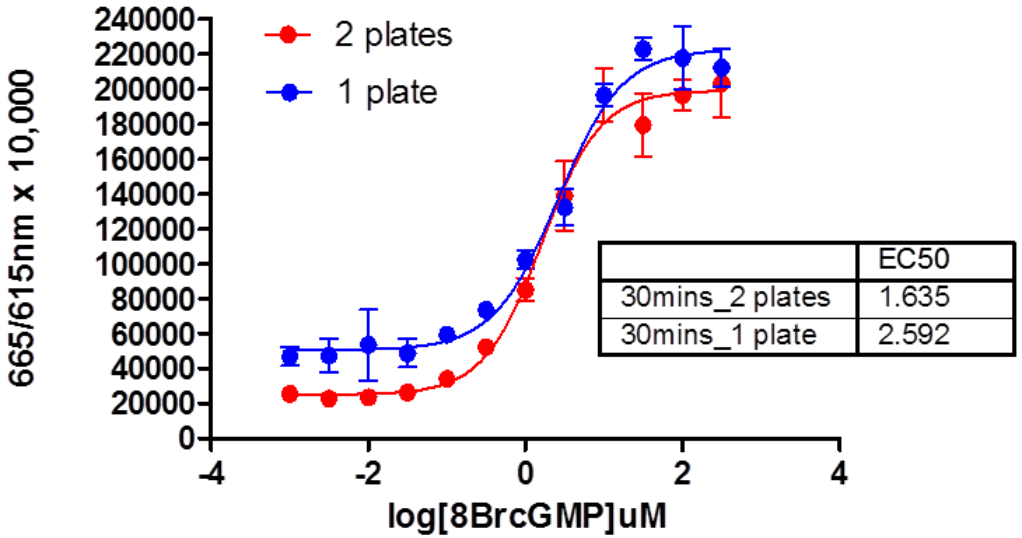
Two vs. One Plate Assay Format



One Plate Assay Format



Phos-Ser239 VASP: 2 vs. 1 Plate



	EC50
30mins_2 plates	1.635
30mins_1 plate	2.592

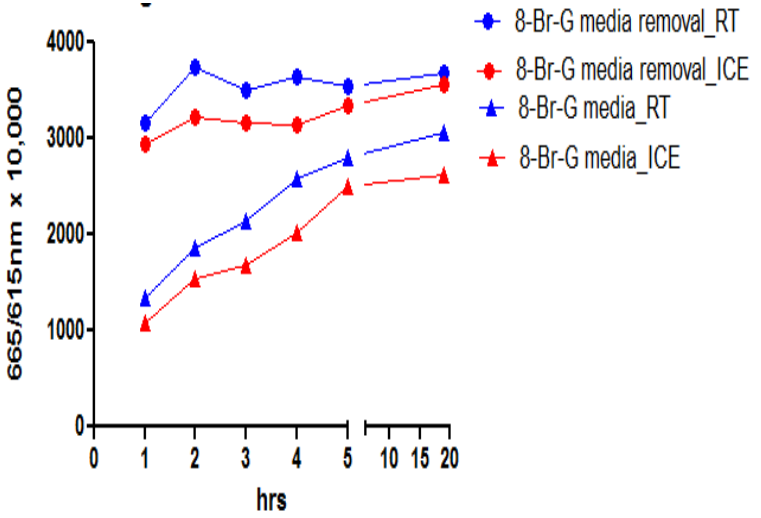
- Cells were treated with 8Br-cGMP for 30 minutes
- Media removed and cells lysed at RT and then transferred to new second plate or
- Cells lysed directly in media and assay continued in same plate

