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APPLICATION NOTE

Simple Western Unravels the Signaling Web in Stem Cells for Regenerative Medicine

Quantitative Signal Pathway Analysis with Sensitive Multiplexing

The reprogramming of human-induced pluripotent stem cells (iPSCs) has revolutionized regenerative medicine, but gaining an accurate and quantitative understanding of signaling networks that regulate stem cell differentiation is crucial and remains a daunting challenge.[1] The complexity of signaling networks stems from the many proteins and phosphorylated protein isoforms involved, and researchers are often challenged with limited material for analysis. Traditional methods like Western blot lack throughput, multiplexing ability, detection sensitivity, and reproducible quantification needed to gain a complete understanding of stem cell biology.

Simple Western™ is an advanced capillary immunoblotting platform that addresses the limitations of current methods for protein expression and cell signaling pathway analysis in stem cells for regenerative medicine.

- Multiplex in chemiluminescence and Stellar™ NIR/IR Modules with unmatched sensitivity
- Only 3 µL of your precious sample is needed - often less than 1 µg of total protein
- Fully quantitative protein expression using conventional Western blot antibodies
- Eliminate the unavoidable variation of traditional Western blots with intra-assay CVs <15%
- Hands-free, walk-away analysis. Come back in 3 hours to publication-ready results
- Superplex across chemiluminescence and NIR/IR fluorescence channels with simultaneous total protein normalization
- Reuse your sample with RePlex™ sequential immunoassays and total protein detection

Monitoring the Differentiation of Induced Pluripotent Stem Cells to Human Germ Layers

Here, we used the advanced automated Western blot analysis of Simple Western to monitor iPSC differentiation into three primary germ layers of human embryos. Using GMP proteins and GMP small molecules, we differentiated iPSCs into endoderm, ectoderm, and mesoderm layers. As these cells differentiated, we applied high sensitivity multiplex assays on Simple Western to reveal and quantify changes in the expression of pluripotent factors and cell signaling proteins that regulate stem cell differentiation. With only 3 µL of starting material, we show how Simple Western has unrivaled multiplex flexibility, sensitivity, and reproducibility for accurate protein expression measurements that scale with manufacturing workflows for stem cell therapy and regenerative medicine.

Materials and Methods

Reagents used in this study are listed in TABLE 1. Antibodies used in this study are listed in TABLE 2.

REAGENT	VENDOR	P/N
12-230 kDa Fluorescence Separation Module	Bio-Techne	SM-FL004
12-230 kDa Separation Module		SM-W004
12-230 kDa Peggy Sue or Sally Sue Separation Module		SM-S001
Total Protein Detection Module		DM-TP01
Stellar NIR/IR Modules		DM-013 – DM-016
Stellar Total Protein Module		DM-TP03
RePlex Module		RP-001
Anti-Rabbit Detection Module		DM-001
Anti-Mouse Detection Module		DM-002
Phalloidin-FITC		5782
CHIR 99021 (GMP)		TB4423-GMP
FGF basic (GMP)		3718-GMP
BMP-4 (GMP)		314E-GMP
Activin A (GMP)		338-GMP
Wnt-3a (GMP)		5036-GMP
Noggin (GMP)		6057-GMP
SB 431542 (GMP)		TB1614-GMP
ExCellerate™ iPSC Expansion Medium		CCM036

TABLE 1. Reagents used in this study.

ANTIBODY	VENDOR	P/N
Anti-OCT4 Antibody	Novus Biologicals, a Bio-Techne Brand	NB100-2379
Anti-SOX17 Antibody	Bio-Techne	AF1924
Anti-Brachyury Antibody		AF2085
Anti-Otx2 Antibody		AF1979
Anti-ERK1/2 Antibody	Cell Signaling Technology	4696S
Anti-Phospho-ERK1/2 Antibody		9101S
Anti-Akt Antibody		58295S
Anti-Phospho-Akt Antibody		9271S
Anti-RS6 Antibody		2317S
Anti-Phospho-RS6 Antibody		4856S
Anti-Phospho-β-Catenin Antibody		5651S
Anti-Nanog Antibody		4903S
Anti-SOX2 Antibody		23064S

TABLE 2. Antibodies used in this study.

Stem Cell Differentiation

iBJ6 human-induced pluripotent stem cells (iPSCs) were maintained and expanded in a naïve state in ExCellerate™ iPSC Expansion Medium, and were differentiated into mesoderm, ectoderm, and endoderm lineages using GMP proteins and small molecules. Mesoderm differentiations were performed through two protocols, (1) FGF basic and BMP-4 alone, and (2) with FGF basic, BMP-4, and CHIR 99021. Endoderm differentiations were performed via a bi-phasic protocol with FGF basic, Activin A, and Wnt-3a, and analyzed after 24 hours and 72 hours of treatment. Ectoderm differentiations were performed in 3 ways, (1) through growth factor withdrawal, (2) Noggin treatment, and (3) with Noggin and SB 431542.

