Total Protein Analysis the Simple Western Way

Introduction

The Simple Western Immunoassay is the gel-free, blot-free and hands-free solution for researchers looking for a better way to get their Western blot data. The simple fact that you get analyzed data in just three hours with only 30 minutes of hands-on time changes things forever! So, since we think change is a *good* thing, we packed all that Simple Western speed, simplicity and data quality into our new Total Protein Assay.

The Simple Western Total Protein Assay for Wes, Sally Sue and Peggy Sue is actually pretty similar to a Simple Western Immunoassay except it doesn't use an antibody (**Figure 1**). You have the same flexibility to use either the 12–230 kDa or the 66–440 kDa separation matrix and sample preparation is the same too, so you'll get an identical number of data points with just 5 µL of sample. Compass software still analyzes your data automatically but as an added bonus, versions 2.6 and higher now also let you easily compare Total Protein Assay and Immunoassay data side-by-side. And cleanup is still as simple as ever — you'll never dispose of a single staining or destaining solution. Now you've got a truly gel-free, hands-free, waste-free Total Protein Assay — which means you'll really never have to run a gel again!

How Does the Total Protein Assay Work?

Sample preparation, separation, and immobilization in the Total Protein Assay are exactly the same as the Simple Western Immunoassay. But, instead of specifically detecting your protein of interest with a primary antibody, the Total Protein Assay attaches biotin to the protein in your sample using a PFP-biotin labeling reagent. Incubation with streptavidin-HRP followed by Luminol/ Peroxide generates a chemiluminescent signal wherever protein is captured to the capillary (**Figure 1**).

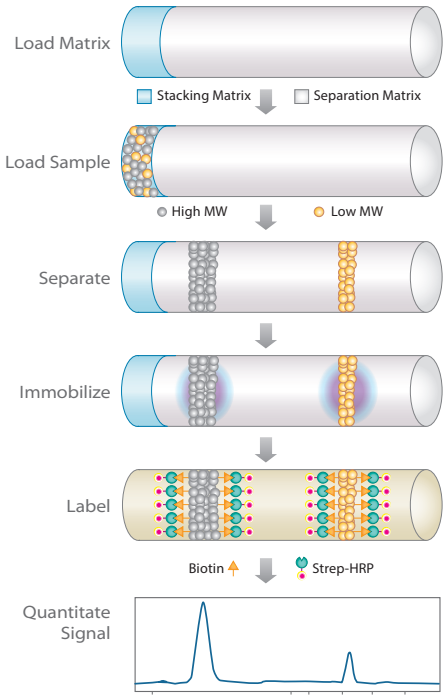

FIGURE 1. How the Total Protein Assay works.

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Setting up a Total Protein Assay

Setting up a Total Protein Assay on Wes, Sally Sue or Peggy Sue is simple. You just prepare your sample, re-suspend the lyophilized Total Protein Labeling Reagent with a couple of reconstitution buffers, mix your Luminol and Peroxide together and then pipette reagents into the plate (**Figure 2**). All the other assay reagents you need come ready to use.