Preferred Method: One plate format

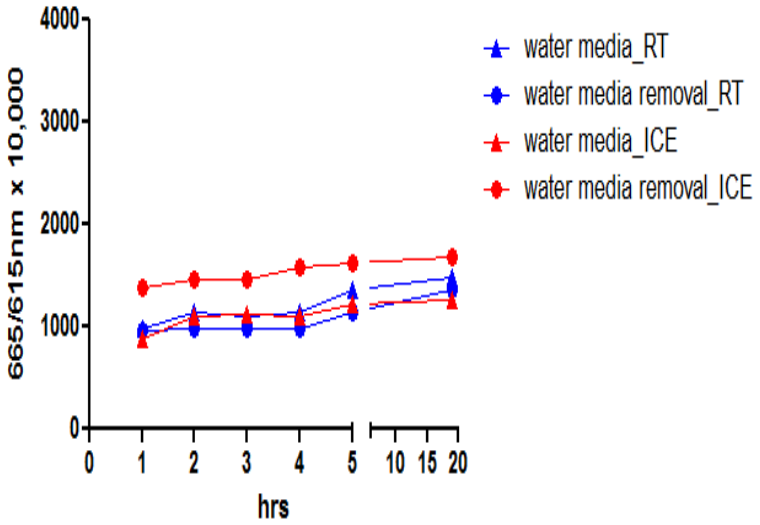
Optimizing Lysis Conditions
Exploring Signal Stability Over Time

Phos-Ser239 VASP

Cells treated with 8Br-cGMP



Cells treated with vehicle (water)



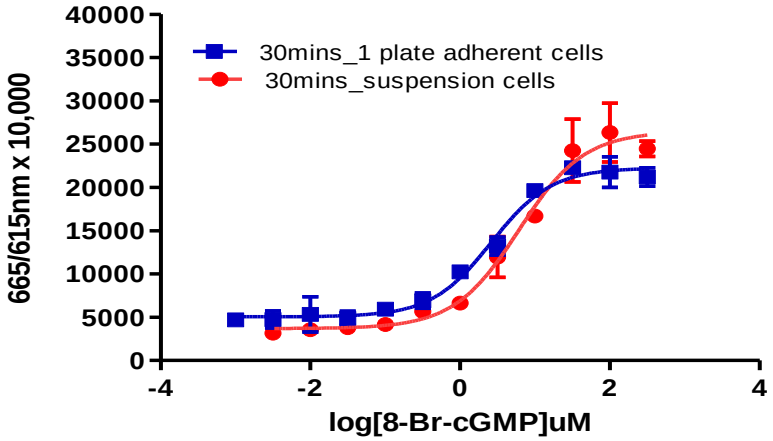
- Cells treated with 100 uM 8Br-cGMP
- Cells lysed by adding lysis buffer directly into the media, or the media was removed and then added to the cells
  - Later switched to treat cells in HBSs instead of media and were able to get maximum signal by 2 hours
- Cells lysed either on ice or RT for 30 minutes
- Signal read over time between 1 and 20 hours

Preferred Method: Lyse cells at RT and read plate after 2 hours

Assay Ready Frozen Cells in Suspension DMSO Tolerability

Phos-Ser239 VASP

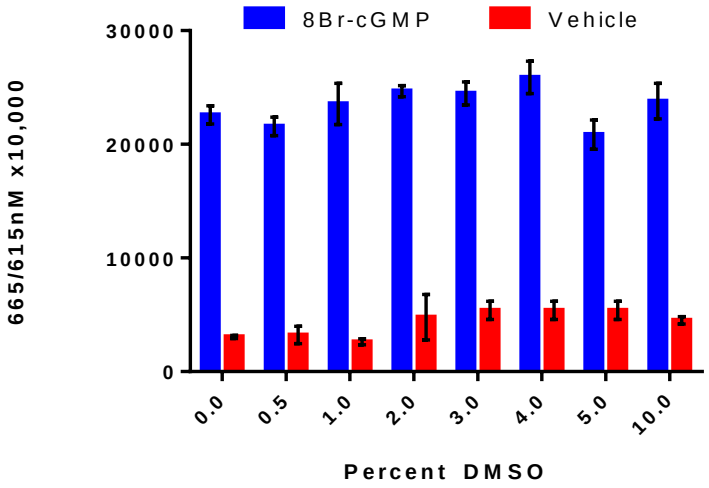
Cultured cells: Plated day before or day of assay



