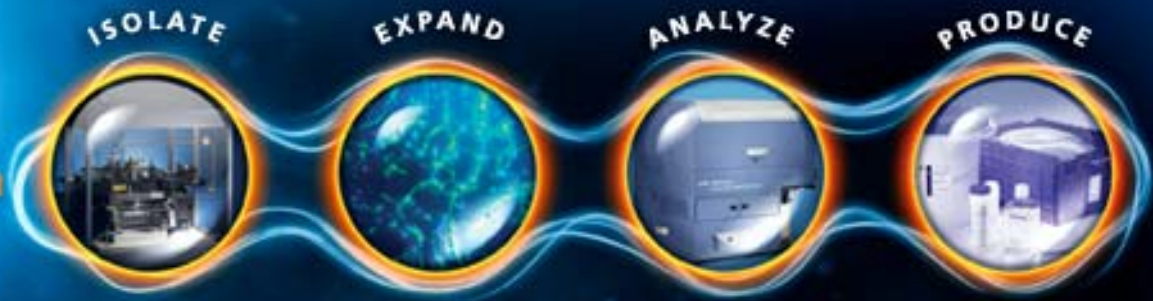


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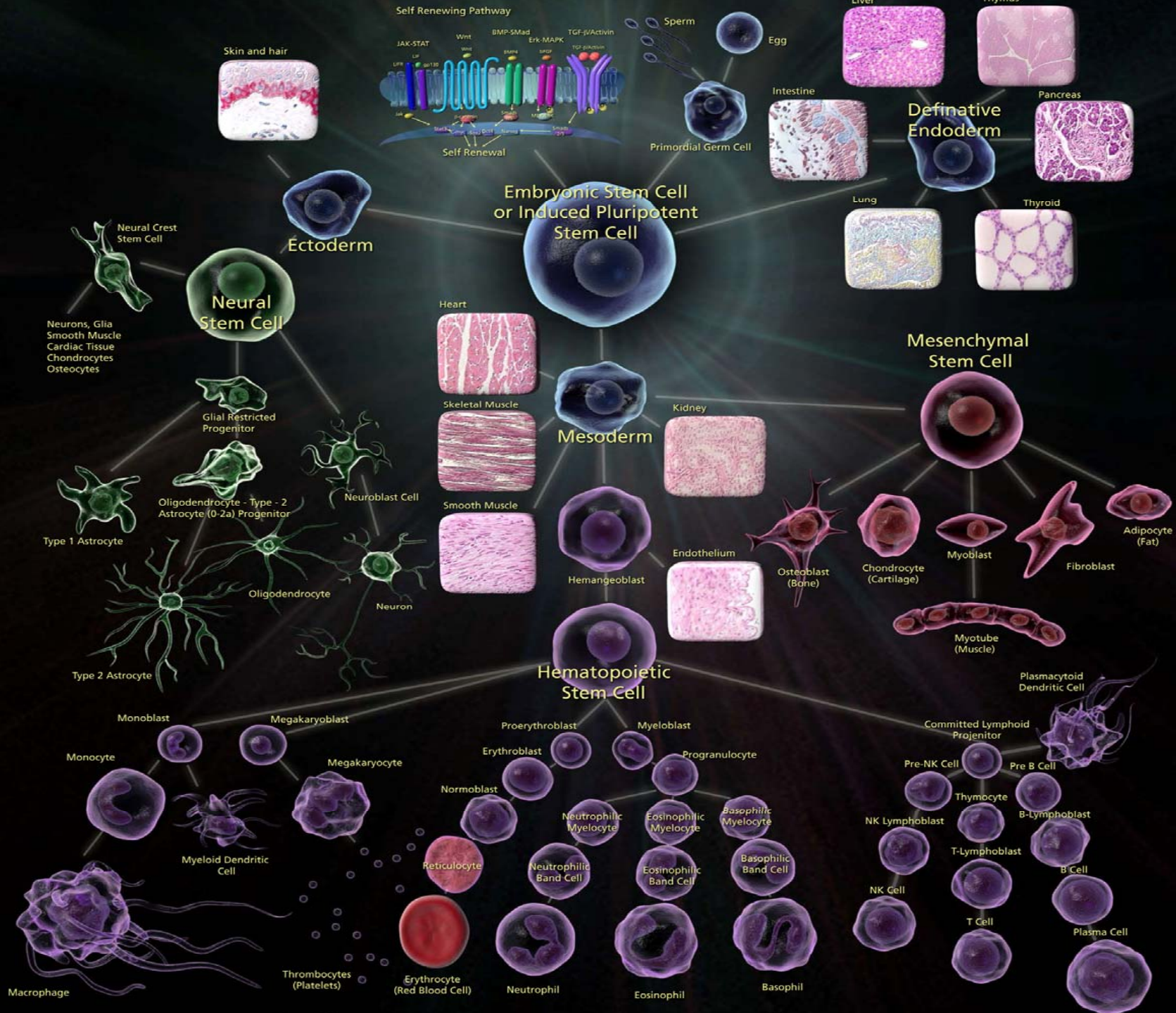
Quantitative Flow Cytometric Analysis of Kinases and Transcription Factors Involved in Maintaining Pluripotency in Human Embryonic Stem Cells

Stephanie Widmann
BD Biosciences

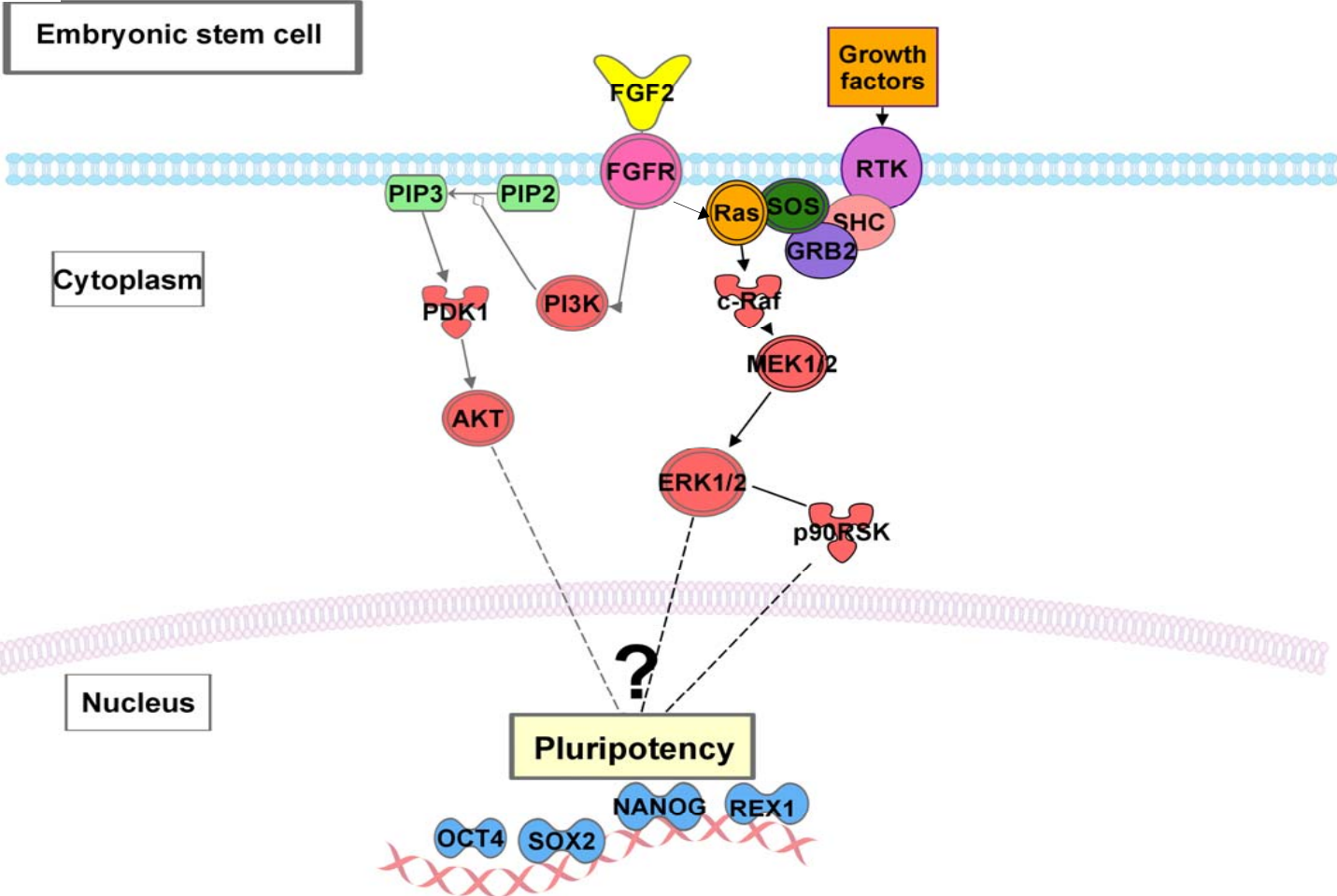
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Stem cell signaling for the maintenance of pluripotency