Simple Western Analysis

After lysis, several concentrations of each sample were interrogated with a variety of monoclonal antibodies using Jess™ and Abby™ with no change in the standard protocols. Peak areas were measured using Compass for Simple Western™ software.

Results

Monitoring the Differentiation of iPSCs to Germ Layers: Pluripotent Marker Analysis

To confirm the successful differentiation of iPSCs into mesoderm, ectoderm, and endoderm lineages, we used two orthogonal immunoassays, immunofluorescence (IF) microscopy and Simple Western. For IF analysis, we stained cells with fluorescent antibodies targeting canonical biomarkers of each primary germ layer, including the mesoderm marker, Brachyury, the endoderm marker, SOX17, and the ectoderm marker, Otx2.[5] Fluorescent staining of F-actin with Phalloidin-FITC and nucleic acid content with DAPI served as positive controls. The results from this analysis showed the presence of each biomarker on corresponding germ layers (shown in red) compared to the undifferentiated control (FIGURE 1). It should be noted that robust expression of endoderm marker SOX17 appeared only after 72 hours post differentiation. Thus, the results of IF analysis confirm successful differentiation of iPSC-derived primary germ layers using antibodies targeting biomarkers of each germ layer.

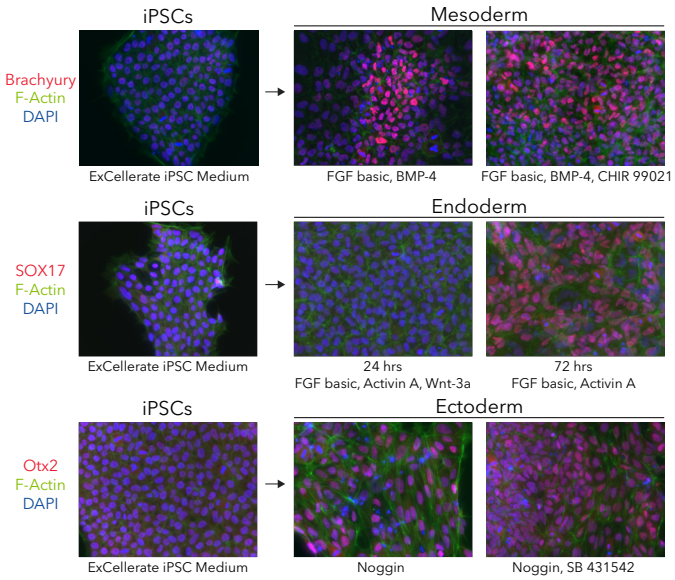

FIGURE 1. Immunofluorescence analysis of undifferentiated iPSCs and iPSC-derived mesoderm, endoderm, and ectoderm germ layers. The biomarkers Brachyury, SOX17, and Otx2 indicate differentiation status (shown in red). Fluorescent staining of F-actin (shown in green) and nucleic acid staining with DAPI (shown in blue) were used as controls.

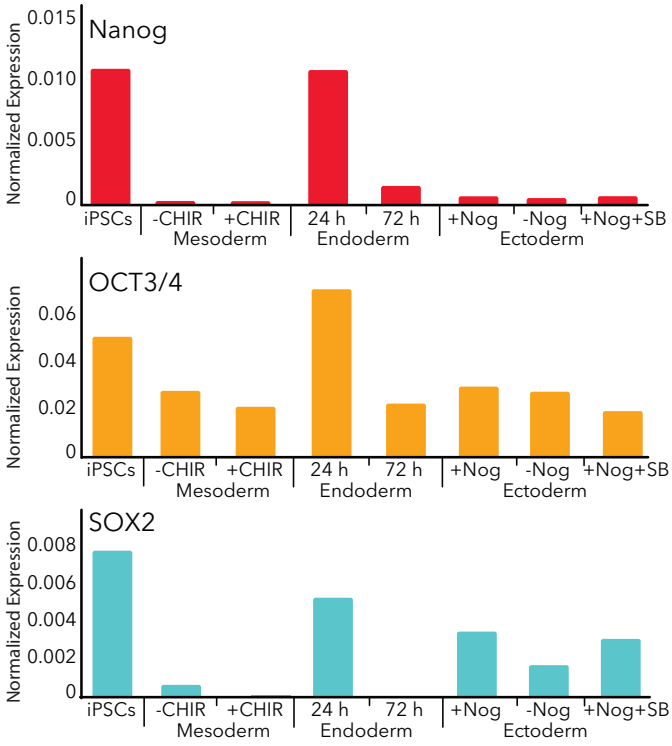

FIGURE 2. Simple Western quantification of 3 biomarkers of cell stemness, Nanog, OCT3/4, and SOX2 in undifferentiated iPSCs and iPSC-derived mesoderm, endoderm, and ectoderm cells. Protein expression values were normalized to total protein. CHIR: CHIR 99021, Nog: Noggin, SB: SB 431542.