	EC50
30mins_suspension cells	6.099
30mins_1 plate adherent cells	2.612

- Cells were plated day before or day of assay
- Cells were treated with 8Br-cGMP for 30 minutes

Frozen cells in suspension: DMSO tolerability

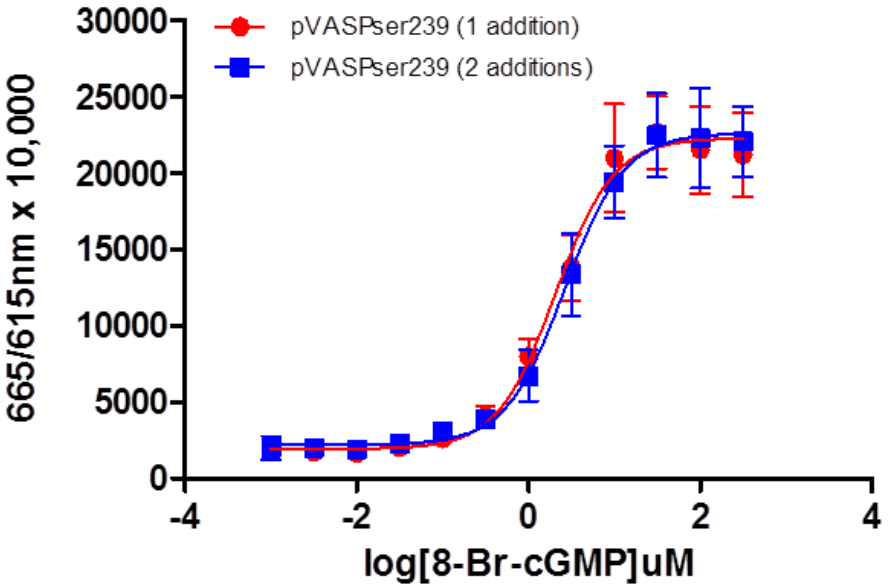


- Assay ready frozen cells in suspension plated day of assay
- Cells were treated with 100 uM 8Br-cGMP for 30 minutes