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Overview

- Tools currently available for protein analysis
- BD™ Cytometric Bead Array (CBA) technology
- Advantages of multiplexing with BD CBA assays
- Specificities for stem cell research
 - Cell signaling molecules and transcription factors
- Human embryonic stem cell (hESC) differentiation and activation data
- BD CBA complements intracellular flow cytometry
- BD CBA analysis correlation to Western blot results



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BD Biosciences protein analysis technologies

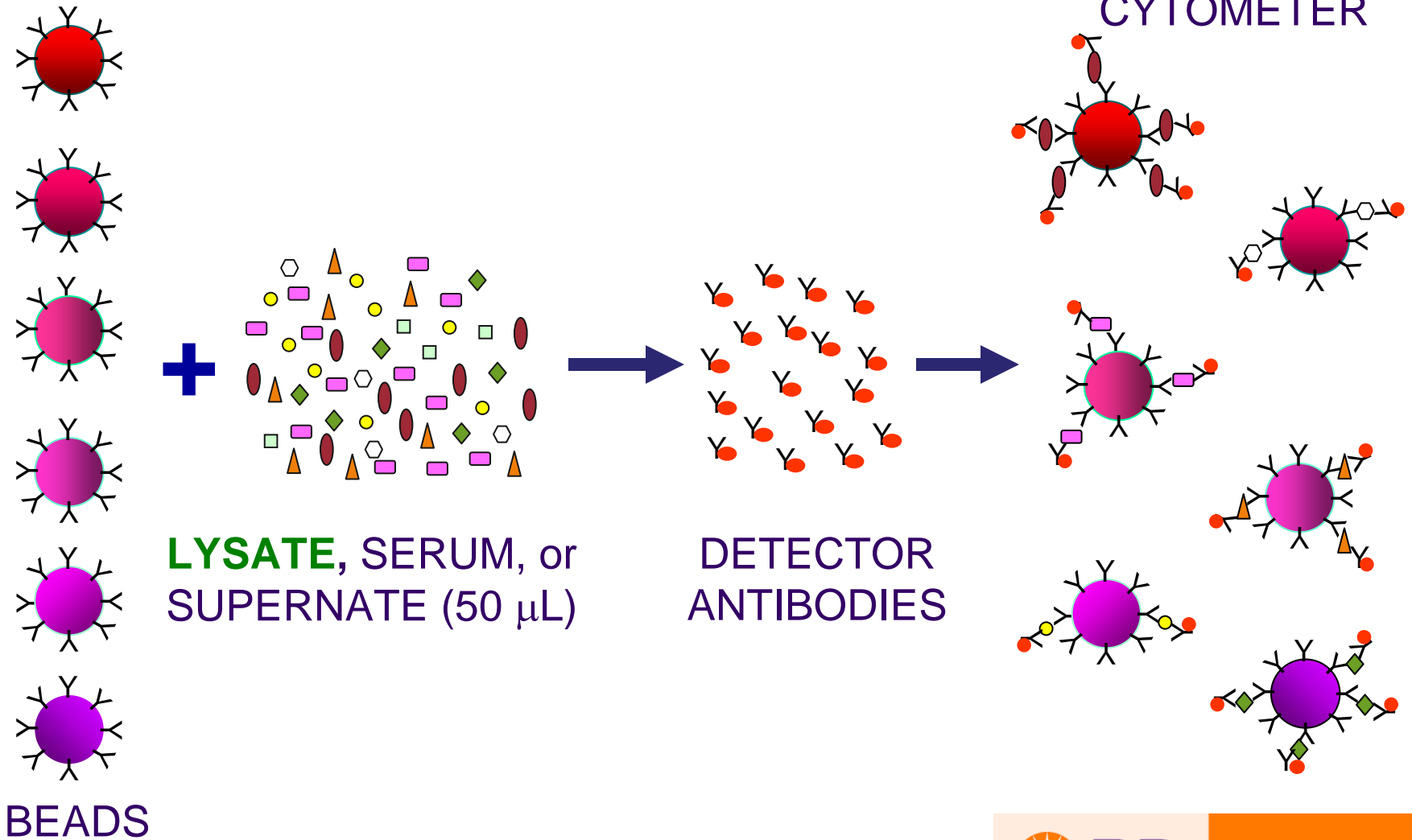
	Intracellular Flow Cytometry	High-Content Cell Imaging	BD Cytometric Bead Array	Western Blot (WB) and Immunoprecipitation
<i>Sample Types</i>	<ul style="list-style-type: none"> • Suspension and adherent cells 	<ul style="list-style-type: none"> • Tissue, adherent cells, immobilized suspension cells 	<ul style="list-style-type: none"> • Lysates from cells or tissues 	<ul style="list-style-type: none"> • Lysates from cells or tissues
<i>Key advantages</i>	<ul style="list-style-type: none"> • Multiplexed protein expression and activation state analysis on single-cell level • Enables phenotyping of small subpopulations using validated cell surface markers • Small sample volume • 96-well plate compatible • 3 to 4 hours to results 	<ul style="list-style-type: none"> • Multiplexed protein expression and activation state analysis on single-cell level • Enables (co-) localization of proteins and phosphorylation events within their subcellular context • Small sample volume • 96-well plate compatible • 3 to 4 hours to results 	<ul style="list-style-type: none"> • Highly multiplexed • Quantification of proteins and phosphorylation events • Small sample volume • Lysates can be collected at different time points, frozen, then assayed together later • 96-well plate compatible • 4 hours to results 	<ul style="list-style-type: none"> • Provides molecular weight information, which can compensate for less specific antibodies • Lysates can be collected at different time points, frozen, then assayed together later
<i>Limitations</i>	<ul style="list-style-type: none"> • Limited use for tissues 	<ul style="list-style-type: none"> • Sophisticated data acquisition and analysis required 	<ul style="list-style-type: none"> • Subpopulation analysis requires cell sorting and/or purification 	<ul style="list-style-type: none"> • Time consuming • Does not support higher throughput • Higher sample consumption • Semi-quantitative • Subpopulation analysis requires cell sorting and/or purification

BD Cytometric Bead Array (CBA)