Next, we quantified the expression of biomarkers of cell stemness (Nanog, OCT3/4, and SOX2) across naïve cells and iPSC-derived germ layers using Simple Western on Jess. In this analysis, antibodies targeting stemness biomarkers were detected using Stellar NIR/IR Modules and total protein was simultaneously detected in the chemiluminescence channel using the Stellar Total Protein Detection Module. In this manner, the protein expression of stemness biomarkers can be normalized to total protein for accurate comparison between samples. Unlike the targets used for IF analysis above, the expression of stemness biomarkers is expected to decrease as iPSCs differentiate. Consistent with this expectation, the expression of stemness biomarkers generally decreased as naïve cells differentiated into germ layers, with a few exceptions (**FIGURE 2**).

The most significant changes were observed with Nanog, which decreased from relatively high expression to little or no detectable expression in differentiated samples. While Nanog expression eventually decreased in all differentiated cells, Nanog expression in endoderm cells was at naïve levels 24 hours post differentiation and diminished significantly 72 hours post differentiation. These results are consistent with IF analysis which showed robust expression of SOX17 at 72 hours post differentiation, and these results indicate that complete endoderm differentiation requires more than 24 hours under the conditions tested here.

The expression of OCT3/4 showed a similar pattern to Nanog, however, the decrease in Oct4 expression was not as pronounced as Nanog, with approximately 50% of native OCT3/4 expression levels remaining in differentiated cells (**FIGURE 2**, middle panel). The persistence of OCT3/4 expression is known to occur in the differentiated germ layers.[5] The expression of SOX2 decreased dramatically in mesoderm and endoderm cells, but SOX2 persisted in ectoderm cells (**FIGURE 2**, bottom panel). These results are consistent with expected results as SOX2 is known to be expressed in ectoderm cells.[6]

Taken together, both immunofluorescence microscopy and Simple Western analysis confirmed the successful differentiation of iPSCs to the 3 primary germ layers of human embryos that are formed during gastrulation. However, the results presented here show that Simple Western is more than just an orthogonal method to validate stem cell differentiation. While IF analysis primarily offers a qualitative assessment of germ layer differentiation, Simple Western provides a fully quantitative analysis of stemness biomarkers for more detailed biochemical insight into monitoring stem cell differentiation.

Quantitative Signal Pathway Analysis with Multiplex and Total Protein Detection

With the validation of iPSC-derived germ layers completed, we next evaluated the signaling activity of differentiated germ layers compared to naïve stem cells. To do so, we leveraged high-sensitivity two-color multiplexed Stellar assays on Jess to measure expression of signaling molecules and their phosphorylation status. We first analyzed the expression of Akt, which plays an important role in regulating stem cell pluripotency.[1] Antibodies that target specifically total-Akt and phospho-Akt isoforms were detected using Stellar NIR/IR Modules, and we measured total protein expression simultaneously using the Stellar Total Protein Module in the chemiluminescence channel. The results from this analysis showed the presence of total-Akt and phospho-Akt isoforms in undifferentiated cells, while phospho-Akt isoforms were virtually undetectable in mesoderm cells, with a complex peak pattern spanning a large molecular weight range corresponding to host cell proteins in both samples (FIGURE 3). The absence of phosphorylated Akt in iPSC-derived mesoderm cells is consistent with the loss of pluripotency in differentiated cells.[1]

While channel-based multiplex assays like Stellar are only compatible on Jess, RePlex sequential chemiluminescence assays are compatible on both Jess and Abby. Therefore, we compared the expression profile of Akt generated by Stellar on Jess and RePlex on Abby. The results obtained by RePlex on Abby showed a similar expression pattern characterized by the absence of phosphorylated Akt in iPSC-derived mesoderm cells, with both total-Akt and phospho-Akt isoforms present in naïve cells (FIGURE 4). Next, we quantified the expression of total-Akt and phospho-Akt isoforms by normalizing expression values to the total protein content of the sample, and we compared the relative expression values obtained from Stellar and RePlex assays. These results showed that Stellar and RePlex were consistent in the quantification of total and phospho-Akt isoforms when normalized to total protein content (FIGURE 5). Thus, Simple Western offers flexible strategies for multi-target analysis using Stellar on Jess or RePlex on Jess and Abby, and these generate quantitative protein expression data that are consistent with one another.

