Multiplexing:
Measure more than cytokines!

AGENDA

- ✓ Module 1: xMAP technology based genomic and proteomic applications
- ✓ Module 2: Lab demo of SNP typing assay

- ❑ **Module 3: Milliplex MAP phospho-signaling**

- ❑ Module 4: Lab demo of a signaling assay
- ❑ Module 5: Wrap up, additional questions/review

Ramsey McIntire, PhD

Multiplex & Cytometry Specialist

EMD Millipore

MILLIPLEX MAP - Market Leader!

Multiplex Assays

Human
Cytokine/chemokine
T lymphocytes
Cytokine receptors
Immunoglobulin
Cardiovascular
Cancer
IGFBPs
Angiogenesis
Metastasis
Neurodegenerative
Neuropeptide
Adipokine/Adipocyte
Metabolic hormone
Liver
Pituitary
Thyroid
Bone
Skin
Kidney Toxicity

Signaling
Y/S/T phosphorylation
Apoptosis
RTK
OxPhos
Fatty Acid Ox
Glycolysis

Primate
Cytokine/chemokine
Metabolic hormone

Rat
Cytokine/chemokine
Immunoglobulin
Adipokine/Adipocyte
Metabolic hormone
Pituitary
Stress hormone
Thyroid
Bone
Cardiovascular
Neuropeptide
Kidney toxicity
Vascular injury

Mouse
Cytokine/chemokine
T lymphocytes
Immunoglobulin
Adipokine/adipocyte
Metabolic hormone
Pituitary
Thyroid
Bone
Cardiovascular
Angiogenesis
Neuropeptide

Canine
Cytokine/chemokine
Metabolism
Pituitary
Kidney Toxicity

Porcine
Cytokine/chemokine

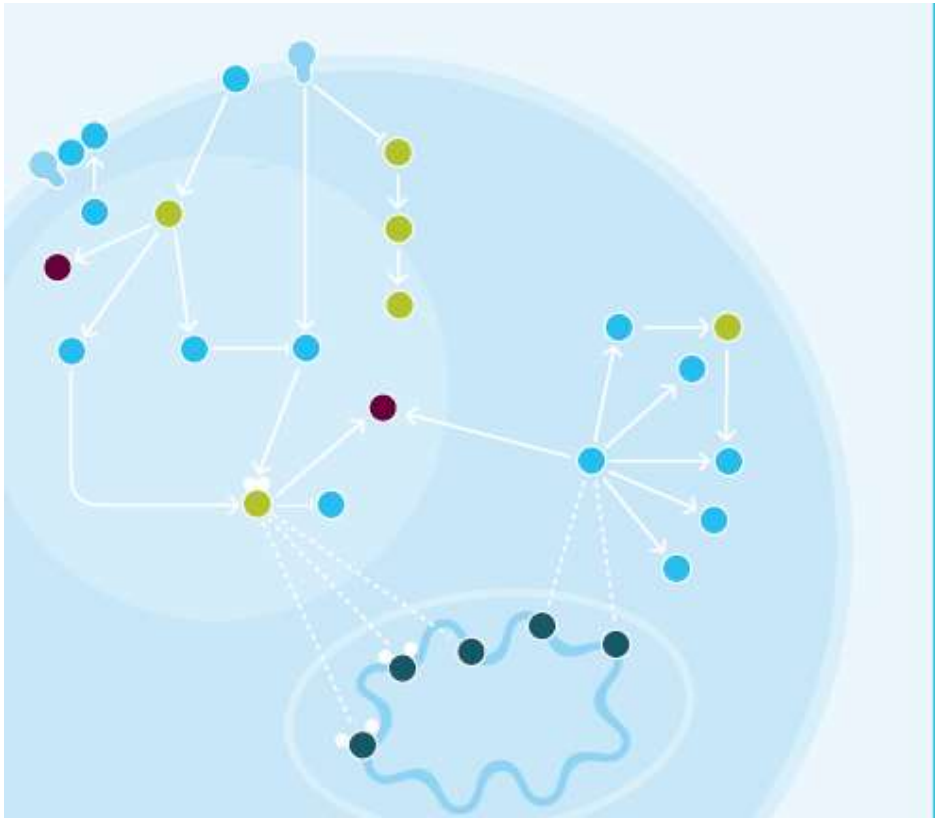
The Challenges of Cell Signaling

Cell signaling is part of a complex system of communication that dictates basic activities in the cell and coordinates cellular functions.

Protein targets of interest are often transient and low expression.

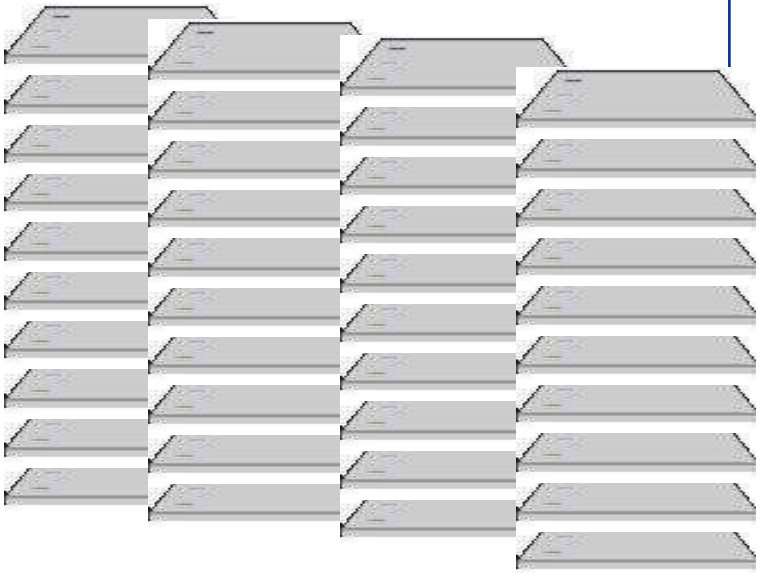
Why multiplex?

- Analyze multiple proteins in a pathway simultaneously
- Analyze multiple pathways in one cell/tissue lysate simultaneously
- Save samples and time!