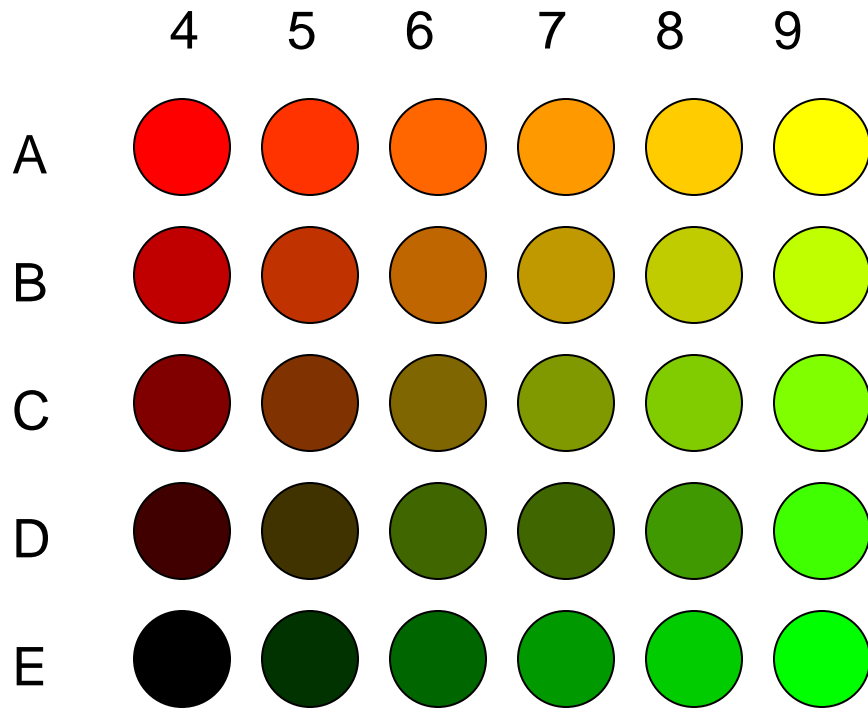
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BD CBA assay protocol

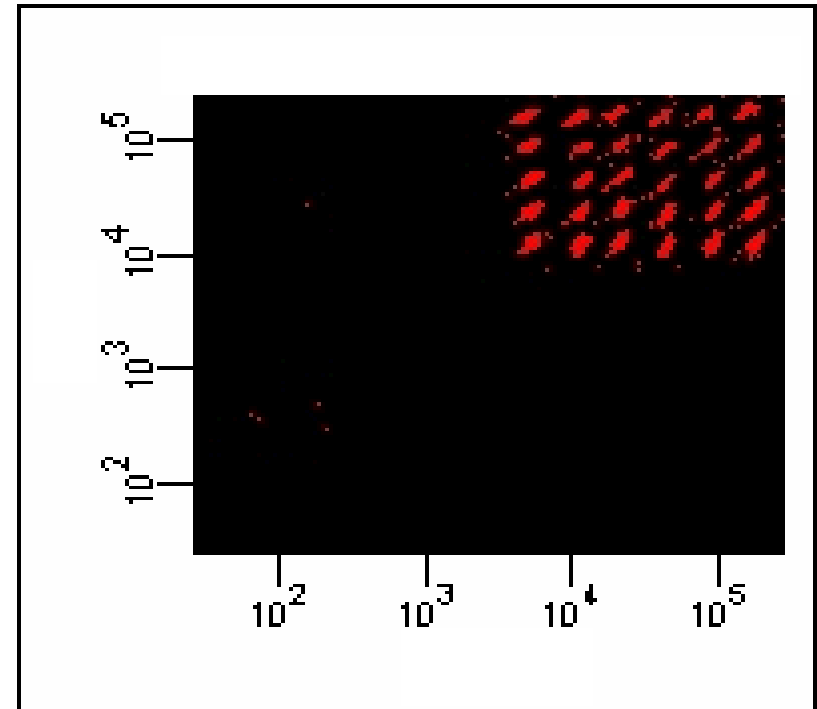


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Flex Set beads allow multiplexing of specificities

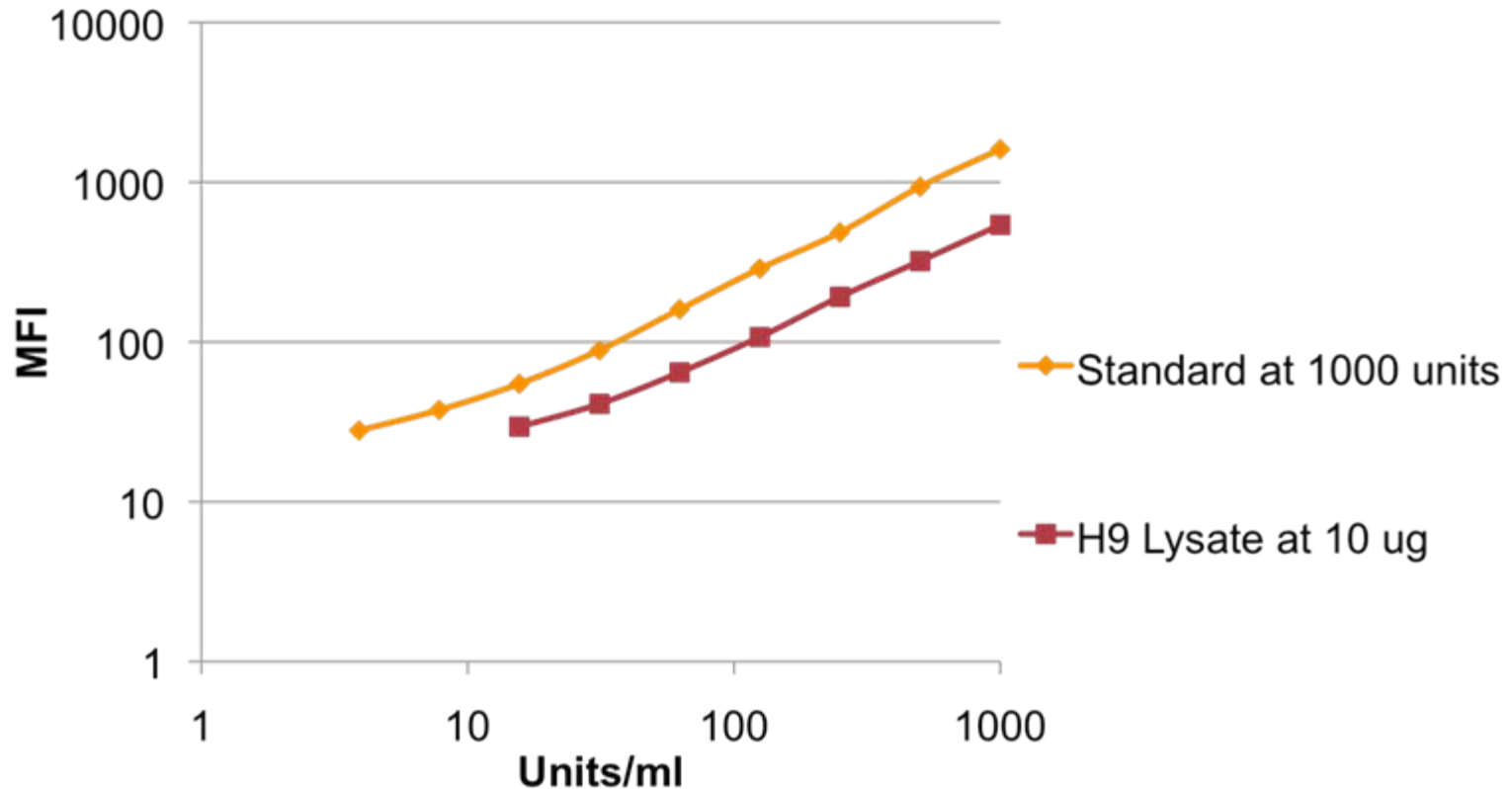


Analyzed by flow cytometry



Two different fluorescent dyes are mixed in different ratios to produce a 30-plex.