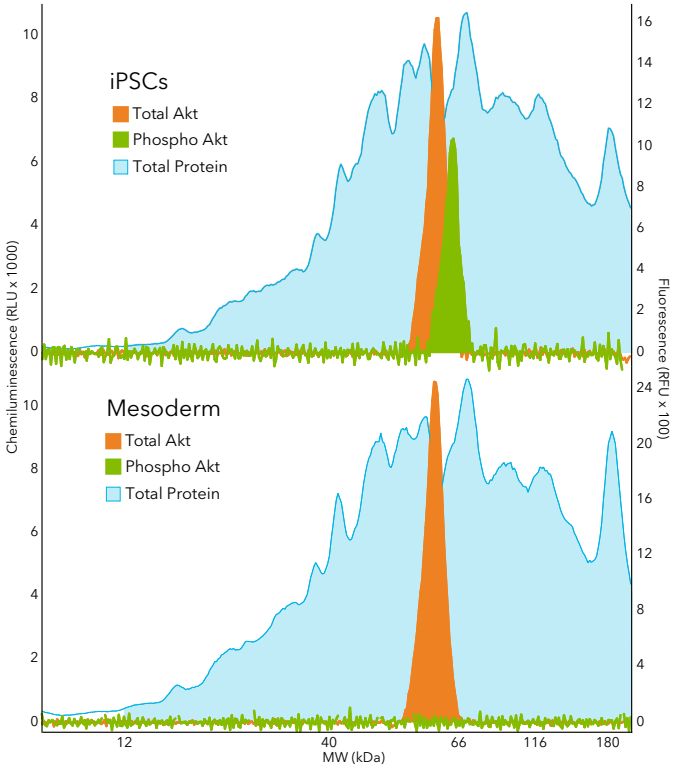

FIGURE 3. Simple Western analysis of total Akt, phospho Akt, and total protein across undifferentiated iPSCs and iPSC-derived mesodermal cells using the Stellar assay on Jess. Antibodies targeting total and phosphorylated Akt isoforms were detected using the Stellar NIR/IR Modules, and total protein was simultaneously detected using the Stellar Total Protein Module in the chemiluminescence channel. The baseline settings for the analysis of electropherograms in Compass for Simple Western were set to 1.0, 15.0, and 7.0 for threshold, window, and stiffness, respectively.

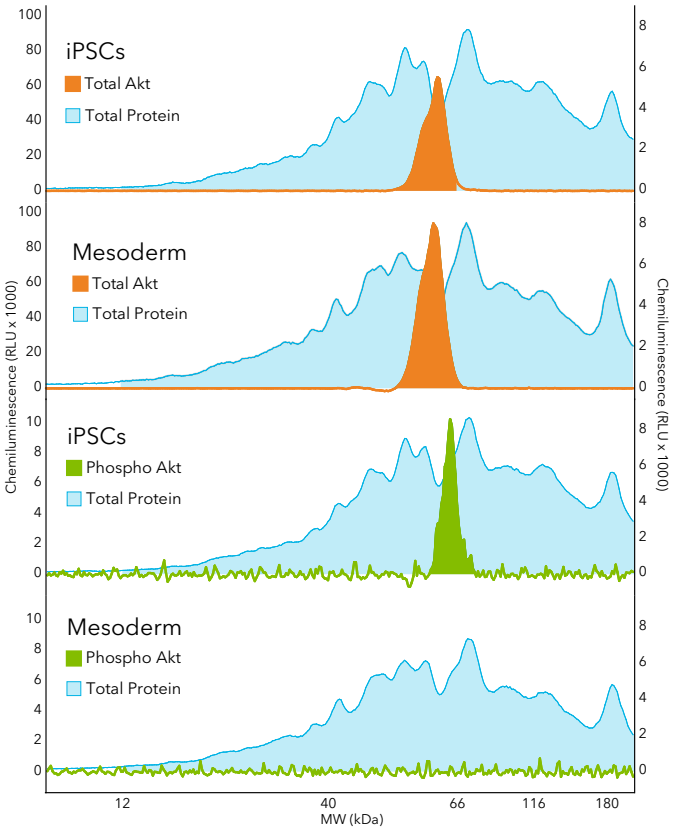

FIGURE 4. Simple Western analysis of total Akt, phospho Akt, and total protein across undifferentiated iPSCs and iPSC-derived mesodermal cells using the RePlex assay on Abby. Total and Phospho-Akt isoforms were detected in Probe 1 of RePlex and total protein was detected in Probe 2 of RePlex. The baseline settings for the analysis of electropherograms in Compass for Simple Western were set to 7.0, 15.0, and 6.0 for threshold, window, and stiffness, respectively.