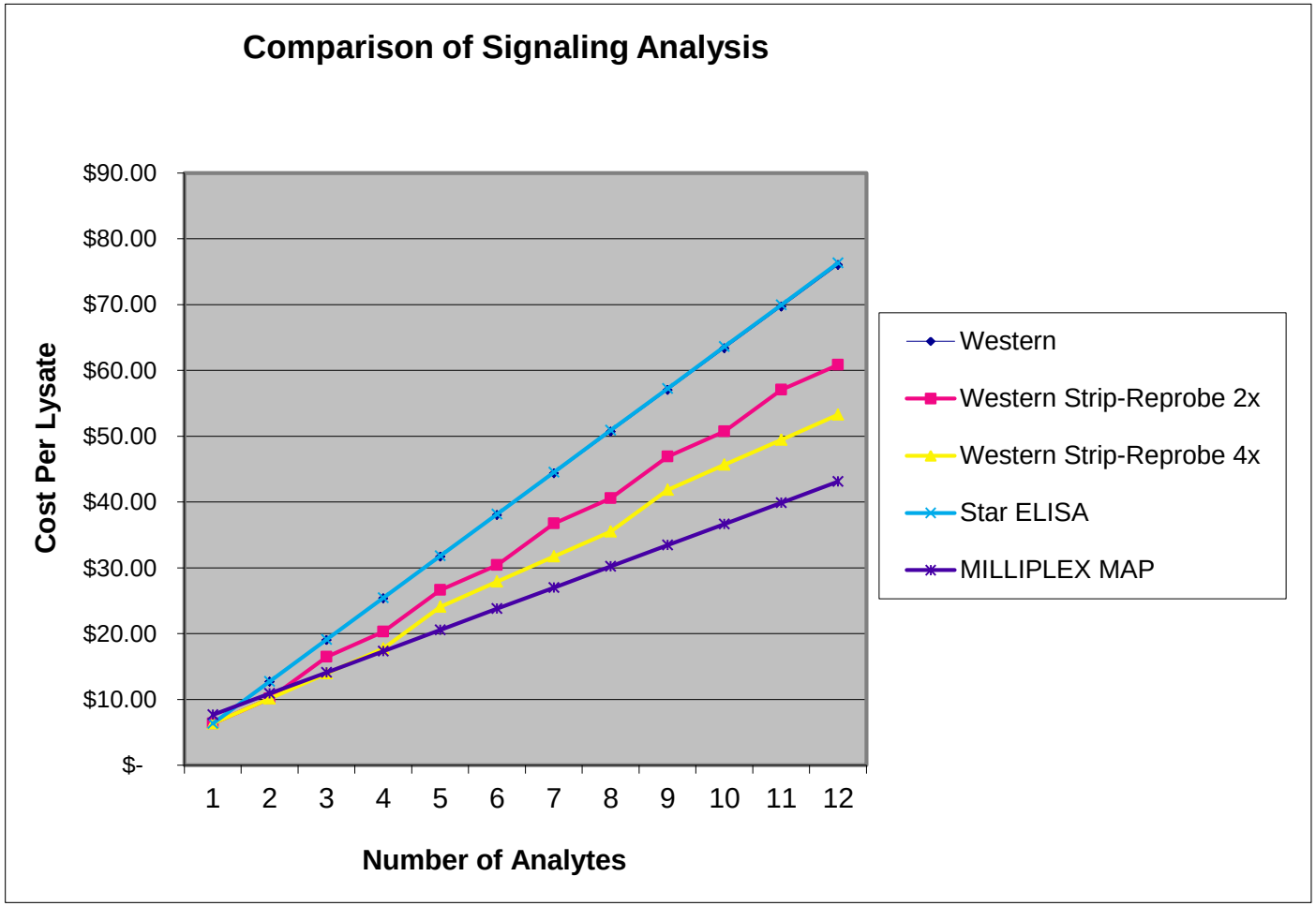


Milliplex vs. Western Blot

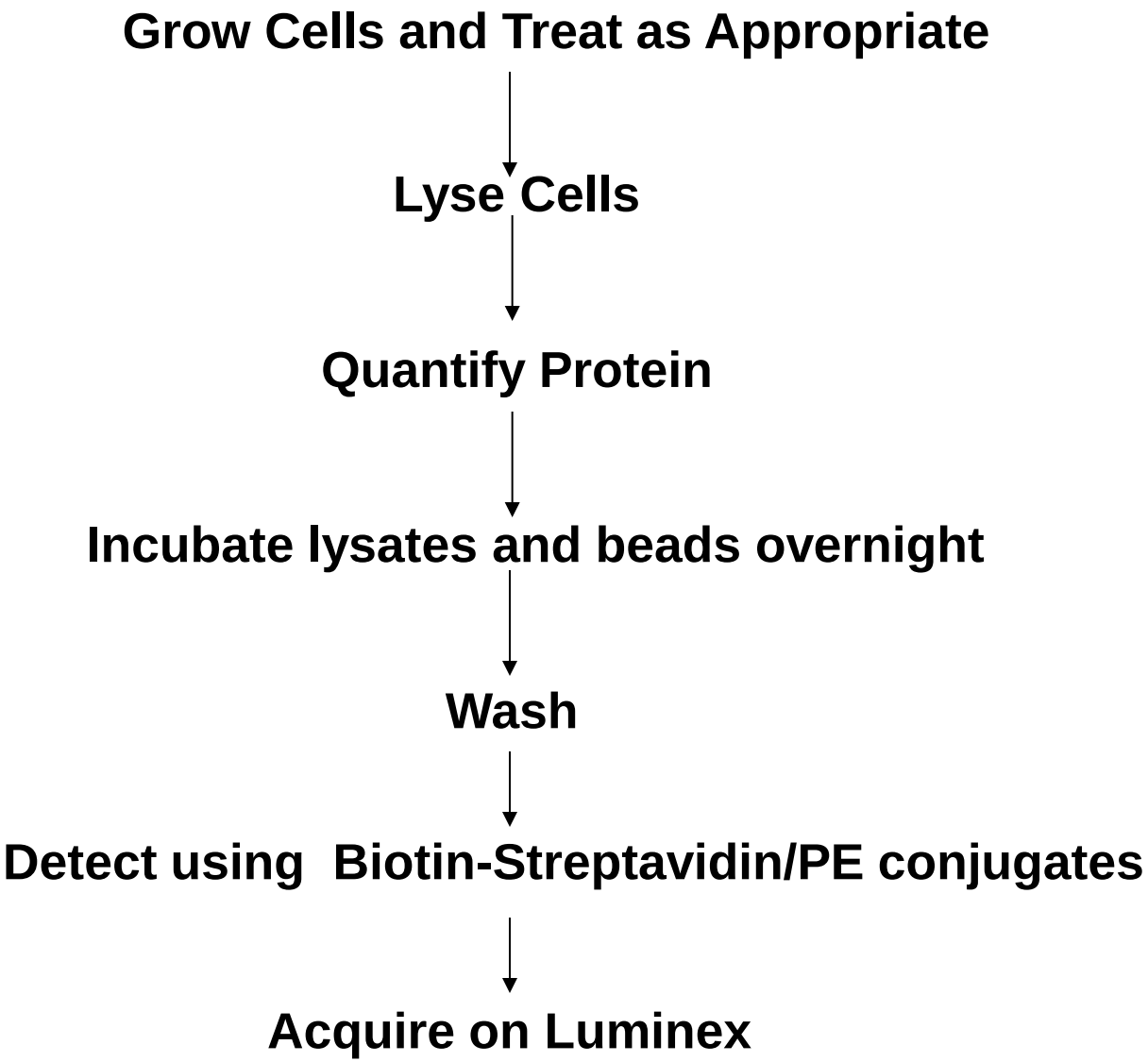
10 proteins
40 samples

	xMAP® Technology	Western blot
Number of assays required		
Total time to result	18 hours	Days/weeks
Results per assay	400	~10
Total sample used	50 µl (1-25 µg)	~200 µl (~100-500 µg)
Internal controls possible?	YES	NO