Nanog standard curve generated from recombinant protein

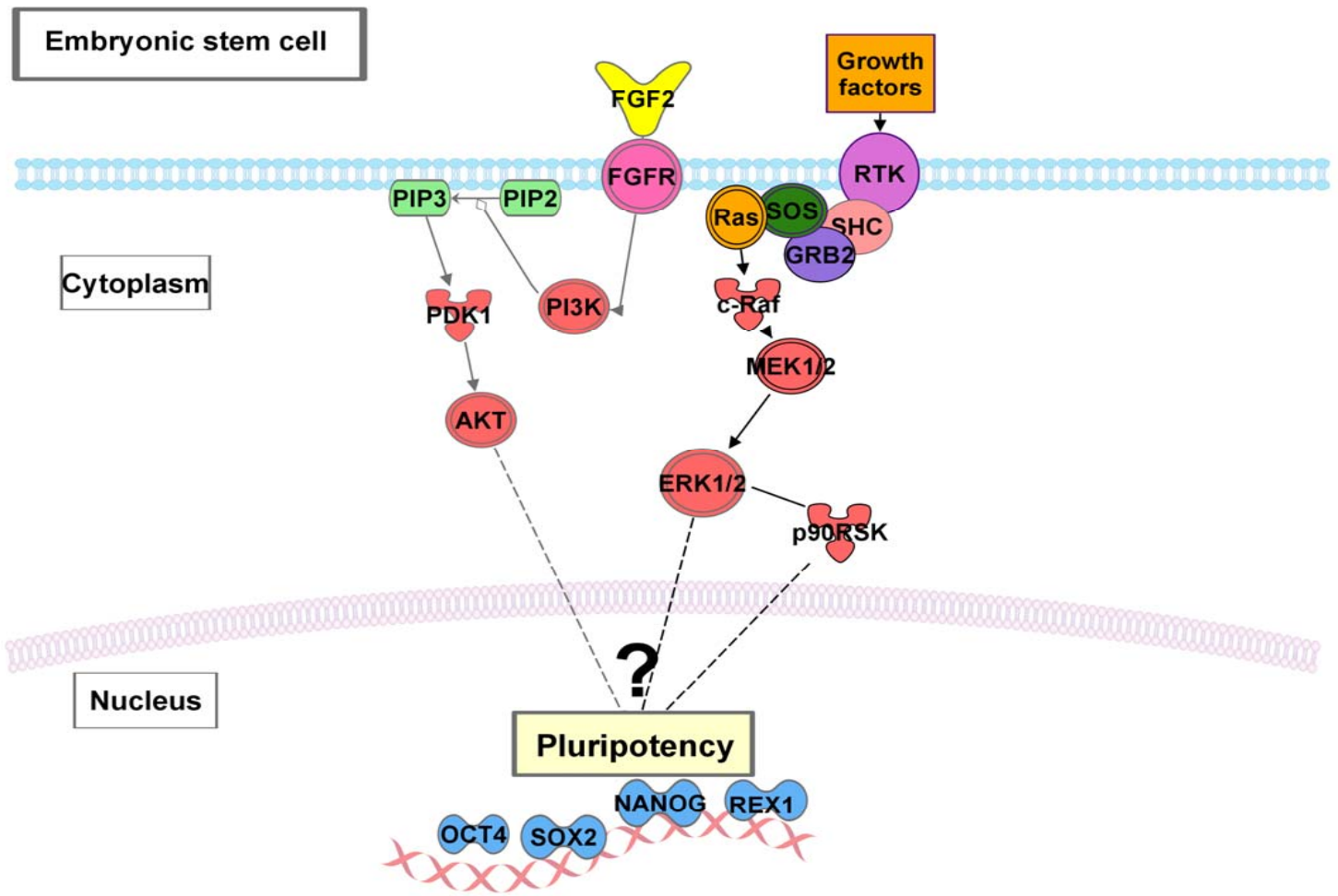


What are the advantages of bead-based immunoassays?

- Analysis of multiple analytes simultaneously
 - Reduces hands-on time by performing parallel analysis
 - Helps eliminate variations from multiple sample loading
- Wide dynamic range
 - Requires fewer sample dilutions to obtain results
- Reduced sample volume requirements
 - For limited samples, full analysis is possible
- Requires only 10 μg of protein lysate for multiple targets



Stem cell signaling and the maintenance of pluripotency



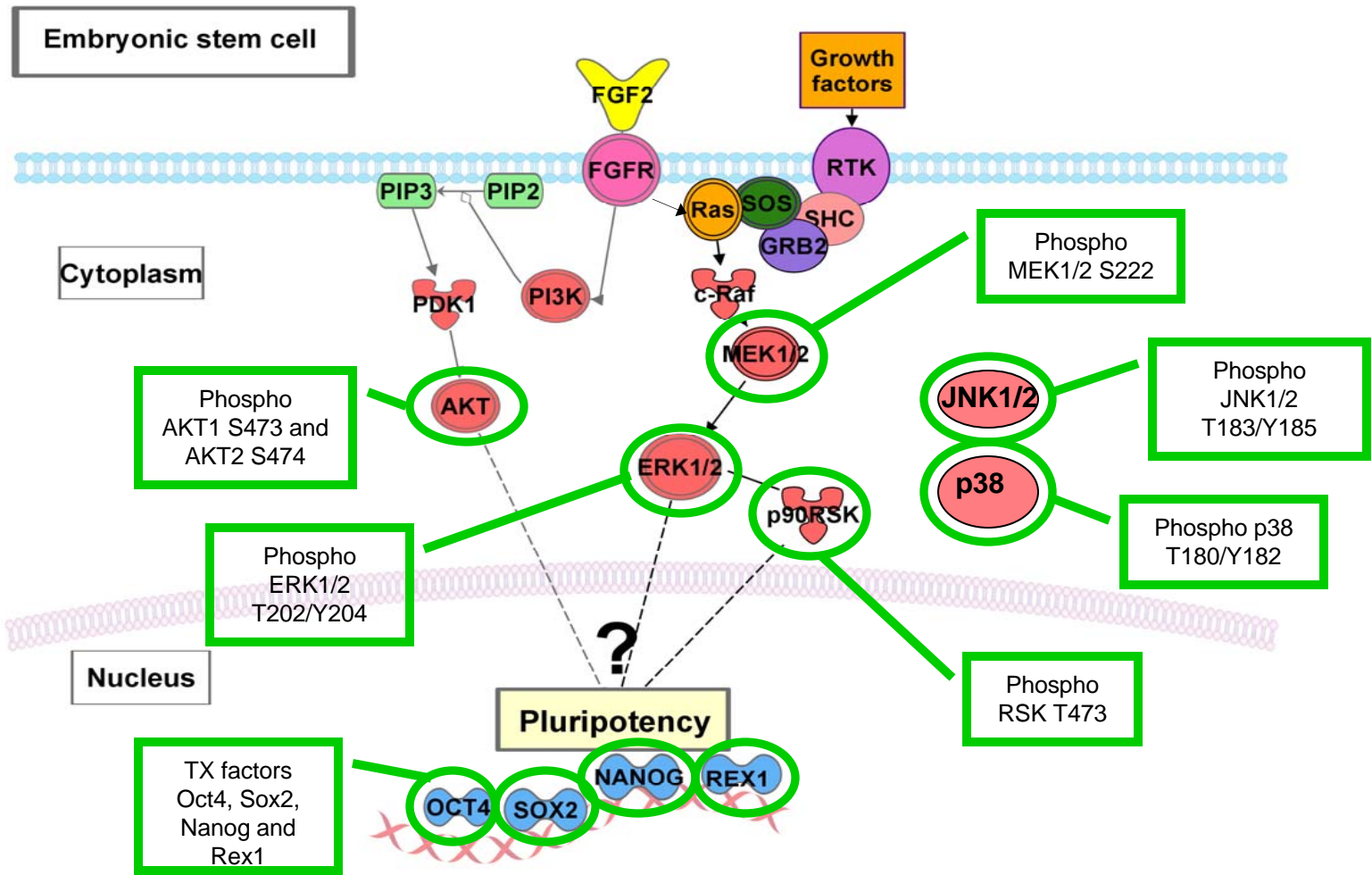
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Stem cell signaling and the maintenance of pluripotency