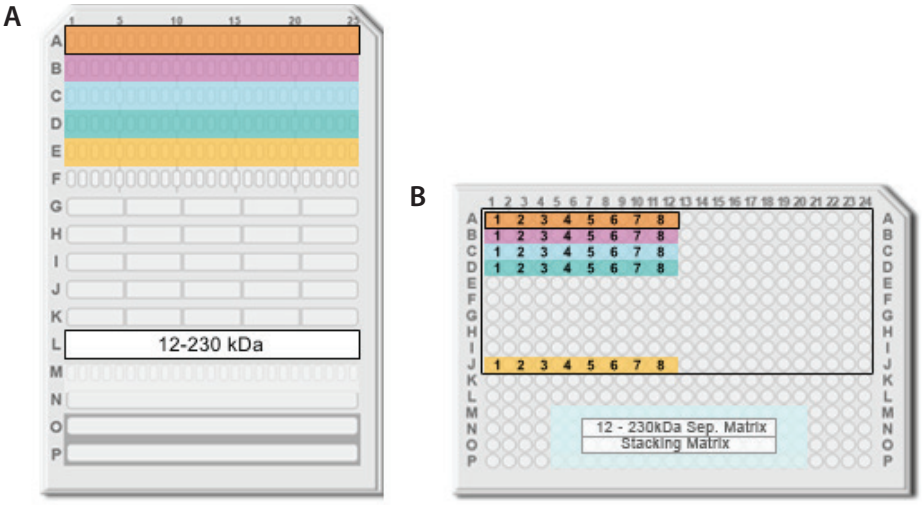


FIGURE 2. Sample plate set up for Wes (A) and Sally Sue/Peggy Sue (B). Samples and ladder go in Row A (orange), Total Protein Labeling Reagent in Row B (purple), Antibody Diluent II (blocking buffer) in Row C (light blue), Total Protein Streptavidin-HRP in Row D (teal), and Luminol/Peroxide in Row E or J (gold).

Using Compass to analyze data is also as simple as ever, but we've thrown in a few extra features for the Total Protein Assay. Protein band colors in Lane View are now blue so you know you're looking at Total Protein Assay data, and you now have the option to fit peaks using either the Total Protein Assay default line-drop fit or the Immunoassay default Gaussian peak fit (**Figure 3**). We've also added an analysis parameter, % Total, which automatically tells you what percentage of the total signal your peak of interest is.

To show you how the Simple Western Total Protein Assay can be easily used to monitor the expression and purification of a recombinant protein, we overexpressed DJ-1 — a protein where loss of function mutations have been found to play a significant role in autosomal recessive Parkinson's disease — in bacterial cells.

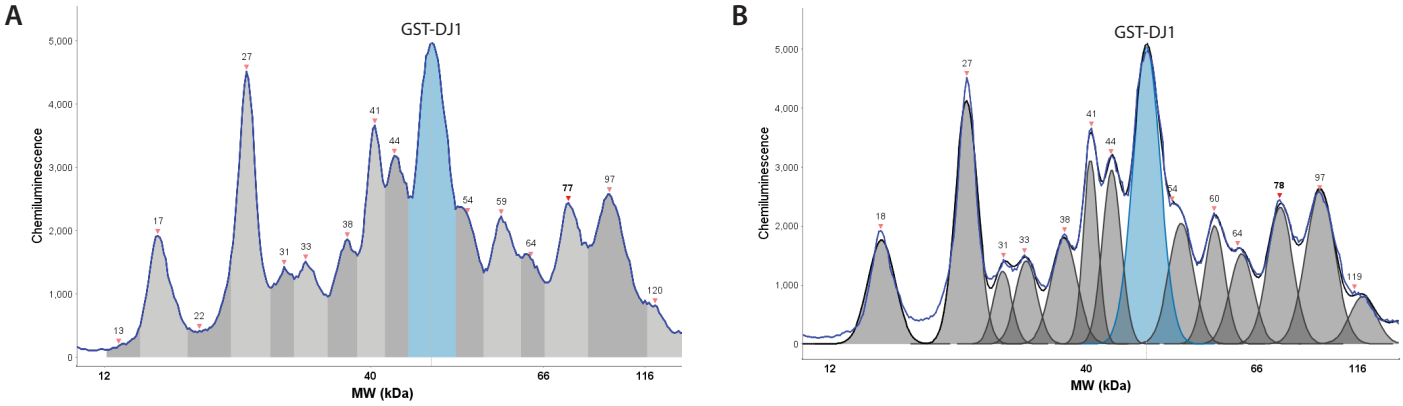


FIGURE 3. New Total Protein Assay peak fit options in Compass. You can analyze data using either the line-drop (A) or Gaussian (B) peak fits.