Western Blot Comparison

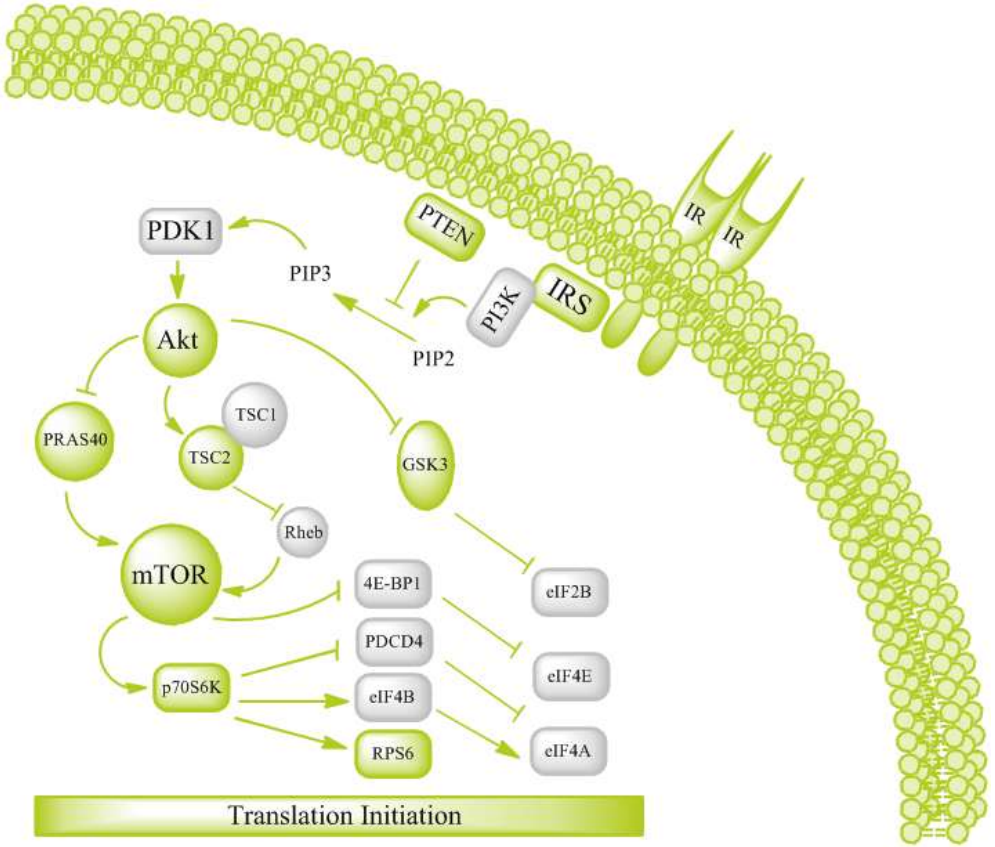


Milliplex Intracellular Assay Procedure



Akt/mTOR Panel: 11-plex

Analytes:
p70S6K (Thr412)
IRS1 (Ser312)
GSK3α (Ser21)
GSK3b (Ser9)
Akt (Ser473)
PTEN (Ser380)
IR (Tyr1162/Tyr1163)
IGF1R (Tyr1135/Tyr1136)
RPS6 (Ser235/Ser236)
TSC2 (Ser939)
mTOR (Ser2448)



The MILLIPLEX MAP Human Akt/mTOR Phosphoprotein Magnetic Bead Panel 11-plex, is used to detect changes in phosphorylated p70S6K (Thr412), IRS1 (Ser312), GSK3a (Ser21), GSK3b (Ser9), Akt (Ser473), PTEN (Ser380), IR Tyr1162/Tyr1163), IGF1R (Tyr1135/Tyr1136), RPS6 (Ser235/Ser236), TSC2 (Ser939), and mTOR (Ser2448) in cell lysates using the Luminex® system. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit has sufficient reagents for one 96-well plate assay.

Akt/mTOR Panel

11-plex Akt/mTOR Panel Analysis of Insulin Treated HepG2 and IGF-1 Treated MCF-7 Cells