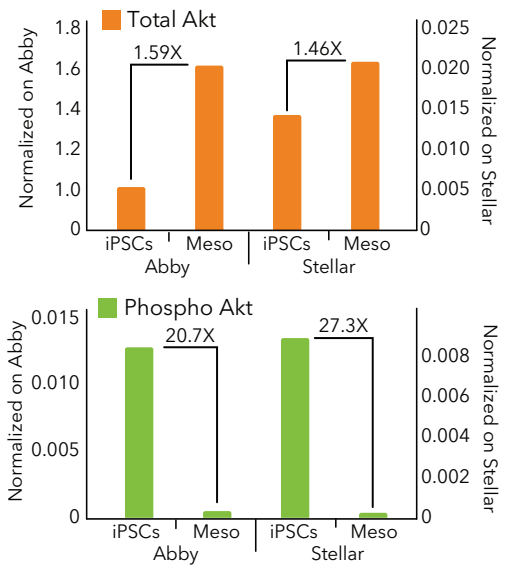

FIGURE 5. Quantification of total and phospho Akt isoforms using RePlex on Abby and Stellar on Jess. The bar graph shows the quantification of total Akt and phospho Akt isoforms normalized to total protein content between iPSCs and iPSC-derived mesoderm samples.

6 Targets in 8 Different Samples plus Total Protein Detection – All on One Run

Next, we evaluated the expression profile of Akt across all three iPSC-derived germ layers using Stellar multiplex immunoassays and total protein detection. We also analyzed several more signaling molecules, including ERK and RS6 which regulate cell proliferation, migration, differentiation, and survival. In a single Simple Western run on Jess, we used Stellar two-color multiplex and total protein detection to quantify total and phospho-protein isoforms of each signaling molecule across undifferentiated and differentiated cells. The results from this analysis showed a decrease in phospho-Akt in differentiated cells compared to the undifferentiated control, as expected (FIGURE 6). Both ERK and RS6 proteins showed increased activation in differentiated cells, as measured by comparing total and phospho-protein isoforms of each target normalized to total protein content of the sample (FIGURE 6). The expression of total and phosphorylated RS6 was strikingly high in the endoderm compared to the other samples. The role of phosphorylated RS6 has been documented in stem cell reprogramming,[7] but its function remains poorly understood.[8,9]