Preferred Method: Assay ready frozen cells in suspension with 1% DMSO

One vs. Two Additions for Antibody Reagents

Phos-Ser239 VASP: 1 vs. 2 step Antibody Addition

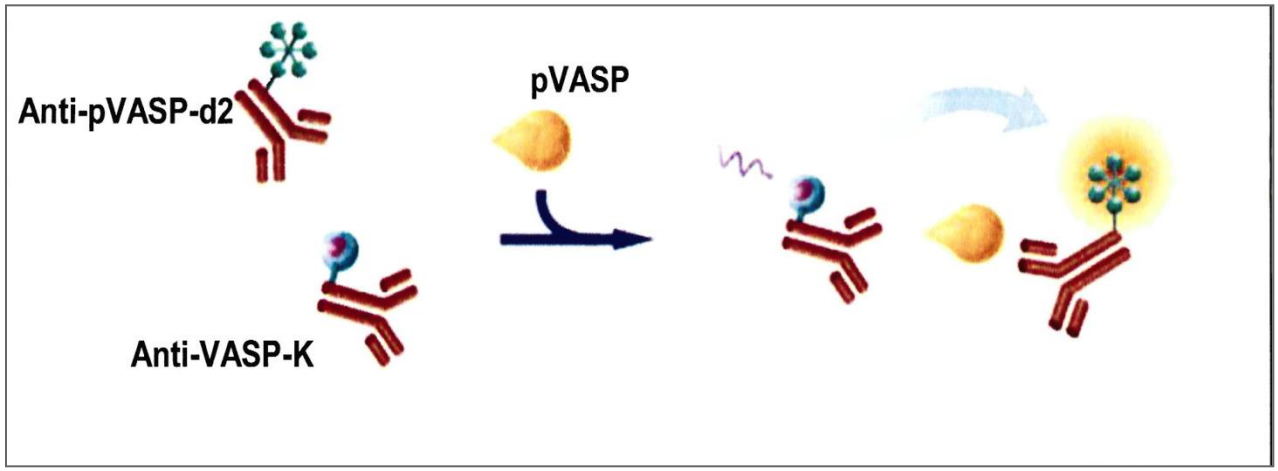


	EC50
pVASPser239 (1 addition)	2.071
pVASPser239 (2 additions)	2.657

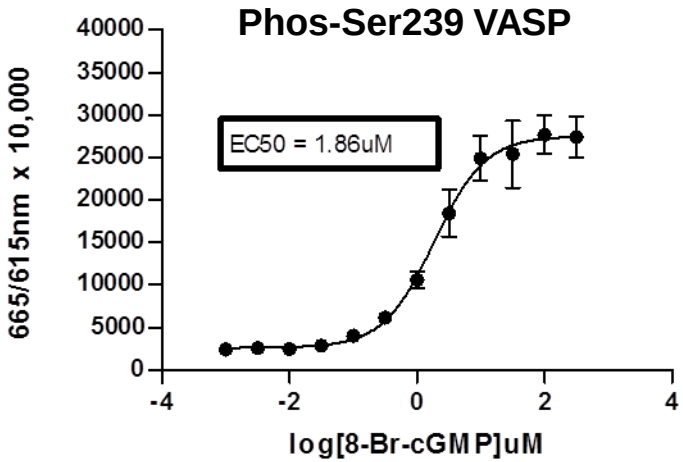
- Cells treated with 8Br-cGMP for 30 minutes;
- Antibodies added one at a time sequentially or pre-mixed and then added together