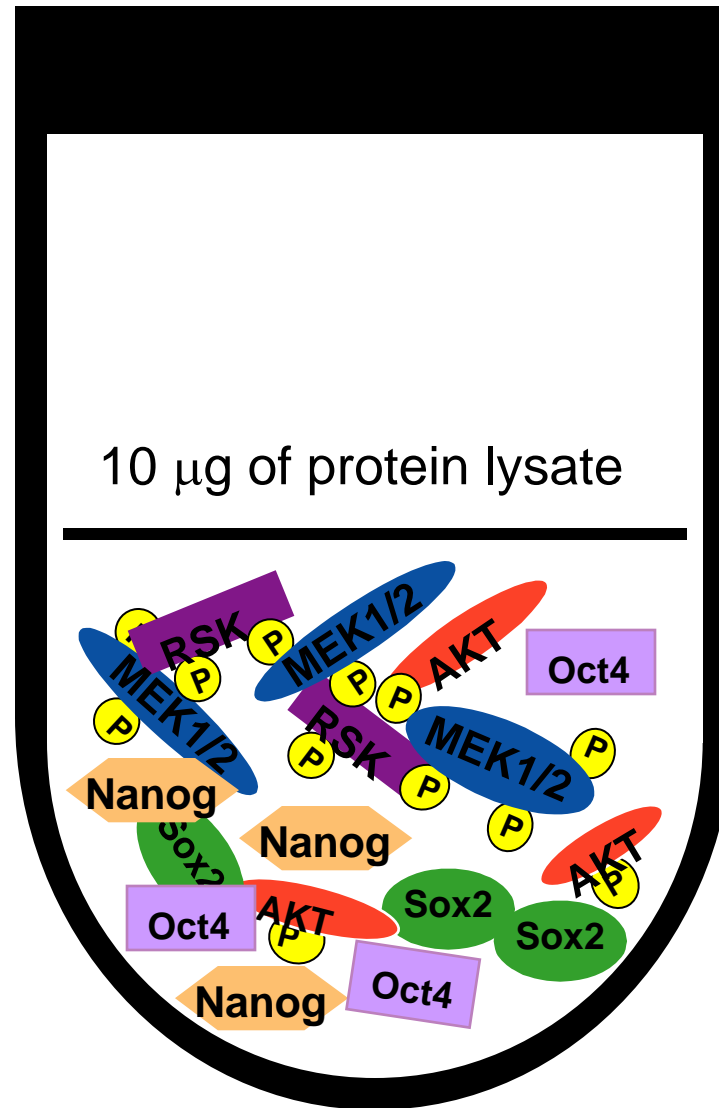
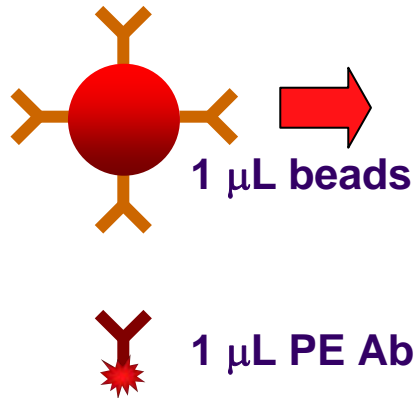
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Multiplexing with BD CBA Flex Set assays

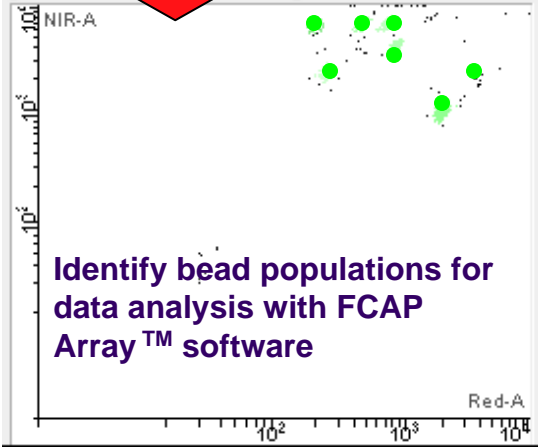
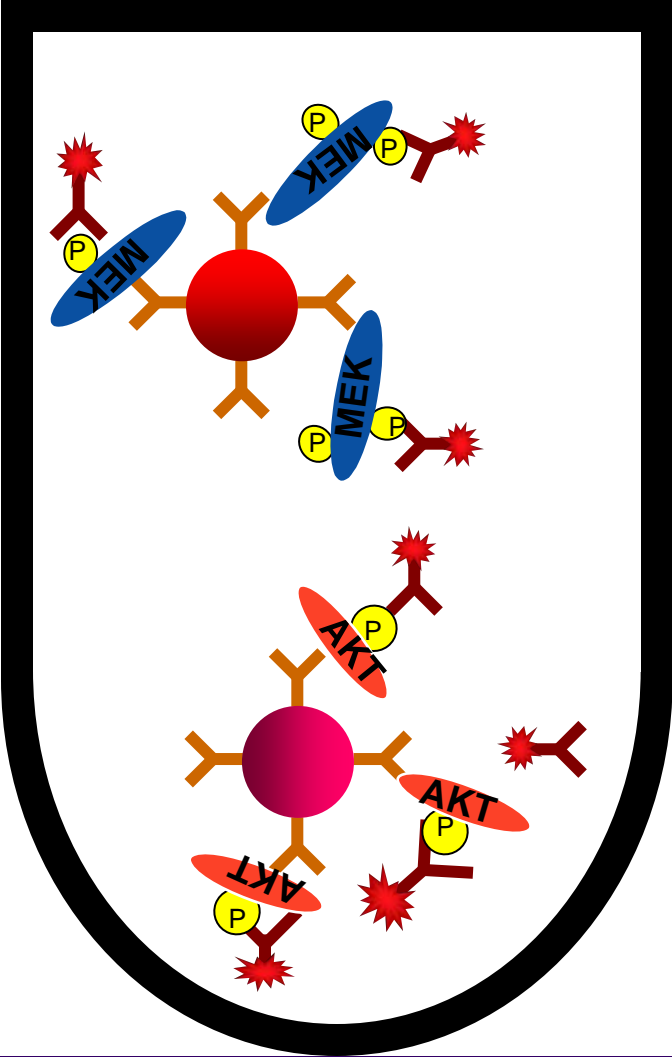


1. Prepare sample.
2. Add beads and sample.
3. Add detector.
4. Wash.



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Analyze the complexes by flow cytometry



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Spontaneous differentiation in human ESC line H9