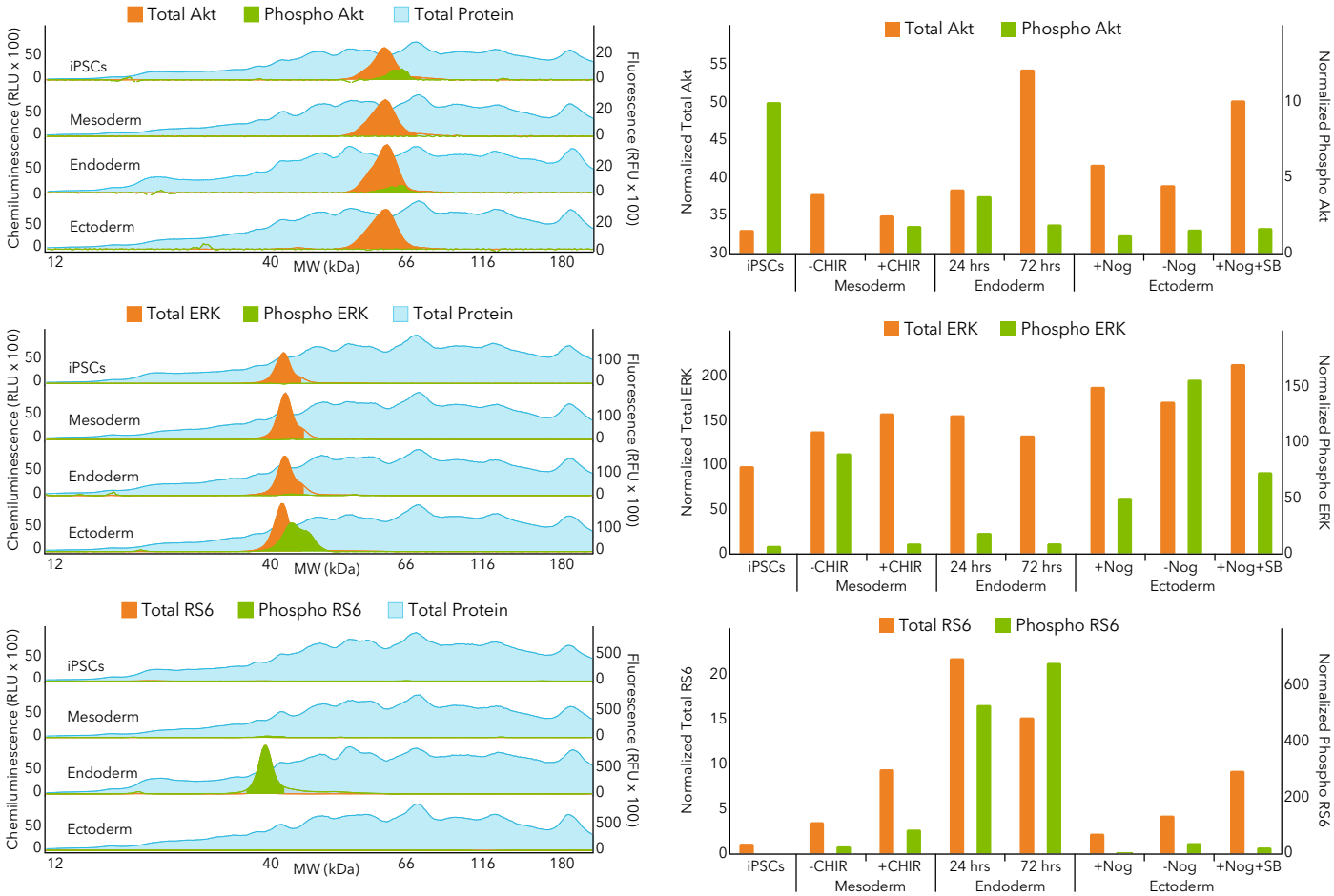

FIGURE 6. Simple Western analysis of total and phospho-protein isoforms of Akt, ERK, and RS6 across undifferentiated iPSCs and iPSC-derived mesoderm, endoderm, and ectoderm samples. Total and phospho-protein isoforms were detected in Stellar NIR/IR channels, and total protein detection was detected using chemiluminescence. Overlaid electropherograms are shown on the left, and protein expression values normalized to total protein are shown on the right. CHIR: CHIR 99021, Nog: Noggin, SB: SB 431542.

It has been reported that Noggin and SB 431542 dually inhibit the Smad pathway for more efficient iPSC conversion.[4] Therefore, we measured the impact of these two inhibitors on Smad1 and Smad5 total and phosphorylated-protein isoforms in iPSC-derived ectoderm cells. Noggin is known to inhibit Smad1/5 activation.[10] Consistent with those observations, ectoderm differentiation showed decreased phosphorylation of Smad1 and Smad5 compared to the undifferentiated iPSC sample (**FIGURE 7**). In the presence of Noggin, total Smad1 expression in iPSC-derived ectoderm cells remained unchanged relative to undifferentiation cells but decreased by more than 2-fold in the absence of Noggin, while phospho Smad1 decreased in all ectoderm samples (**FIGURE 7**).

The results so far have demonstrated how several protein targets plus total protein content can be measured in 24 samples at once using Stellar multiplex assays on Jess and RePlex sequential immunoassays on Jess and Abby. However, Simple Western's throughput capacity may be extended even further. Simple Western instruments like Peggy Sue™, Sally Sue™, and NanoPro™ 1000 can generate 96 data points overnight without user intervention. In addition to size separation, Peggy Sue and NanoPro 1000 can separate proteins by charge (pI), which can reveal protein isoforms with high resolution, including phospho-isoforms of ERK and Akt.[11]

To assess the performance of Peggy Sue for characterizing stem cell differentiation, we analyzed 7 protein targets with replicates across 4 sample types with total protein detection performed on each sample. The targets include stemness marker Nanog, as well as total and phospho-protein isoforms of ERK, Akt, and RS6. The results from this analysis were consistent with the above analysis performed on Jess.