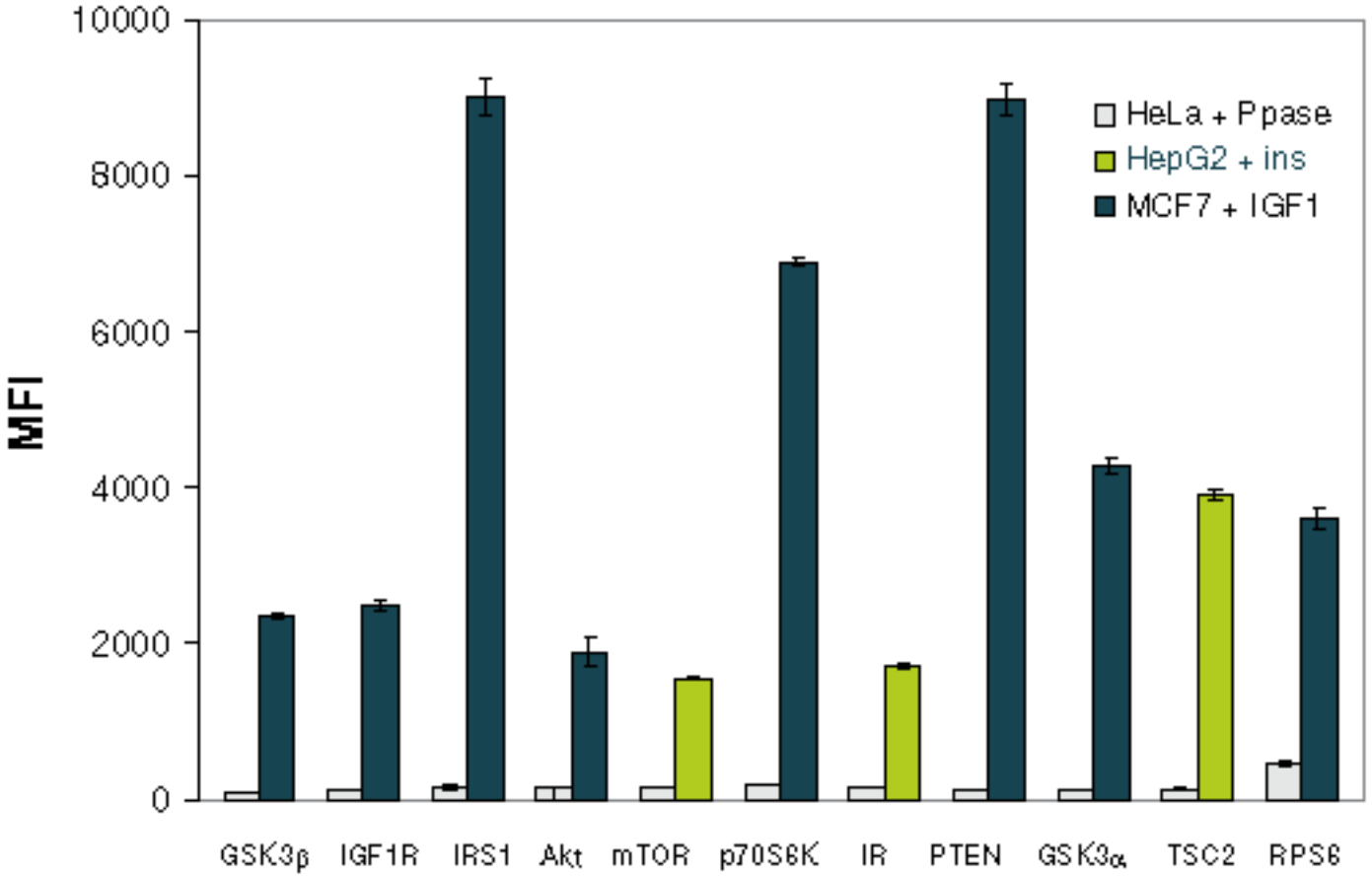
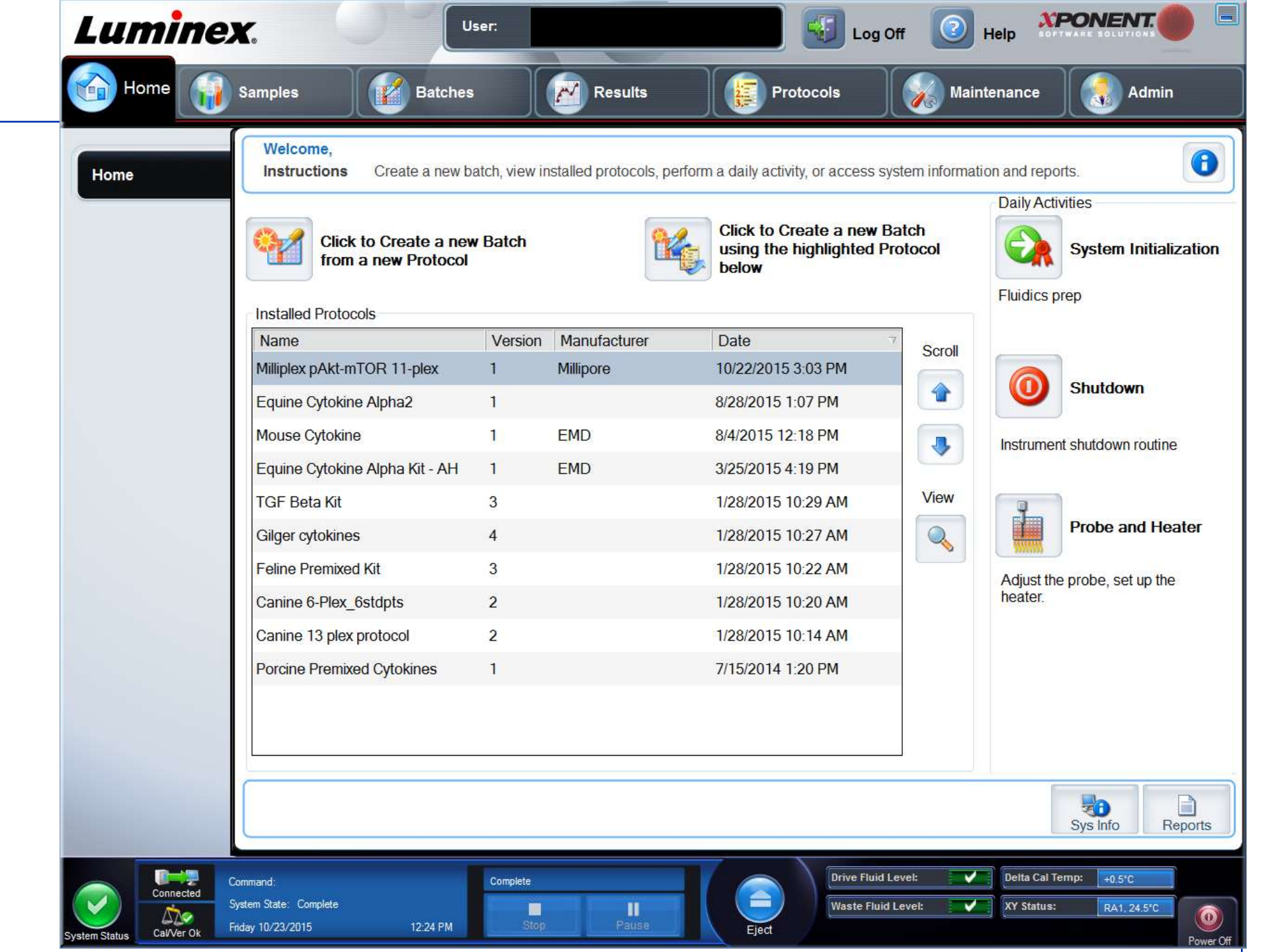


Figure 1. Multiplex analysis of HepG2 and MCF-7 cells treated with insulin or IGF-1. HepG2 cells stimulated with 10 µg/mL of insulin (15 min) or MCF-7 cells stimulated with 50 ng/mL IGF-1 (10 min) were assayed. The cells were lysed in MILLIPLEX MAP Lysis Buffer containing protease inhibitors. 20 µg total protein of each lysate diluted in MILLIPLEX MAP Assay Buffer 2 were analyzed according the Assay protocol (lysate incubation at 4ºC overnight). The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of three replicate

Akt/mTOR Panel: Western to Panel Comparison

1. HeLa untreated
2. HeLa treated with lambda phosphatase
3. HepG2 untreated
4. HepG2 + insulin
5. HepG2 + 0.1 μM wortmannin + insulin *PI3K inh.
6. HepG2 + 0.1 μM rapamycin + insulin
7. HepG2 + 10 μM U0126 + insulin
8. HepG2 + 50 μM LY-294-002 + insulin *PI3K Inh.
9. HepG2 + 1 μM Ro-31-8220 + insulin *Rsk2 inh.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SOFTWARE SOLUTIONS
Home
Samples
Batches
Results
Protocols
Maintenance
Admin
Home
Welcome,
Instructions
Create a new batch, view installed protocols, perform a daily activity, or access system information and reports.
Click to Create a new Batch from a new Protocol
Click to Create a new Batch using the highlighted Protocol below
Installed Protocols
Name
Version
Manufacturer
Date
Milliplex pAkt-mTOR 11-plex 1 Millipore 10/22/2015 3:03 PM
Equine Cytokine Alpha2 1 8/28/2015 1:07 PM
Mouse Cytokine 1 EMD 8/4/2015 12:18 PM
Equine Cytokine Alpha Kit - AH 1 EMD 3/25/2015 4:19 PM
TGF Beta Kit 3 1/28/2015 10:29 AM
Gilger cytokines 4 1/28/2015 10:27 AM
Feline Premixed Kit 3 1/28/2015 10:22 AM
Canine 6-Plex_6stdpts 2 1/28/2015 10:20 AM
Canine 13 plex protocol 2 1/28/2015 10:14 AM
Porcine Premixed Cytokines 1 7/15/2014 1:20 PM
Scroll
View
Daily Activities
System Initialization
Fluidics prep
Shutdown
Instrument shutdown routine
Probe and Heater
Adjust the probe, set up the heater.
Sys Info
Reports
System Status
Connected
Cal/Ver Ok
Command:
System State: Complete
Friday 10/23/2015 12:24 PM
Complete
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Waste Fluid Level:
Delta Cal Temp: +0.5°C
XY Status: RA1, 24.5°C
Power Off

Luminex

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Current Batch

Saved Batches

LIS Results

Reports

Now displaying batch "Milliplex pAkt-mTOR 11-plex".