Making the GST-DJ-1 Expression Clone

We received a pJ404-GST-DJ1 bacterial expression plasmid from Addgene (ID#51409), working in agreement with The Michael J. Fox Foundation for Parkinson's Research. The plasmid came in a bacterial stab of the plasmid in DH5α cells that we used to streak an LB Agar plate with 100 µg/mL ampicillin (Teknova #L1004). After an overnight incubation at 37 °C, one colony was then grown up in LB broth with 100 µg/mL ampicillin (Teknova #L8510) overnight to make a glycerol stock and purify the DNA from the bacteria.

To purify the DNA, we used a QIAGEN Plasmid Mini Kit (QIAGEN #12123) and followed the vendor's protocol. We then heat-shocked the DNA into BL21 expression cells (Sigma #B2685) and streaked the cells onto an LB Agar plate with 100 µg/mL ampicillin.

Optimizing Protein Induction is Simple

A BL21-pJ404-GST-DJ-1 colony was grown in LB broth with ampicillin overnight. The next morning, we diluted the culture 1:10 to make sure the bacteria was in the logarithmic growth phase and grown at 37 °C. After one hour, an uninduced sample was taken before inducing production of GST-DJ-1 for three hours with 0.4 mM IPTG (Sigma #I1284). Next, we ran samples using Total Protein Assay default conditions on Wes so we could monitor the protein induction. A second experiment was also run on Wes where the samples were immunoprobed with anti-DJ-1 and anti-GST, which confirmed the identity of the expressed protein (**Figure 4**) when compared to the Total Protein Assay data. Data comparison and analysis was really simple since Compass let us open multiple run files at the same time to view and analyze both assays side-by-side.