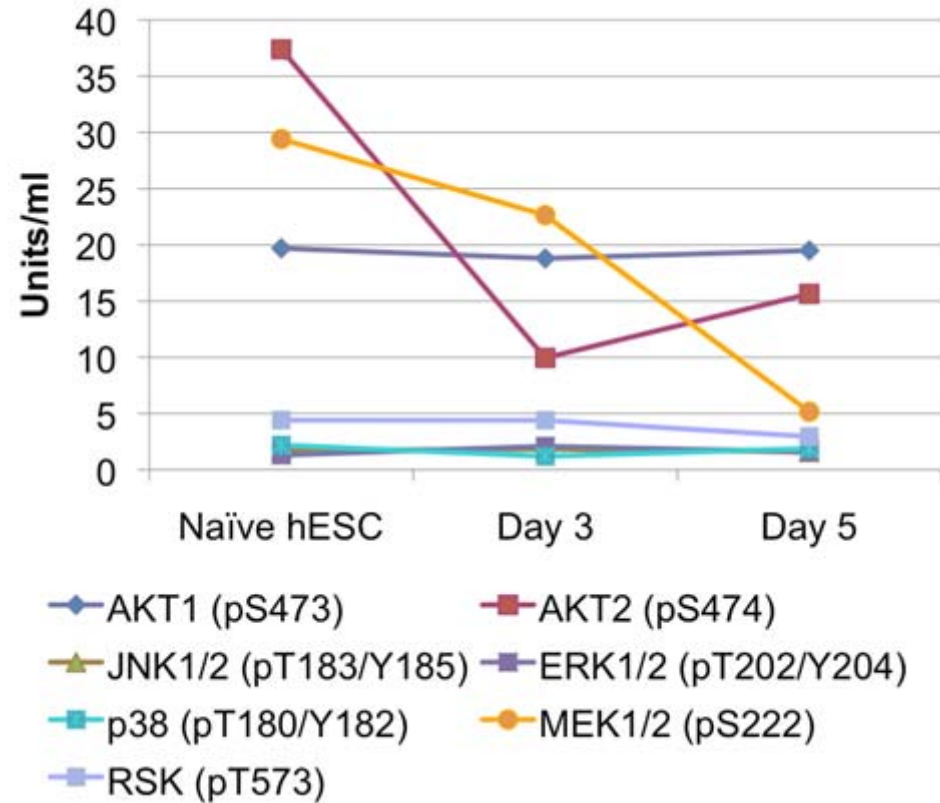
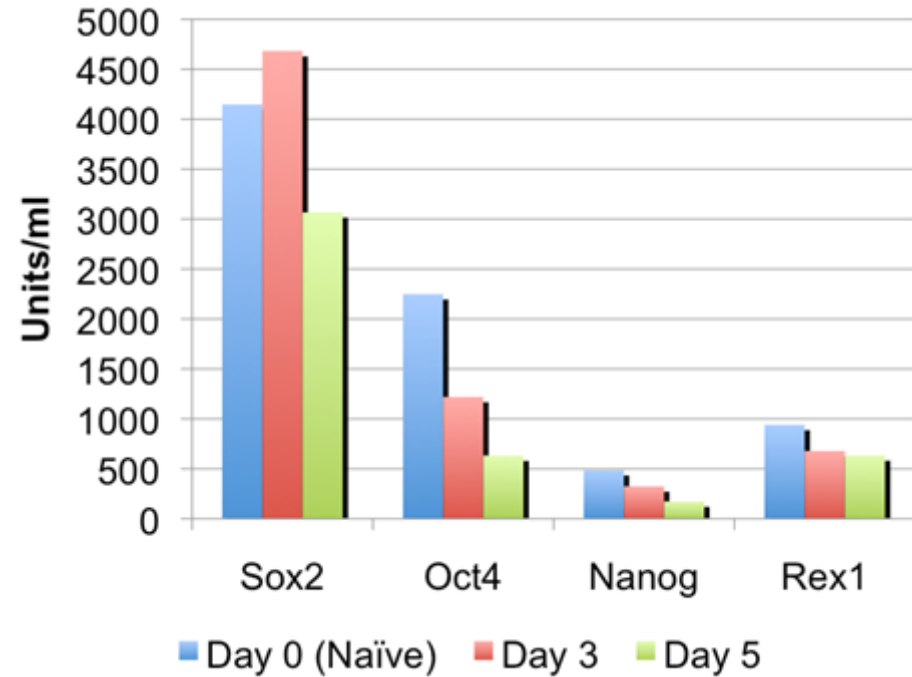
- Cells were maintained on BD Matrigel™ hESC-qualified matrix and mTeSR®1*.
- Cells were then cultured in hESC media without bFGF.
- Cells were lysed for BD CBA testing on days 0, 3, and 5.
- Cell lysate protein concentrations were acquired by bicinchoninic acid assay.
- Lysates were tested using BD CBA for 4 stem cell transcription factors and 7 phospho-signaling proteins.

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BD CBA multiplex for stem cell TX and cell signaling levels in hESC lysate

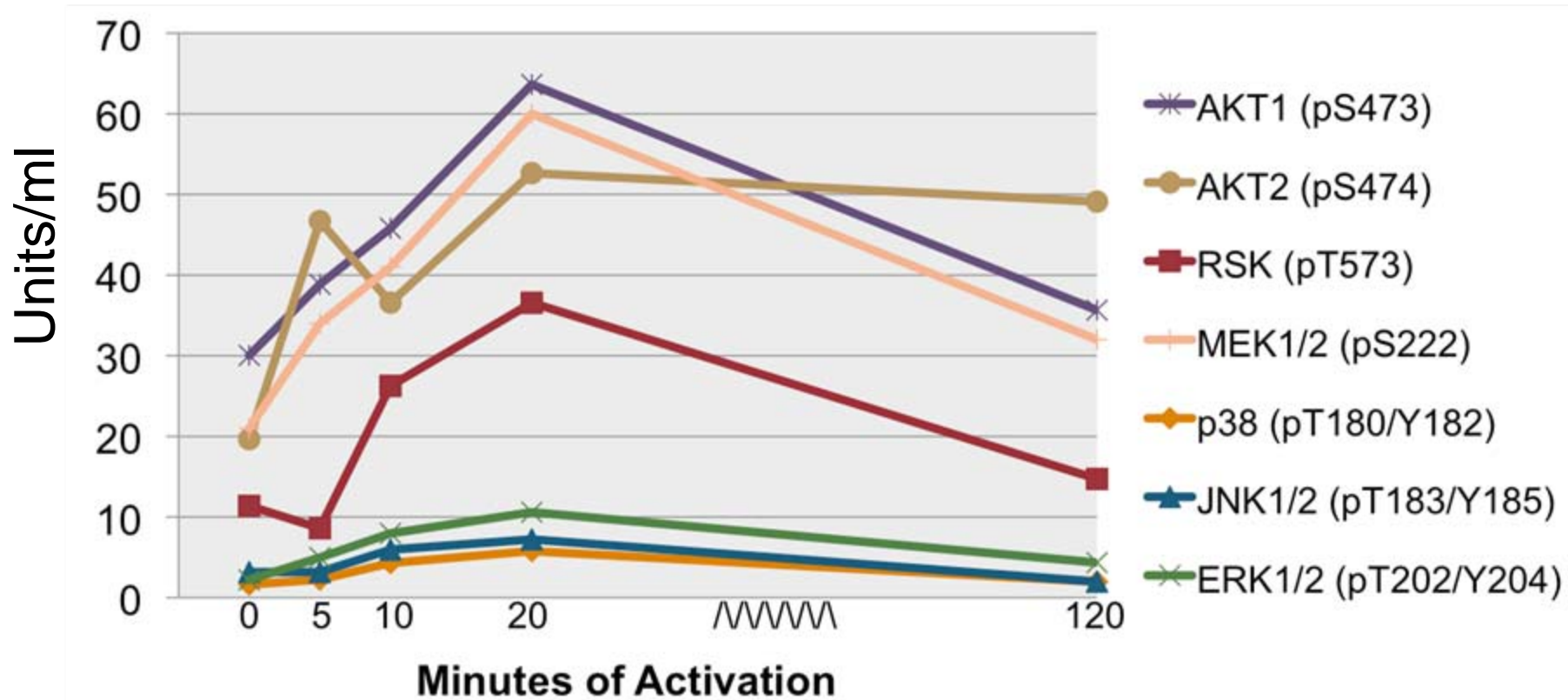


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Phospho-kinetic experiment showing hESCs in response to growth factors

- Cells were maintained on BD Matrigel hESC-qualified matrix and mTeSR®1.
- Confluent hESCs were cultured in hESC media without bFGF overnight.
- Medium was replaced with mTeSR®1 for 5 min, 10 min, 20 min, and 2 h.