Preferred Method: Pre-mix antibodies and one step addition

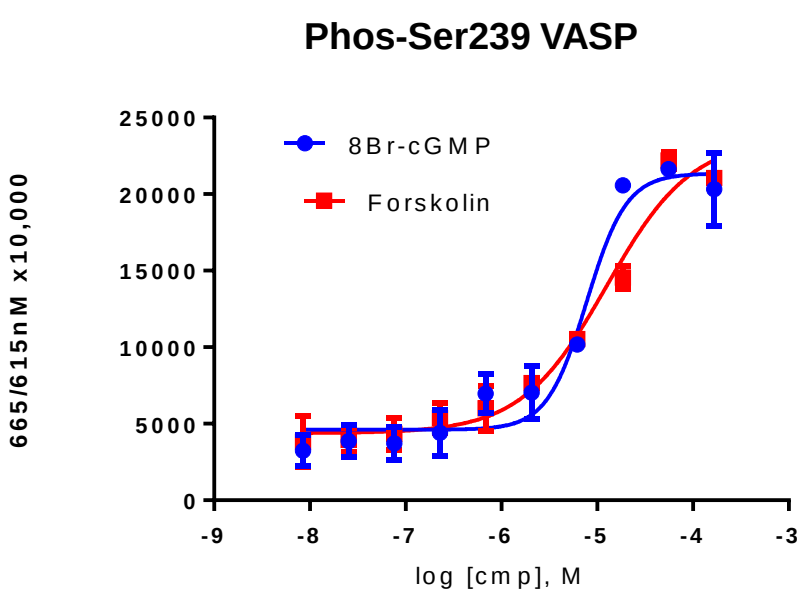
Phos-VASP Ser239 and Ser157 HTRF Assay

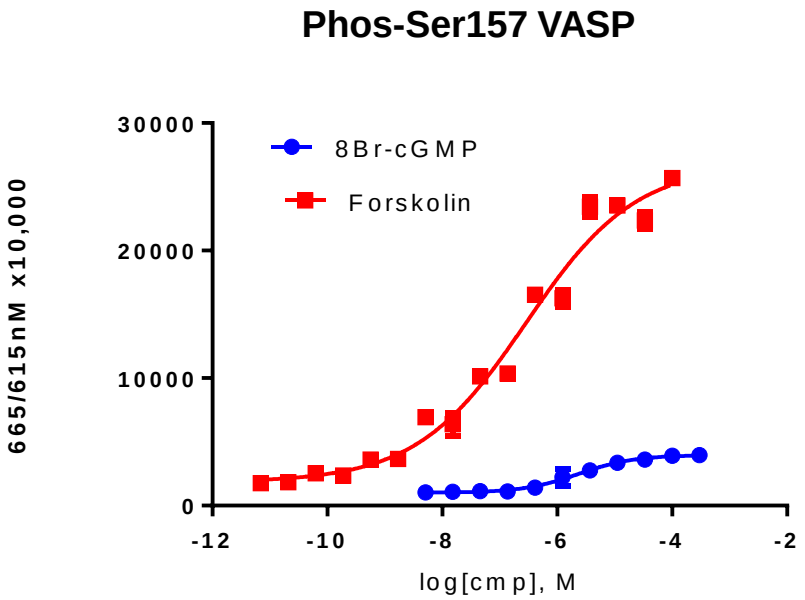


- Jump-In Hek293 or U2OS overexpressing VASP cell lines
- Assay ready frozen cells used in suspension day of assay
- 384 well low volume proxiplate
- 1250 cells per well in 6 ul HBSS
- Compound treatment in 1 % DMSO
- Lyse cells (2 ul) directly into HBSS at room temperature for 30 minutes
- Mix antibodies and add to plate (2 ul)
- Read two hours later, up to 20+ hours later
- ~0.7 Z-prime



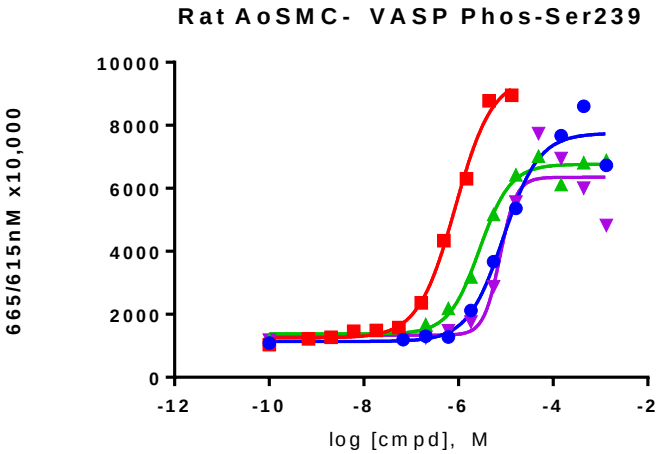
Recombinant Cell Line for Screening: Hek293 Cells Overexpressing VASP