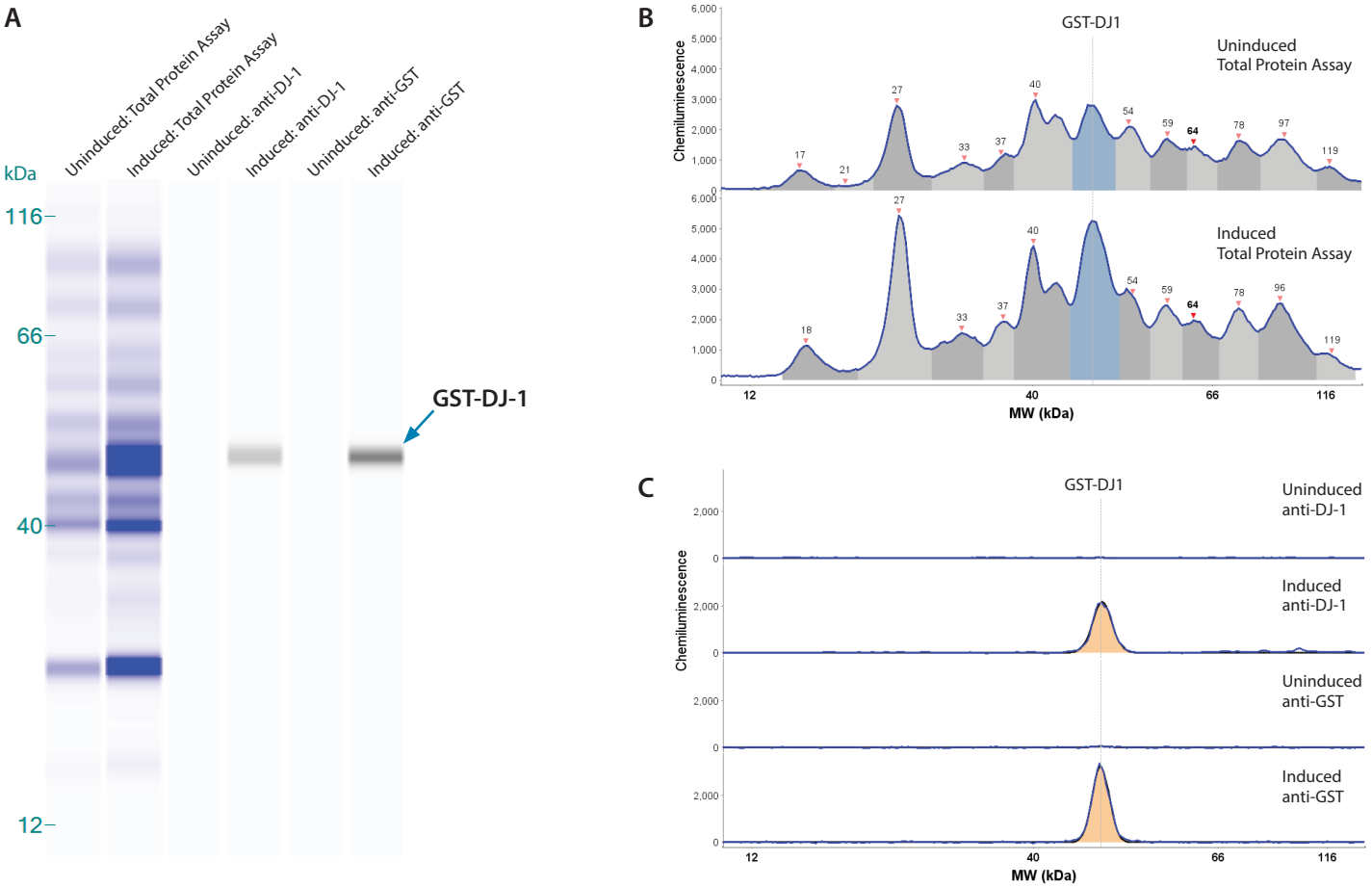
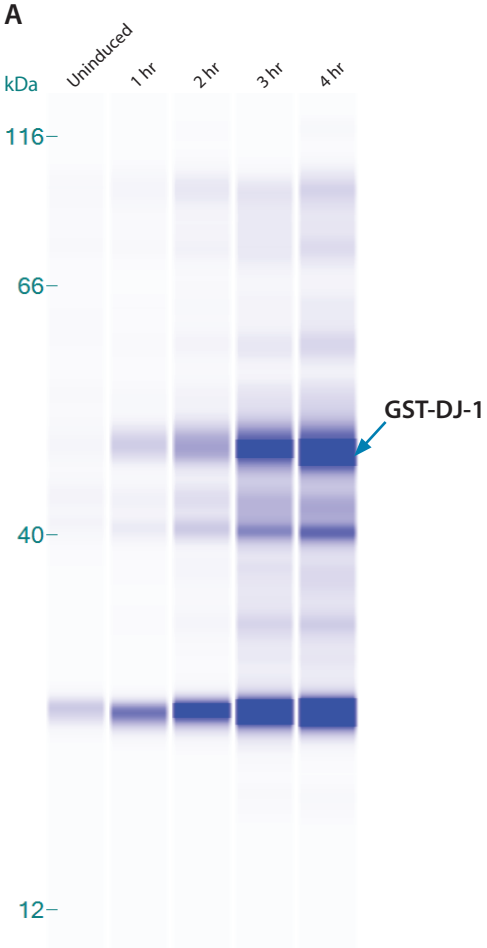


FIGURE 4. Monitoring protein induction using the Total Protein Assay with protein identity confirmation using the Immunoassay. 300 µL of bacterial culture was pelleted pre- and post-IPTG induction and resuspended in 50 µL ProteinSimple Bicine/Chaps Buffer. 2 µL of each sample was used to monitor protein expression with the Total Protein Assay (A, lanes 1-2). Samples were then diluted 1:1000 and 2 µL of the diluted sample was immunoprobed to confirm protein identity (A, lanes 3-6). Total Protein Assay data shown was viewed in Compass using both Lane View (A) and Electropherogram View (B) and immunoassay data shown was viewed in Compass using both Lane View (A) and Electropherogram View (C).

We saw significant induction of our GST-DJ-1 at 49 kDa. And since we induced the protein at 37 °C when the bacteria were in a logarithmic growth phase, we also saw increased expression of some endogenous bacterial proteins. So, we analyzed things further by monitoring the %Total as well as the overall peak area.