Phospho-signaling levels after activation with growth factors

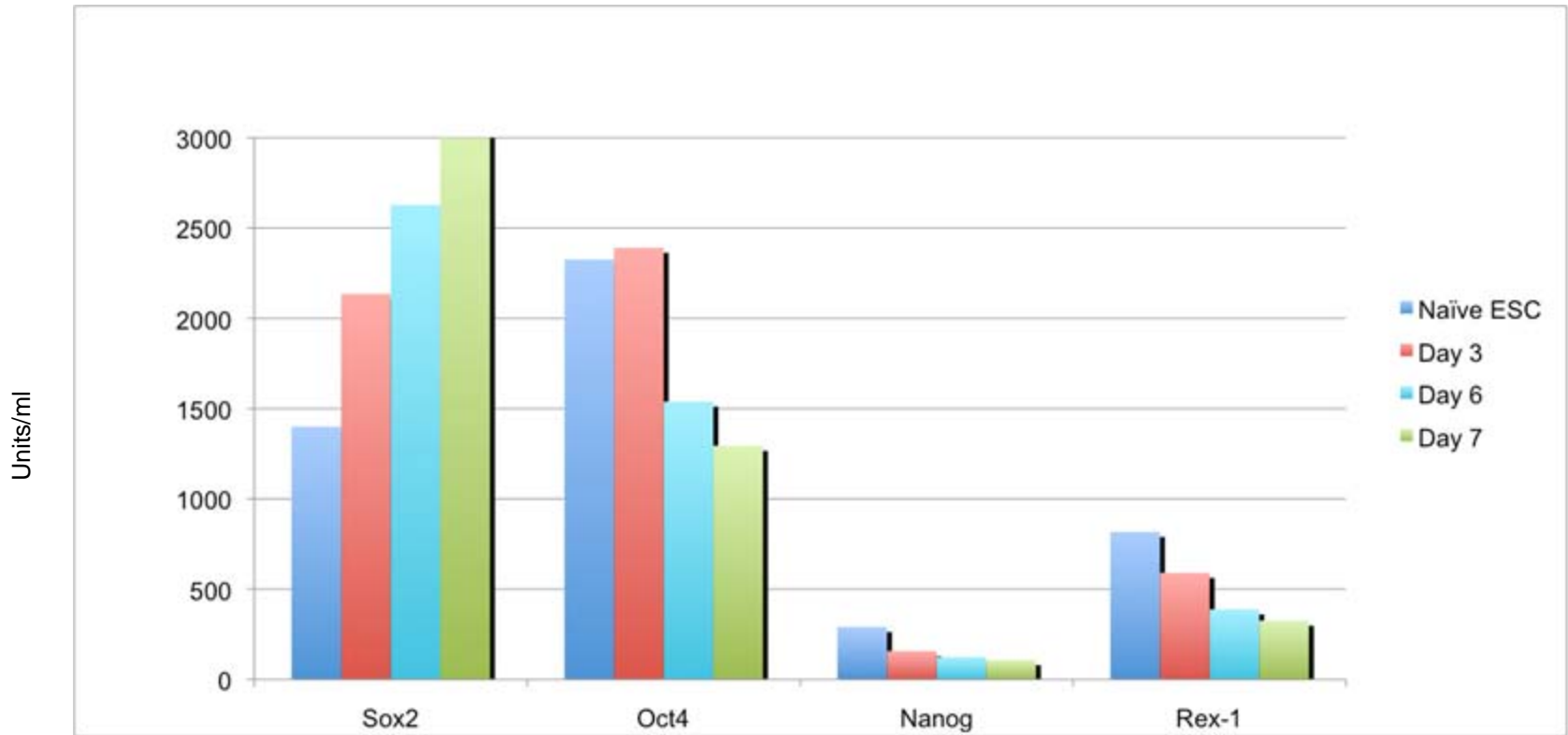


Data showed a steady climb between 5 and 20 min and a decrease between 20 min and 2 h.

Effects of PI3K inhibitor LY294002 on transcription factors involved in maintaining pluripotency

- Naïve hESCs were cultured in 10 mM of the PI3K/AKT inhibitor, LY294002.
- Cell lysates were acquired on days 0, 3, 6, and 7.
- Lysates were tested for the transcription factors Sox2, Oct4, Nanog, and Rex1.

Effects of PI3K inhibitor LY294002 on transcription factors involved in maintaining pluripotency



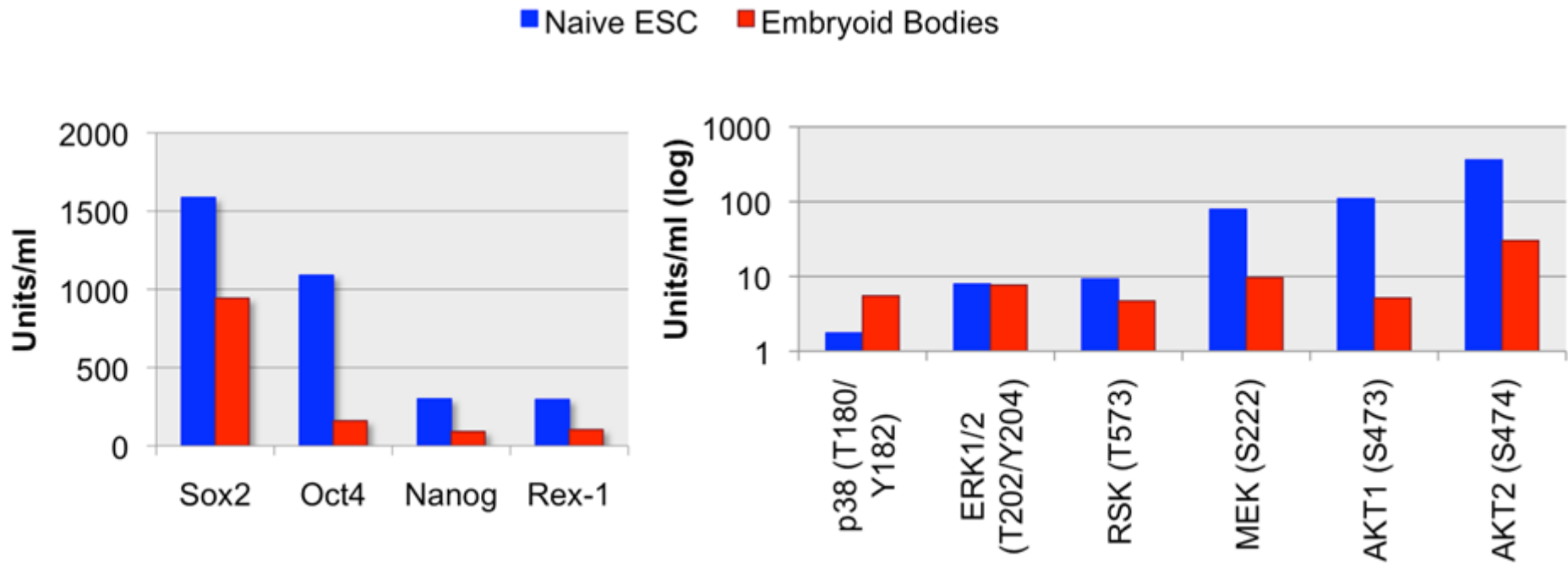
Naïve hESCs and embryoid bodies (EBs) analyzed for kinase phosphorylation and transcription factor levels

- hESC colonies were manually isolated and placed in hESC media without bFGF on low adhesion plates for a period of 7 to 10 days. EBs were subsequently cultured on BD Matrigel-coated 10-cm dishes for an additional 4 to 7 days.
- BD Cytometric Bead Array and BD Phosflow™ intracellular flow cytometry assays were performed on protein lysates or cells, respectively.