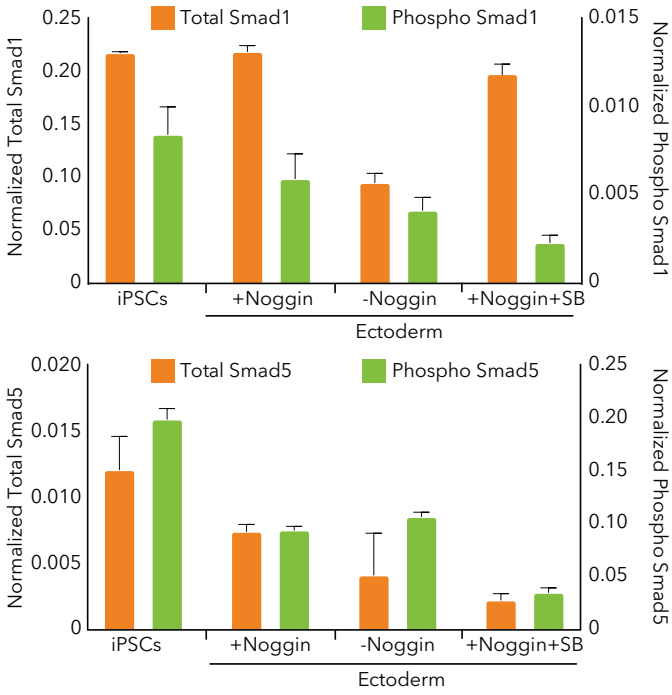

FIGURE 7. Simple Western analysis of Smad1 and Smad5 total and phospho-protein isoforms in iPSC-derived ectoderm cells. Antibodies targeting total and phospho-protein isoforms were detected in NIR/IR Stellar channels, and total protein detection was detected using chemiluminescence. The bar graph shows averages from 3 replicates, each normalized by total protein, with error bars representing the standard error of the means. CHIR: CHIR 99021, Nog: Noggin, SB: SB 431542.

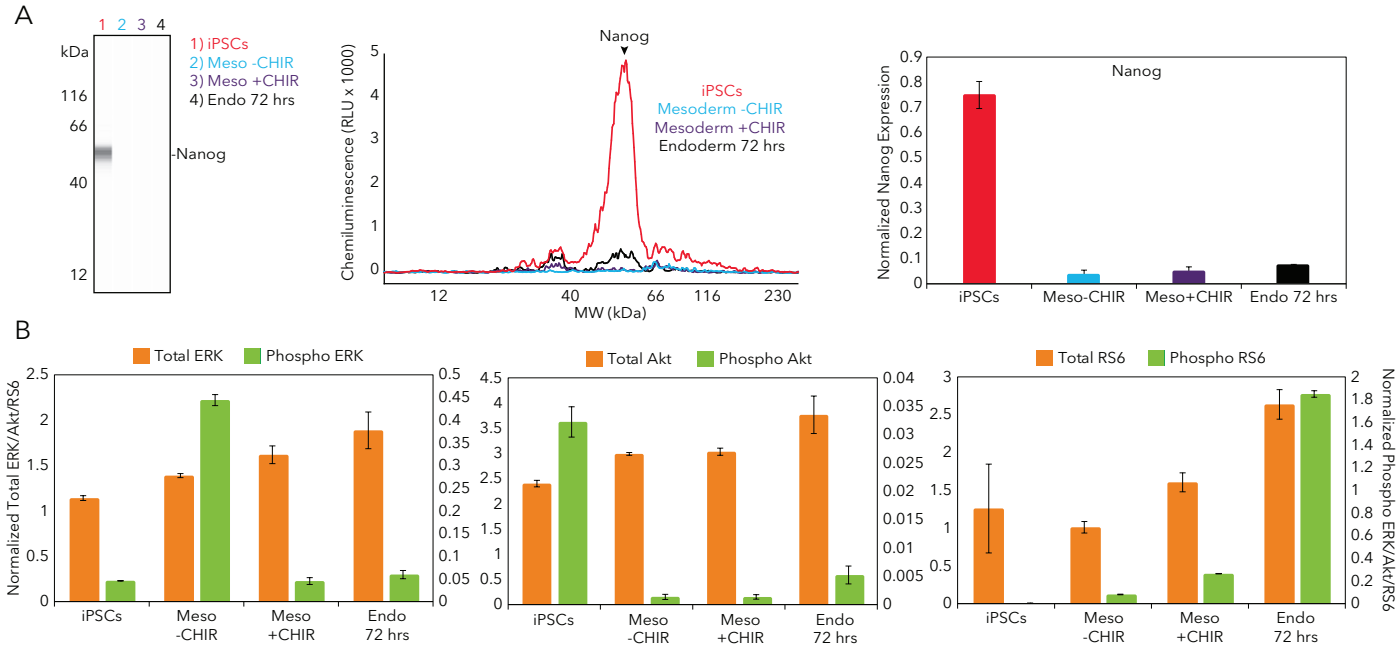

FIGURE 8. Peggy Sue analysis of stemness biomarker Nanog (A) and signaling molecules ERK, Akt, and RS6, both total and phospho-protein isoforms (B). All expression values were normalized by total protein assays performed in the same run. Corrected peak area values represent the means of 3 replicates, and the error bars represent the standard error of the means. CHIR: CHIR 99021, Nog: Noggin, SB: SB 431542.