To optimize the timing of protein expression, we grew up a 3 mL culture using the same method used earlier, induced with IPTG, and collected 300 µL aliquots to pellet every hour to confirm we were getting optimal protein expression (**Figure 5**). By assessing the %Total and the peak area, we decided to induce all subsequent cultures for 3 hours since expression of the GST-DJ-1 started plateauing by 4 hours.

A



B

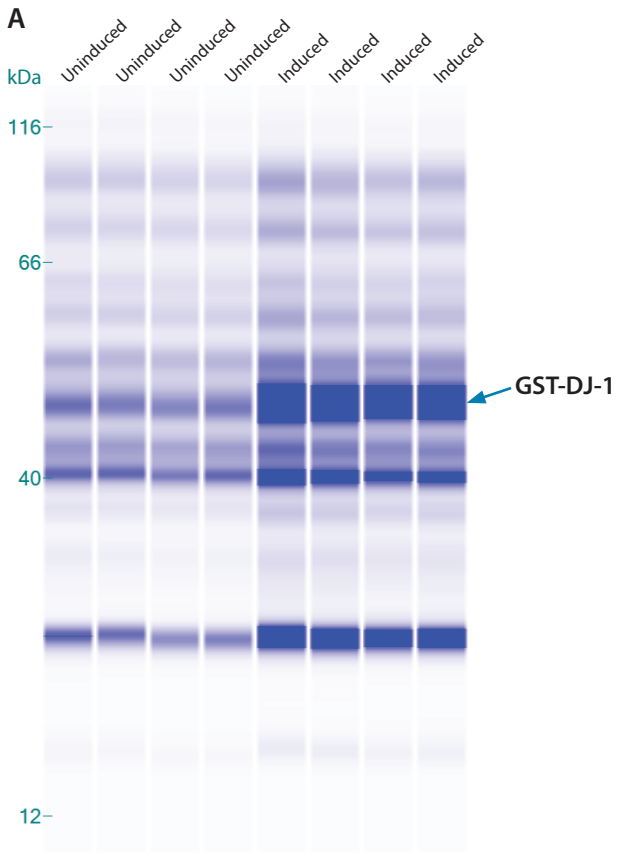
Sample	Name	MW (kDa)	Area	% Total
Uninduced	GST-DJ-1	49	9550	11.11
1 hr	GST-DJ-1	49	21705	19.28
2 hr	GST-DJ-1	49	36863	20.07
3 hr	GST-DJ-1	49	59163	20.81
4 hr	GST-DJ-1	48	65154	20.15

FIGURE 5. Optimizing the timing of protein induction with the Total Protein Assay. A bacteria culture was induced with 0.4 mM IPTG and 300 µL of bacterial culture was collected and pelleted every hour. 2 µL of each sample was then prepared for monitoring of GST-DJ-1 expression on Wes. The protein induction was monitored in Compass using Lane View (A) and the % Total values in the Peaks Table (B).

Data Precision that's First-rate

We then ramped up our protein purification process and induced a 15 mL culture based on the conditions optimized earlier. To show that you can get the same amazing data reproducibility that you've come to expect with Simple Western assays, we ran uninduced and induced samples in quadruplicate capillaries (**Figure 6**).

We still saw a notable induction of the GST-DJ-1 protein, but it was slightly lower than before. This could be due to differences in BL21 cell confluence between the two experiments since we didn't normalize using the culture OD. But as expected, the CVs were fantastic — area CVs for the uninduced and induced sample were 5.2% and 6.6% respectively, and %Total CVs were 5.8% and 3.3%!