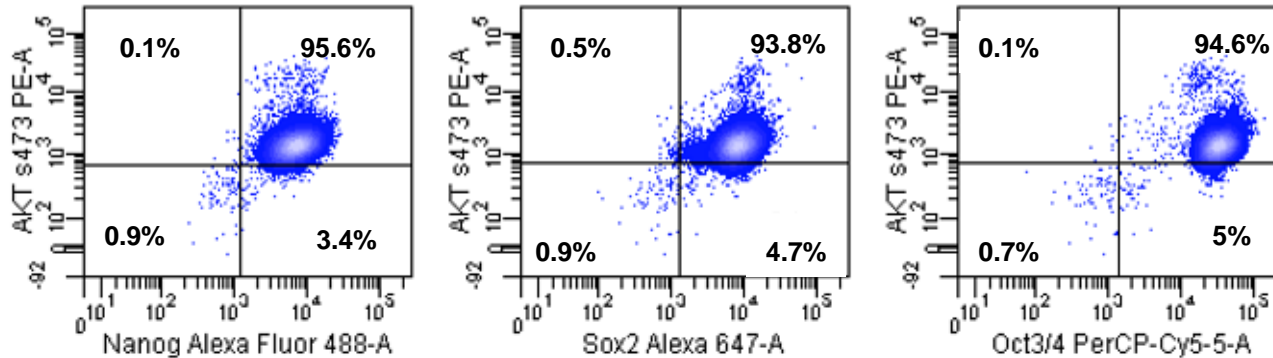
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Multiplexing of BD CBA cell signaling Flex Sets and BD CBA stem cell Flex Sets permits the simultaneous quantitation of hESC and EB protein lysates

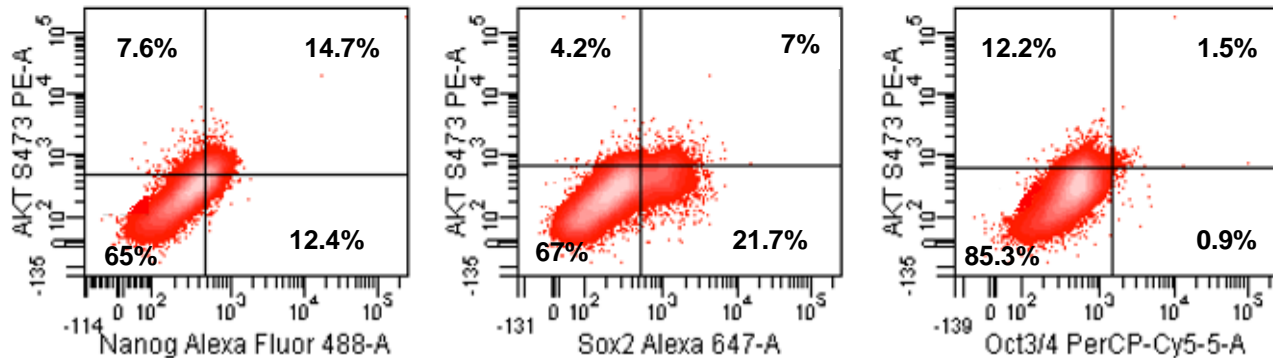


Multicolor flow analysis of hESCs and EBs for AKT (pS473), Nanog, Sox2, and Oct4

hESCs



EBs



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Flow cytometric measurement of intracellular signal transduction in mesenchymal stem cells

Patrick C. Baer¹, PhD and Ralf Schubert², PhD

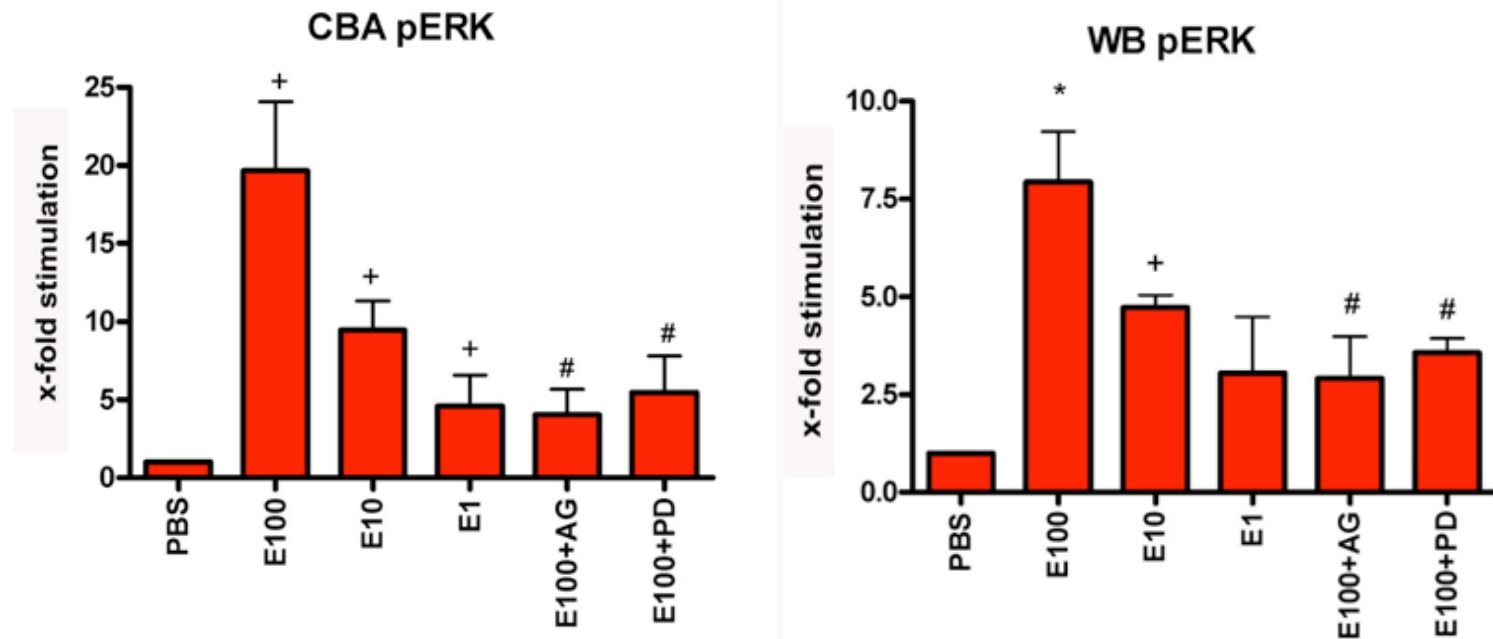
¹Division of Nephrology, Department of Internal Medicine, and ²Children's Hospital, Goethe University, Frankfurt am Main, Germany



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MAPK activation with and inhibition of EGF in adipose-derived stem cells analyzed by BD CBA and WB

(Baer P, Schubert R)



Western blots were measured by densitometry. Abbreviations: PBS=PBS buffer, as negative control; E100, E10, E1=EGF at 100, 10, 1 ng/mL; AG=EGF receptor inhibitor AG1478; PD=Inhibitor PD98059; pERK=phospho-ERK. n=4–5, *p<0.01 compared to PBS; + p<0.05 compared to PBS; # p<0.05 compared to E100.

Results

- Data clearly showed that the BD CBA and WB data were significantly correlated ($p < 0.0001$).
- WB is very time consuming.
- Simultaneous measurement of protein phosphorylation with BD CBA technology represents a convenient quantitative technique with the possibility of high-throughput analysis.

Summary

BD Cytometric Bead Array Flex Sets

Quantitative, multiplexing, and small sample size allow broader analysis of precious samples.

Flexible system allows customized multiplex analysis.

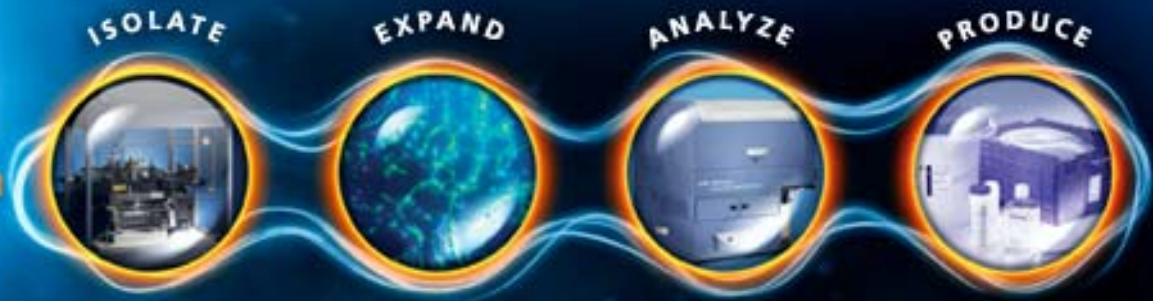
Enable measurement of intracellular proteins in cell lysates using cell signaling BD CBA Flex Sets and extracellular proteins from serum, plasma, and supernatants using soluble protein Flex Sets.

Ideal for analyzing multiple samples simultaneously.



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Acknowledgments

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Rosanto Paramban
Nil Emre

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