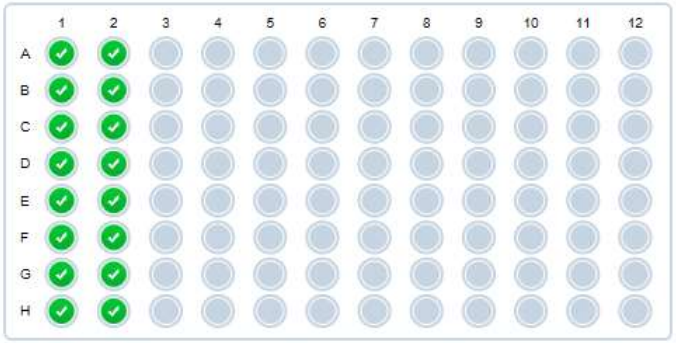
Instructions View current run statistics and analyte progress per well. For more analysis and result options for this batch, select Details or go to Saved Batches when batch acquisition completes.

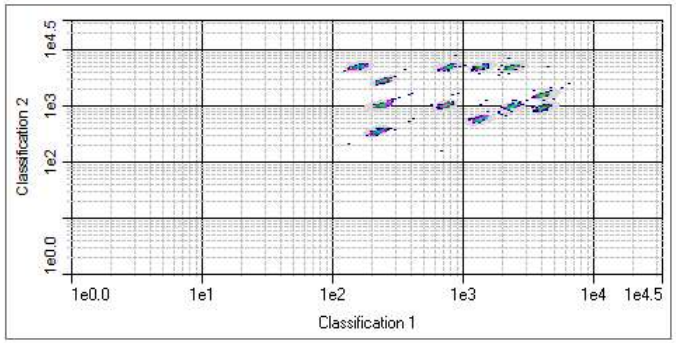
Results

Statistic: Median Analyte: Select an Analyte Current Well 1,H2 Single Step

	Well	Sample	Run Sta...	b-tubulin	GSK3b	IGF1R	IRS1	Akt	mTOR	p70S6K
	1,A1	Background0	Ok	82	30	42.5	31	127	73	544
	1,B1	Background0	Ok	93	35	50	36	147	78	497
	1,C1	Unknown1	Ok	1203	101	69	155	154	437.5	180
	1,D1	Unknown1	Ok	1201.5	100	68	152	155	437	178

Plate 1





Date	Message	Code
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10/23/2015 12:17:20 PM	Rinse completed at RD1.	54D
10/23/2015 12:17:21 PM	Clean started at RC1.	54B

Save Image | Chg. Vol | Progress

System Status | Connected | Cal/Ver Ok | Command: Clean at RC1 | System State: Active | Friday 10/23/2015 12:19 PM | Batch Running | Stop | Pause | Eject | Drive Fluid Level: | Waste Fluid Level: | Delta Cal Temp: +0.6°C | XY Status: RC1, 24.6°C | Power Off

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Current Batch

Saved Batches

LIS Results

Reports

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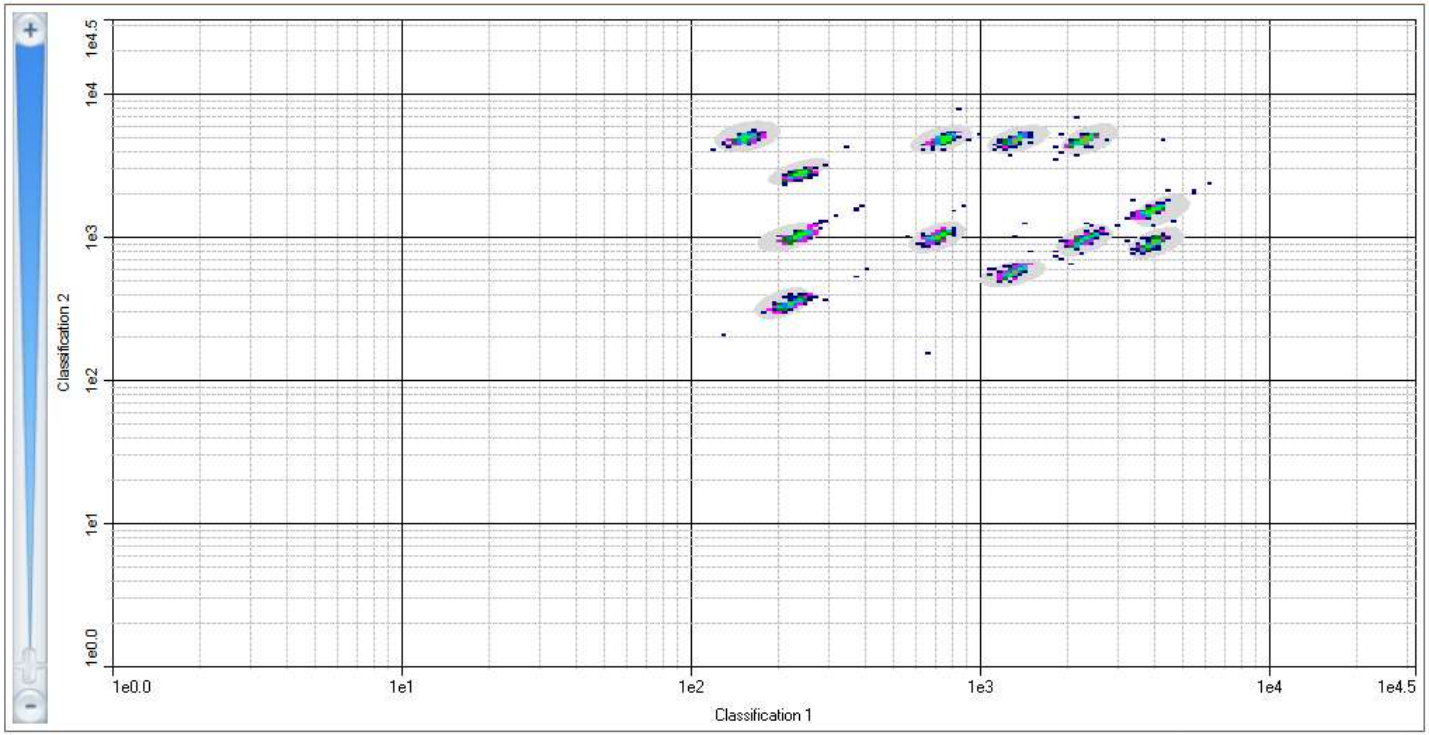
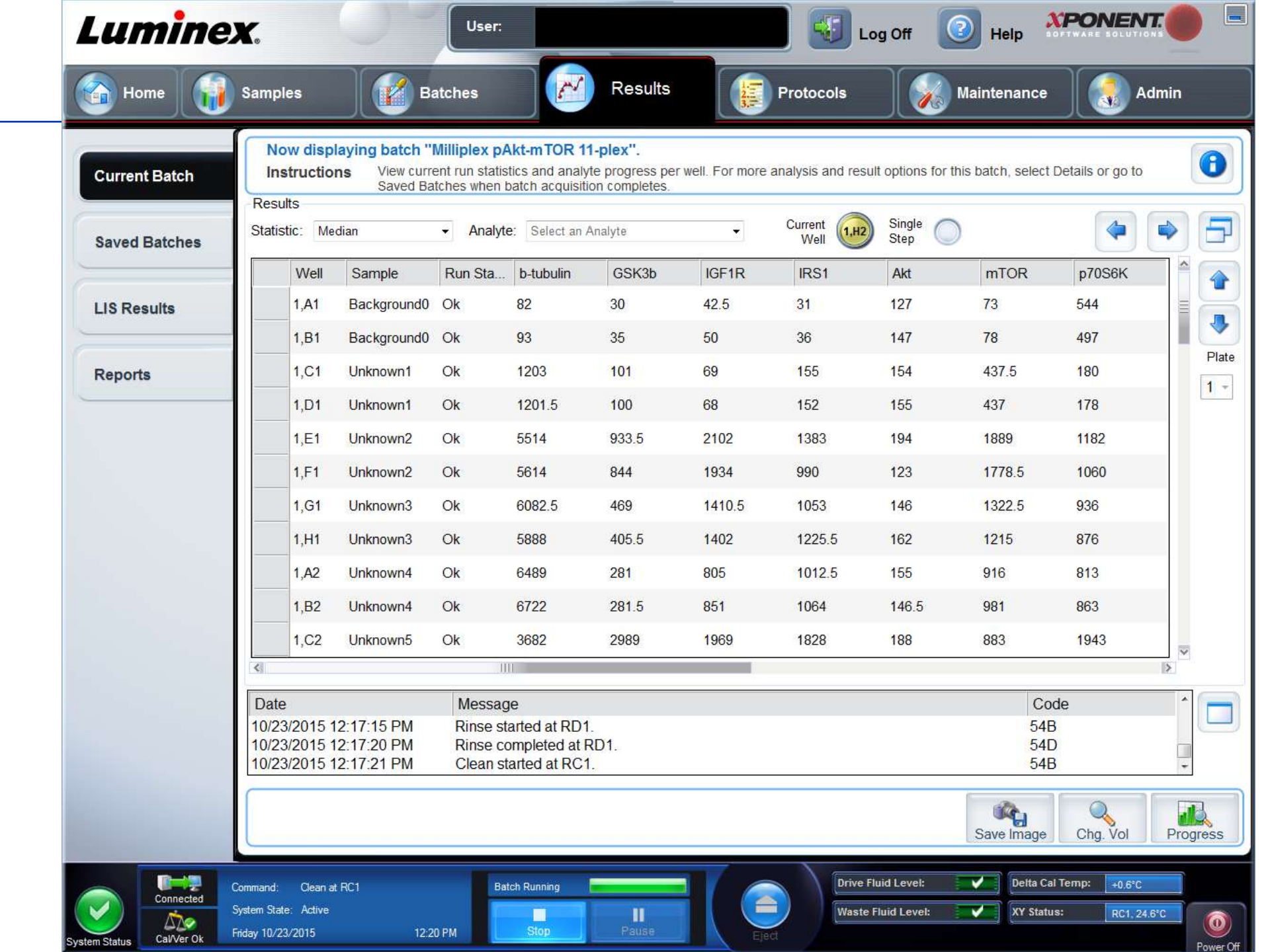
Instructions View current run statistics and analyte progress per well. For more analysis and result options for this batch, select Details or go to Saved Batches when batch acquisition completes.