B

Sample	Name	MW (kDa)	Area	% Total
Uninduced	GST-DJ-1	49	50578	13.61
Uninduced	GST-DJ-1	49	45108	12.65
Uninduced	GST-DJ-1	49	46620	13.85
Uninduced	GST-DJ-1	49	49450	14.30
Induced	GST-DJ-1	49	93850	17.93
Induced	GST-DJ-1	49	84985	17.56
Induced	GST-DJ-1	49	83092	18.73
Induced	GST-DJ-1	49	80876	17.93

C

Sample	Name	Area	Std.Dev.	% CV
Induced (4)	GST-DJ-1	85701	5687	6.6
Uninduced (4)	GST-DJ-1	47939	2517	5.2

D

Sample	Name	%Total	Std.Dev.	% CV
Induced (4)	GST-DJ-1	18.04	0.4910	2.7
Uninduced (4)	GST-DJ-1	13.60	0.6968	5.1

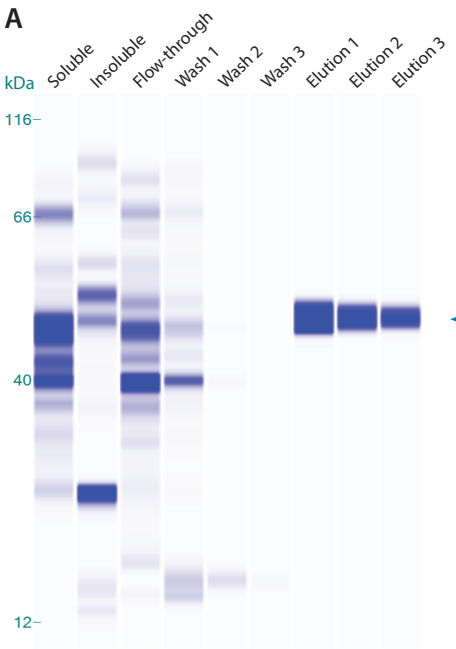
FIGURE 6. Data precision for both uninduced and induced samples. 300 µL of bacterial culture was collected pre and post-IPTG induction and pelleted. Pellets were resuspended with 50 µL ProteinSimple Bicine/CHAPS Buffer and 2 µL of sample was prepared and run in quadruplicate capillaries (A). The line-drop peak fit in Compass was used to generate raw data (B), statistical data from the peak area (C), and statistical data from the %Total (D). All CVs were ≤6.6%.

Monitoring a GST Affinity Purification with Ease

Because the Total Protein Assay is also really useful for monitoring the purification of proteins from expression clones, we also affinity purified the GST-DJ-1 from the induced bacterial culture. The bacteria was pelleted and lysed with PBS containing lysozyme, Tx-100, DTT, and PMSF. Samples were frozen and thawed multiple times and then centrifuged to separate the soluble and insoluble fractions. We pre-washed glutathione sepharose beads (Sigma #GE17-0758-01) twice with PBS/1% Tx-100 and once with PBS, then applied the soluble fraction to 50% glutathione sepharose beads. The lysate and beads were incubated for two hours at 4 °C on a rotator to allow the GST-DJ-1 to bind to the beads. Next, we collected the flow-through and washed the beads twice with PBS/1% Tx-100 followed by a wash with PBS. Finally, GST-DJ-1 was eluted with glutathione (Sigma #G4251) to compete the protein off the beads.

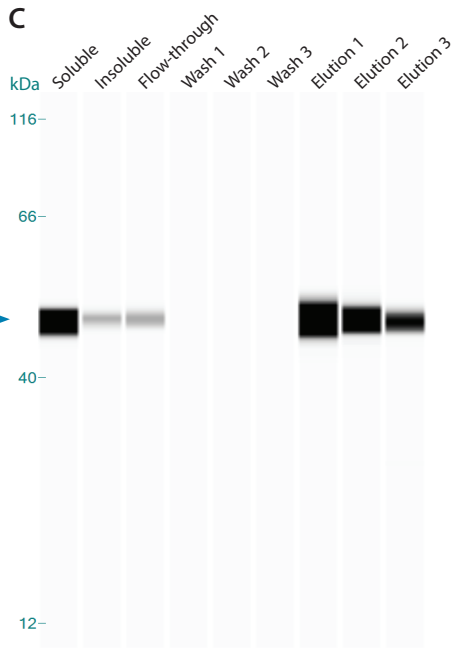
We then ran each sample using the Total Protein Assay and in less than three hours with only 30 minutes of hands-on time we were able to monitor the different steps of our affinity purification (**Figure 7**). To properly quantify the data, we needed to make sure all the samples were within the linear range of the assay. To do this, we ran different volumes of each sample and found that 2 µL of each sample kept us in the linear range except for the soluble sample and the elutions, where we used 1 µL and 0.5 µL sample, respectively.