Specifically, stemness marker Nanog expression dropped precipitously in differentiated mesoderm and endoderm layers compared to naïve iPSCs (FIGURE 8A). The elevated expression of phospho-RS6 in endoderm cells and phospho-ERK in mesoderm (–CHIR) cells, and the reduced expression of phospho-Akt in differentiated cells compared to iPSCs (FIGURE 8B) were also consistent with the above analysis on Jess (FIGURE 6). Thus, stem cell analysis on Simple Western can be scaled up to 96 data points per overnight run without compromising data quality.

Industry-Leading Fluorescence Detection Sensitivity with Stellar

Finally, we sought to compare the sensitivity of Stellar detection with chemiluminescence detection on Jess. To do so, we created a serial dilution series of iPSC-derived endoderm cells 24 hours post differentiation and analyzed each sample dilution using an antibody targeting β-Catenin, which regulates cellular proliferation, adhesion, and migration. The results from this analysis showed a clear band corresponding β-Catenin that had an apparent MW of 88 kDa which is consistent with the theoretical MW (FIGURE 9). With the quantitative results generated from this analysis, we calculated the limit of detection (LOD) and the limit of quantification (LOQ), which showed that Stellar NIR detection had lower LOD and LOQ values than chemiluminescence (TABLE 3). Therefore, Stellar NIR/IR detection can measure target protein expression with sensitivity that rivals chemiluminescence. Stellar NIR/IR detection on Jess vastly outperforms traditional fluorescence Western blotting workflows, and it sets a new industry standard in fluorescence detection sensitivity.

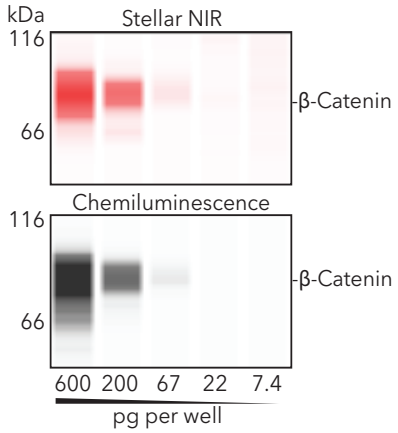

FIGURE 9. Simple Western analysis of phosphorylated protein β-Catenin in a serial dilution series of iPSC-derived endoderm cells using Stellar NIR and chemiluminescence detection on Jess.

	STELLAR	CHEMILUMINESCENCE
LOD	3.81 µg/mL	5.07 µg/mL
LOQ	11.55 µg/mL	15.35 µg/mL

TABLE 3. Comparison of Stellar NIR and chemiluminescence LOD and LOQ values of β-Catenin detection on Jess.

Comprehensive Molecular and Cellular Characterization for Efficient Stem Cell Manufacturing

Historically, the methods used to study stem cell pluripotency and differentiation have been inadequate to gain a complete, quantitative understanding of the complex signaling cascades that regulate these processes.[1] While RT-PCR measures gene expression at the transcript level, transcripts are only partially predictive of protein expression and do not shed light on post-translational regulation. Immunofluorescence assays can detect markers of cell stemness and differentiation, but they lack the throughput and quantitative ability to study the intracellular signaling networks at play. While flow cytometry offers high-throughput single-cell analysis, detecting intracellular proteins is challenging, and antibodies that discriminate between different protein isoforms (like phospho-proteins that are central to signaling cascades) are often not available. Traditional Western blot needs a prohibitively large amount of starting material for stem cell research and is poorly reproducible and only semi-quantitative. Finally, mass spectrometry is challenging to perform, requiring specialized equipment and training. As a result, there remains a major gap in the proteomic understanding of stem cell signaling, and this has been a challenge in developing effective stem cell therapies for regenerative medicine.

In this Application Note, we show that Simple Western provides a highly quantitative, sensitive, and reproducible protein analysis method for regenerative medicine. Using as little as 3 µL of starting material with flexible multiplex strategies, Simple Western assays generated protein expression profiles in stem cells with unprecedented sensitivity and reproducibility, revealing new insight into intracellular signaling cascades in response to iPSC differentiation. Simple Western's automation and high throughput enable at-line analysis, and Simple Western is emerging as a central component of large-scale stem cell manufacturing workflows for stem cell therapy and regenerative medicine.[12]

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