Date	Message	Code
10/23/2015 12:17:15 PM	Rinse started at RD1.	54B
10/23/2015 12:17:20 PM	Rinse completed at RD1.	54D
10/23/2015 12:17:21 PM	Clean started at RC1.	54B

Save Image | Chg. Vol | Progress

System Status | Connected | Cal/Ver Ok | Command: Clean at RC1 | System State: Active | Friday 10/23/2015 12:20 PM | Batch Running | Stop | Pause | Eject | Drive Fluid Level: | Waste Fluid Level: | Delta Cal Temp: +0.6°C | XY Status: RC1, 24.5°C | Power Off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Results
Protocols
Maintenance
Admin
Current Batch
Saved Batches
LIS Results
Reports
Now displaying batch "Milliplex pAkt-mTOR 11-plex".
Instructions
View current run statistics and analyte progress per well. For more analysis and result options for this batch, select Details or go to Saved Batches when batch acquisition completes.
Results
Statistic: Median
Analyte: Select an Analyte
Current Well
1,H2
Single Step
Plate
1
Well | Sample | Run Sta... | b-tubulin | GSK3b | IGF1R | IRS1 | Akt | mTOR | p70S6K
1,A1 | Background0 | Ok | 82 | 30 | 42.5 | 31 | 127 | 73 | 544
1,B1 | Background0 | Ok | 93 | 35 | 50 | 36 | 147 | 78 | 497
1,C1 | Unknown1 | Ok | 1203 | 101 | 69 | 155 | 154 | 437.5 | 180
1,D1 | Unknown1 | Ok | 1201.5 | 100 | 68 | 152 | 155 | 437 | 178
1,E1 | Unknown2 | Ok | 5514 | 933.5 | 2102 | 1383 | 194 | 1889 | 1182
1,F1 | Unknown2 | Ok | 5614 | 844 | 1934 | 990 | 123 | 1778.5 | 1060
1,G1 | Unknown3 | Ok | 6082.5 | 469 | 1410.5 | 1053 | 146 | 1322.5 | 936
1,H1 | Unknown3 | Ok | 5888 | 405.5 | 1402 | 1225.5 | 162 | 1215 | 876
1,A2 | Unknown4 | Ok | 6489 | 281 | 805 | 1012.5 | 155 | 916 | 813
1,B2 | Unknown4 | Ok | 6722 | 281.5 | 851 | 1064 | 146.5 | 981 | 863
1,C2 | Unknown5 | Ok | 3682 | 2989 | 1969 | 1828 | 188 | 883 | 1943
Date | Message | Code
10/23/2015 12:17:15 PM | Rinse started at RD1. | 54B
10/23/2015 12:17:20 PM | Rinse completed at RD1. | 54D
10/23/2015 12:17:21 PM | Clean started at RC1. | 54B
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Chg. Vol
Progress
System Status
Connected
Cal/Ver Ok
Command: Clean at RC1
System State: Active
Friday 10/23/2015 12:20 PM
Batch Running
Stop
Pause
Eject
Drive Fluid Level:
Waste Fluid Level:
Delta Cal Temp: +0.6°C
XY Status: RC1, 24.6°C
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Akt/mTOR Panel

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PLoS one

Tissue-Specific Responses of IGF-1/Insulin and mTOR Signaling in Calorie Restricted Rats