A



GST-DJ-1

C



B

Sample	Name	MW (kDa)	Area	% Total
Soluble	GST-DJ-1	49	139152	29.38
Insoluble	GST-DJ-1	49	44245	14.56
Flow-through	GST-DJ-1	49	74161	15.61
Wash 1	GST-DJ-1	48	37747	14.94
Wash 2	GST-DJ-1	48	4404	12.99
Wash 3	GST-DJ-1	49	57	0.79
Elution 1	GST-DJ-1	49	127217	98.10
Elution 2	GST-DJ-1	49	88026	98.70
Elution 3	GST-DJ-1	49	68788	98.72

D

Sample	Primary	Name	MW (kDa)	Area
Soluble	anti-DJ-1	GST-DJ-1	49	27515
Insoluble	anti-DJ-1	GST-DJ-1	49	4033
Flow-through	anti-DJ-1	GST-DJ-1	49	4679
Wash 1	anti-DJ-1	GST-DJ-1	49	830
Wash 2	anti-DJ-1	GST-DJ-1	50	101
Wash 3	anti-DJ-1	GST-DJ-1	49	0
Elution 1	anti-DJ-1	GST-DJ-1	49	59966
Elution 2	anti-DJ-1	GST-DJ-1	49	24763
Elution 3	anti-DJ-1	GST-DJ-1	49	11670

FIGURE 7. GST affinity purification monitoring. Samples were affinity purified using glutathione sepharose beads. 2 µL of sample was run on Wes using the Total Protein Assay (A) and analyzed using Compass (B). Samples were diluted 1:1000 and then immunoprobed with anti-DJ-1 to specifically detect the amount of GST-DJ-1 in the sample (C) then analyzed using Compass (D).

Monitoring both the Total Protein Assay and the Immunoassay lets us easily evaluate the different steps in our purification process. For example, the data from the flow-through samples indicate we could improve the GST-DJ-1 binding efficiency to the glutathione sepharose beads, either by increasing the incubation time for the beads with the lysate or by increasing the volume of beads used. We can also gauge the purity of our final product after purification to determine if we also non-specifically bound and eluted protein. It's important to note that in these experiments, we had extremely pure sample and the eluted samples were >98% DJ-1 protein.

Conclusion

Now you can have all the things you've come to love about the Simple Western Immunoassay in a Total Protein Assay too! To show you how great the data is, we overexpressed and affinity purified GST-DJ-1 in BL21 cells and ran the numbers. As you'd expect, data reproducibility was off the charts good with CVs for area and %Total of less than 6.6%. We also used the Total Protein Assay data to optimize the protein induction, monitor the different steps of an affinity purification, and determine purity of the purified sample. To confirm identity of the protein band, all we had to do was immunoprobe samples for anti-GST and anti-DJ-1 using the Simple Western Immunoassay.

Only 30 minutes of hands-on time was needed for each experiment, each was completed in three hours, and the data generated was easily analyzed in Compass software. And at the end of each experiment, we just threw the cartridge and plate away to be ready for the next run. The new Total Protein Assay gives you simple setup, simple analysis, and simple cleanup with no harmful waste to dispose of — making it an environmentally friendly way to go, too. So now with the Simple Western you can analyze total protein and run immunoassays on one platform, and never run a gel again!

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Toll-free: (888) 607-9692
Tel: (408) 510-5500
Fax: (408) 510-5599
info@proteinsimple.com
proteinsimple.com

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