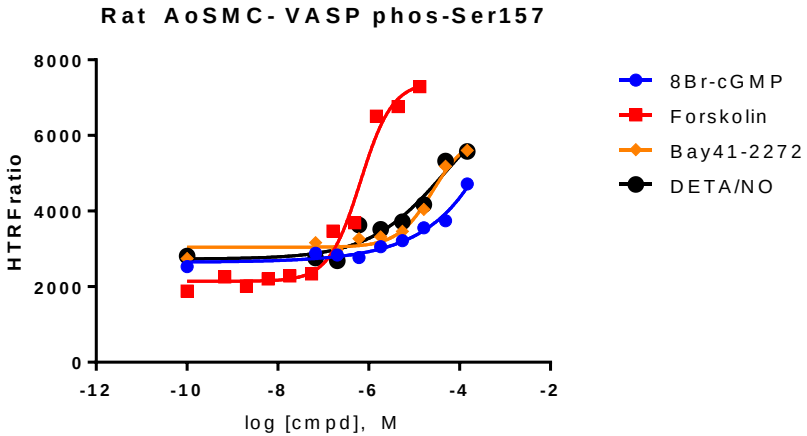




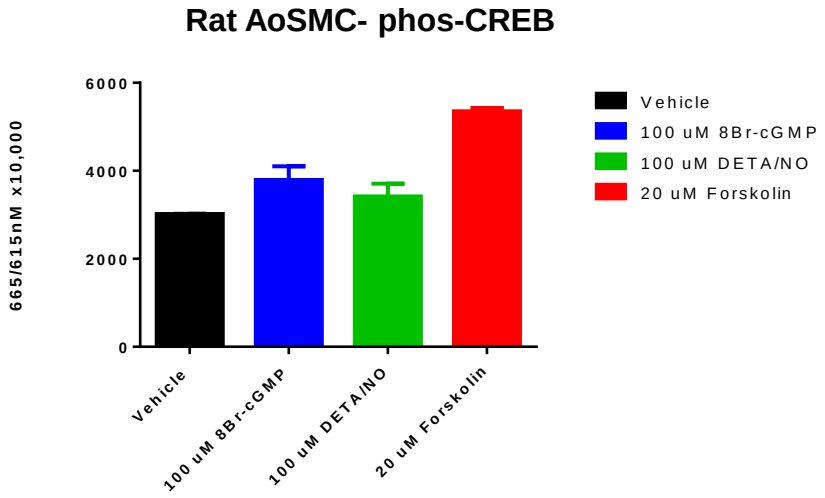
cAMP pathway leads to Ser239 and Ser157 VASP phosphorylation
cGMP pathway leads to Ser239 VASP phosphorylation

Physiological Relevant Cells: Rat Aortic Smooth Muscle Cells

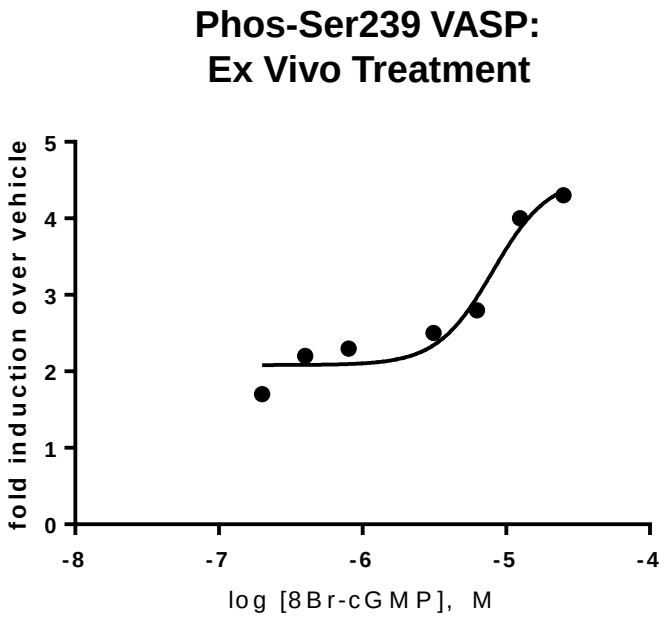




- cAMP pathway leads to Ser239 and Ser157 VASP phosphorylation and ~2-fold induction phos-CREB
- cGMP pathway leads to Ser239 VASP phosphorylation