Naveen Sharma[1], Carlos M. Castorena[1], Gregory D. Cartee[1,2,3]*

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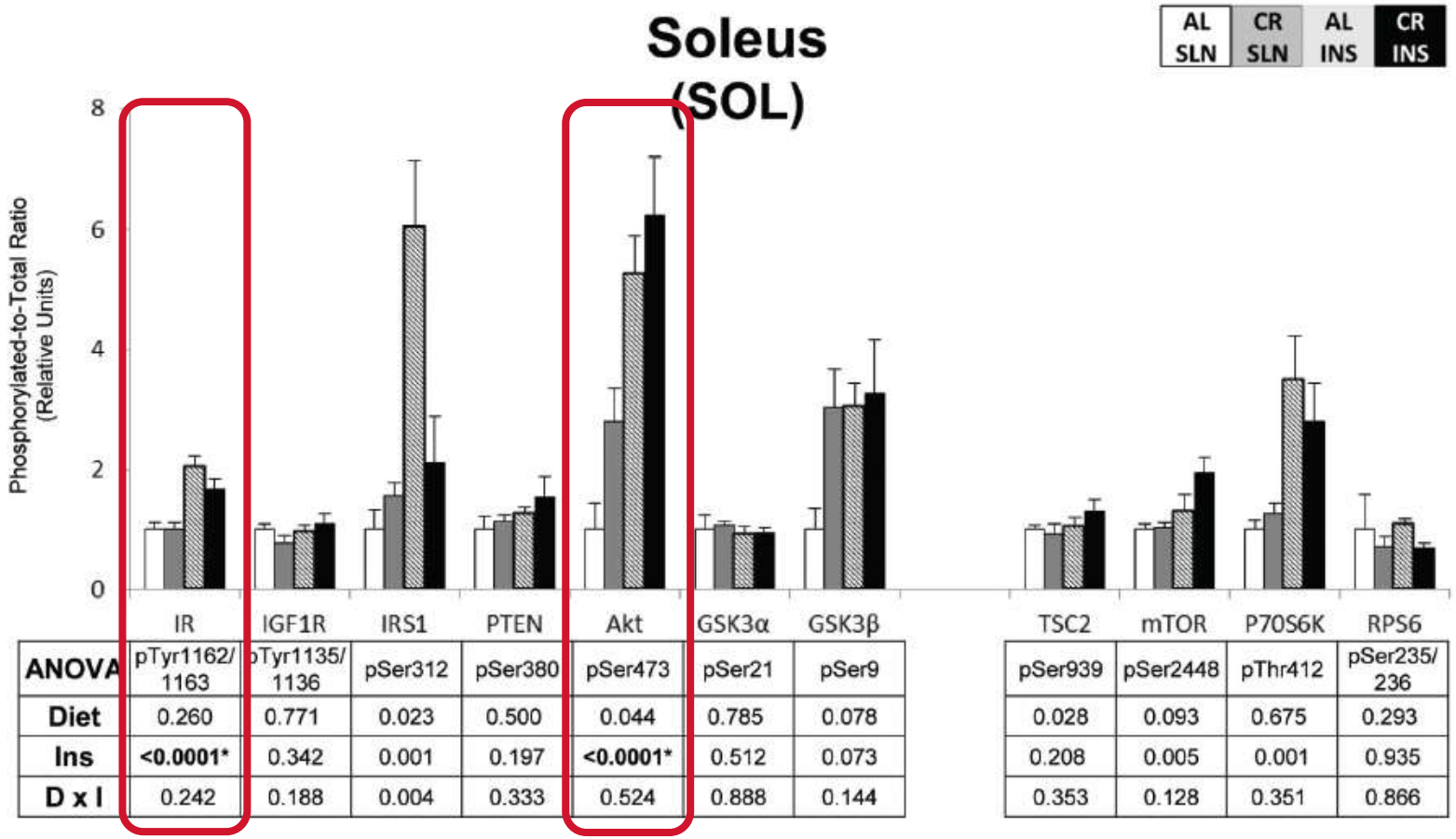
Abstract

Moderate calorie restriction (CR) (~60% of ad libitum, AL, intake) has been associated with numerous favorable physiological outcomes in many species, and the insulin/IGF-1 and mTOR signaling pathways have each been proposed as potential mediators for many of CR's bioeffects. However, few studies have assessed the widely held idea that CR induces the down-regulation of the insulin/IGF-1 and/or mTOR pathways in multiple tissues. Accordingly, we analyzed the phosphorylation status of 11 key signaling proteins from the insulin/IGF-1 ($IR^{Tyr1162/1163}$, $IGF\text{-}1R^{Tyr1135/1136}$, $IRS\text{-}1^{Ser312}$, $PTEN^{Ser380}$, Akt^{Ser473}, $GSK3\alpha^{Ser21}$, $GSK3\beta^{Ser9}$) and mTOR ($TSC2^{Ser939}$, $mTOR^{Ser2448}$, $P70S6K^{Thr412}$, $RPS6^{Ser235/236}$) pathways in 11 diverse tissues [liver, kidney, lung, aorta, two brain regions (cortex and cerebellum), and two slow-twitch and three fast-twitch skeletal muscles] from 9-month-old male AL and CR Fischer 344 x Brown Norway rats. The rats were studied under two conditions: with endogenous insulin levels (i.e., AL>CR) and with insulin infused during a hyperinsulinemic-euglycemic clamp so that plasma insulin concentrations were matched between the two diet groups. The most striking and consistent effect of CR was greater pAkt in 3 of the 5 skeletal muscles of CR vs. AL rats. There were no significant CR effects on the mTOR signaling pathway and no evidence that CR caused a general attenuation of mTOR signaling across the tissues studied. Rather than supporting the premise of a global downregulation of insulin/IGF-1 and/or mTOR signaling in many tissues, the current results revealed clear tissue-specific CR effects for the insulin signaling pathway without CR effects on the mTOR signaling pathway.

Measured effects of Calorie Restriction and Insulin treatment in multiple skeletal muscles, brain, liver, kidney, heart, and lung



ANOVA	pTyr1162/1163	pTyr1135/1136	pSer312	pSer380	pSer473	pSer21	pSer9	pSer939	pSer2448	pThr412	pSer235/236
Diet	0.260	0.771	0.023	0.500	0.044	0.785	0.078	0.028	0.093	0.675	0.293
Ins	**<0.0001***	0.342	0.001	0.197	**<0.0001***	0.512	0.073	0.208	0.005	0.001	0.935
D x I	0.242	0.188	0.004	0.333	0.524	0.888	0.144	0.353	0.128	0.351	0.866

Overall finding: Calorie restriction showed tissue-specific effects, but more consistently impacted insulin/IGF rather than mTOR

Src Family Kinase Panel

Cancer Cell
Article

Integrative Genomic and Proteomic Analyses Identify Targets for *Lkb1*-Deficient Metastatic Lung Tumors

Julian Carretero,[1,2,13] Takeshi Shimamura,[1,3,13] Klarisa Rikova,[4] Autumn L. Jackson,[5] Matthew D. Wilkerson,[5] Christa L. Borgman,[1] Matthew S. Buttarazzi,[1,7] Benjamin A. Sanofsky,[1,7] Kate L. McNamara,[1,7] Kathleyn A. Brandstetter,[1,7] Zandra E. Walton,[1,7] Ting-Lei Gu,[4] Jeffrey C. Silva,[4] Katherine Crosby,[4] Geoffrey I. Shapiro,[1,3] Sauveur-Michel Maira,[8] Hongbin Ji,[9] Diego H. Castrillon,[10] Carla F. Kim,[11] Carlos Garcia-Echeverria,[8] Nabeel Bardeesy,[12] Norman E. Sharpless,[5,6] Neil D. Hayes,[5] William Y. Kim,[5,6] Jeffrey A. Engelman,[12] and Kwok-Kin Wong[1,3,7,*]