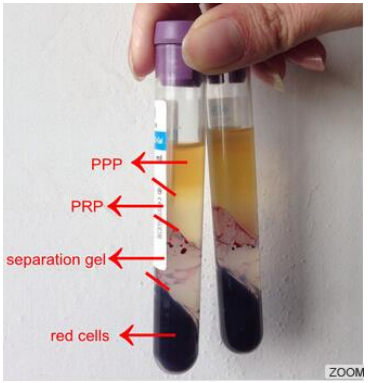


Mouse Whole Blood Ex Vivo Assay



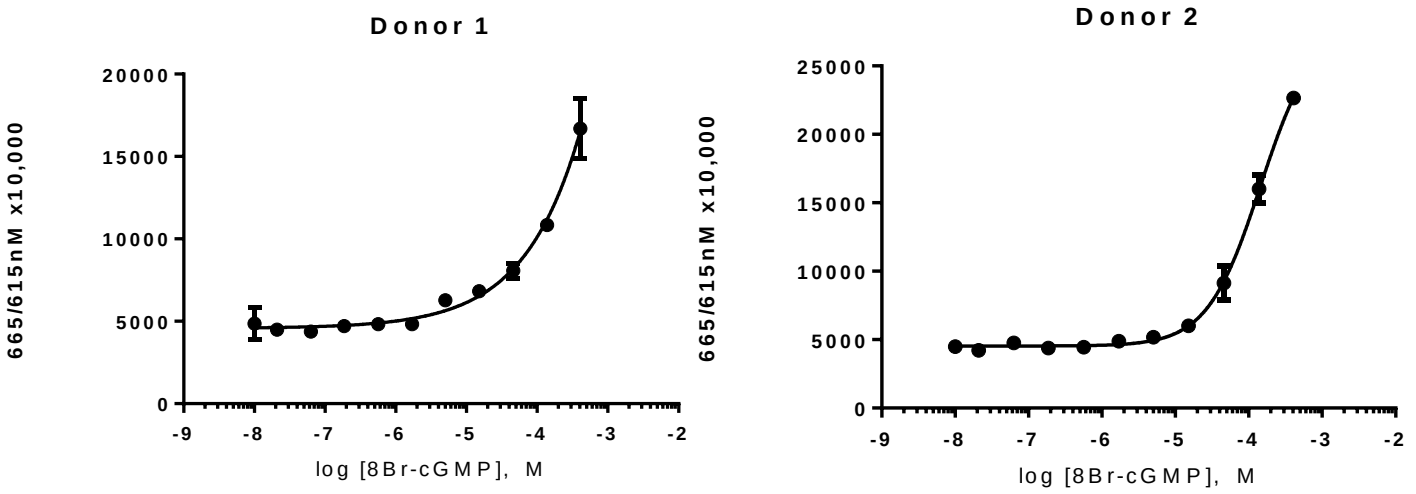
VASP HTRF can be used as target engagement assay to help establish in vitro in vivo correlations

Human Platelet Ex Vivo Assay



- Human donor blood collected in citrate tubes
- Blood centrifuged to separate RBCs and plasma
- Plasma centrifuged in a second step
- Remove plasma and retain platelet pellet
- Platelets treated with Prostagladin E1
- Platelets counted and plated in 384-well plate
- Platelets treated ex-vivo
- HTRF assay performed

Phos-Ser239 VASP: Ex Vivo Treatment



VASP HTRF can be used on human platelet samples for potential biomarker
Potential to look at PBMC and whole blood

Conclusion

- We developed VASP HTRF assays to measure phos-Ser239 and phos-Ser157 in cells.
  - 384 well format, 10 ul final volume, robust signal
- Assay can be used to differentiate compounds affecting cGMP and cAMP pathways

- Advantages of HTRF for Target Engagement
  - Assay can be run in high throughput in a streamlined fashion with few liquid additions, no liquid exchanges and with minimal steps
  - Robust assay in recombinant cells for routine screening
  - Assay translates to physiologically relevant cells (ex. Rat AoSMCs)
  - Assay can be performed on ex vivo and in vivo animal model samples and utilized to help establish in vitro in vivo correlations
  - Assay has potential as biomarker assay for human clinical samples