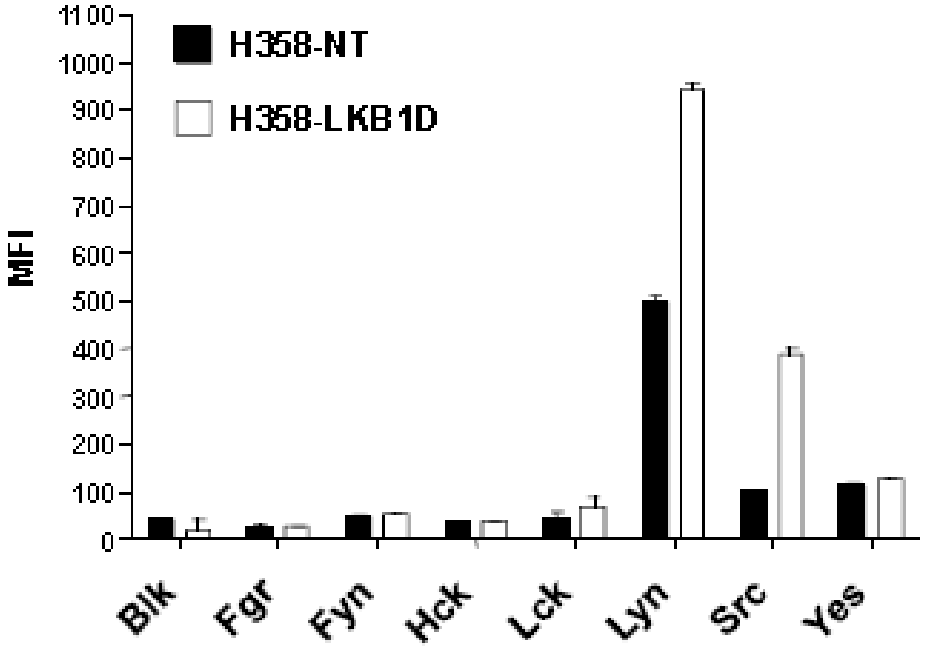
SUMMARY

In mice, *Lkb1* deletion and activation of $Kras^{G12D}$ results in lung tumors with a high penetrance of lymph node and distant metastases. We analyzed these primary and metastatic de novo lung cancers with integrated genomic and proteomic profiles, and have identified gene and phosphoprotein signatures associated with *Lkb1* loss and progression to invasive and metastatic lung tumors. These studies revealed that SRC is activated in *Lkb1*-deficient primary and metastatic lung tumors, and that the combined inhibition of SRC, PI3K, and MEK1/2 resulted in synergistic tumor regression. These studies demonstrate that integrated genomic and proteomic analyses can be used to identify signaling pathways that may be targeted for treatment.

Src Family Kinase Panel

Because Phosphoscan and western blot analyses detect a phosphorylation site (Y416) that is common to all SRC family kinases (SFKs) including SRC, FYN, LYN, LCK, HCK FGR, and YES, we interrogated the phosphorylation status of SFKs affected by LKB1 loss in NCI-H358 and A549 cell lines using a Luminex bead assay.

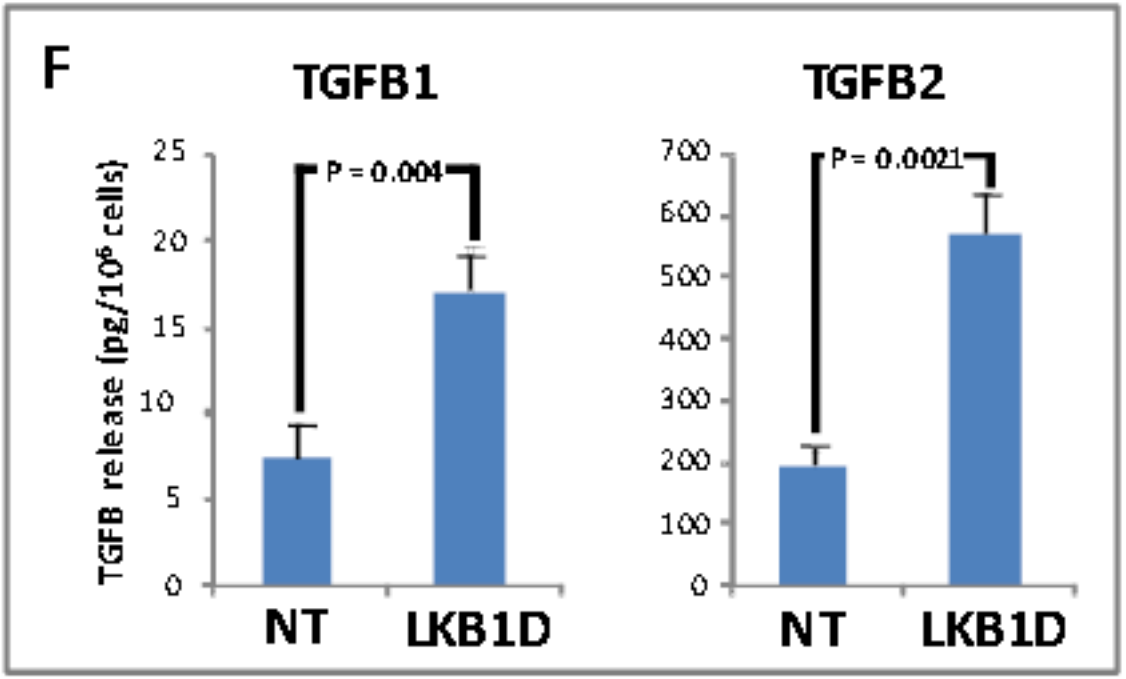
These changes in SRC and FAK suggest impaired adhesion and increased cellular motility (Yeatman, 2004).



D. Phosphoprotein profile of NCI-H358 cells analyzed by protein arrays.

NCI-H358 cells stably expressing shRNA to *LKB1* (LKB1D) or a non-specific shRNA (NT) were subjected to a kinase array as described in materials in methods.

Increased TGFbeta secretion: EMT?



F. TGF-β release analyzed by multiplex bead assay. *In vitro* secretion of TGFB1, TGFB2 and TGFB3 (below detection limit, not shown) were carried out in NCI-H358 cells stably expressing shRNA to *LKB1* (LKB1D) or a non-specific shRNA (NT). Conditioned media were collected after 24h of cell culture and incubated with beads coated with specific antibodies for TGFB1, TGFB2 and TGFB3 as described in Methods. Data is graphed as mean of 3 replicates (pg/10^6 cells ± SD).

MILLIPLEX MAP Cell Signaling Portfolio

Pathway Panels:

Glycolysis
Oxidative Phosphorylation
Oxidative Stress
Pyruvate Dehydrogenase
Akt/mTOR
Early Apoptosis
Late Apoptosis
DNA Damage
Heat Shock Protein
MAPK/SAPK
Mitogenesis RTK
Multi-Pathway
NFkB
Src Kinase
STAT
Human T Cell Receptor
TGFbeta

Phospho-Total 2 Plexes:

Akt/PKB
CREB
Erk/MAPK ½
IRS1
JNK
mTOR
p38
STAT3
(all of above can be plexed)

Individual MapMates:

46 and counting!

Thank you!

Ramsey McIntire: Multiplex & Cytometry

Karen Tamul: Field Application Scientist

Michelle Dennis: All things EMD Millipore!