

Total Protein Normalization using RePlex and NIR Detection

A Protocol for Protein Expression Analysis of Low Abundance Targets in High Concentration Lysate Samples

Housekeeping proteins are unreliable standards for protein normalization (PN). With Simple Western Technology, total protein normalization (TPN) is fast and reproducible, even with high concentration lysate samples.

TPN using the PN channel allows the detection of target proteins by an immunoassay with chemiluminescent or fluorescent probes and total protein in the same capillary. Alternatively, TPN using RePlex and Chemiluminescence detection allows total protein detection following RePlex to remove antibodies from an immunoassay with chemiluminescent or fluorescent probes.

Introduction

The **Jess™ System**, powered by **Simple Western™ Technology**, provides fully quantitative protein expression measurements in cell and tissue lysates. Jess users benefit from several flexible methods to implement TPN seamlessly in their workflows for accurate protein expression measurements.

This protocol describes a third method, TPN using RePlex and NIR detection, that allows total protein detection following RePlex to remove antibodies from an immunoassay with chemiluminescent or fluorescent probes. TPN using RePlex and NIR detection is linear for high sample concentrations (0.5–2.5 mg/mL), facilitating the analysis of low abundance targets (FIGURE 1). Like all Simple Western assays, this method is fully automated, and results are ready in just a few hours.

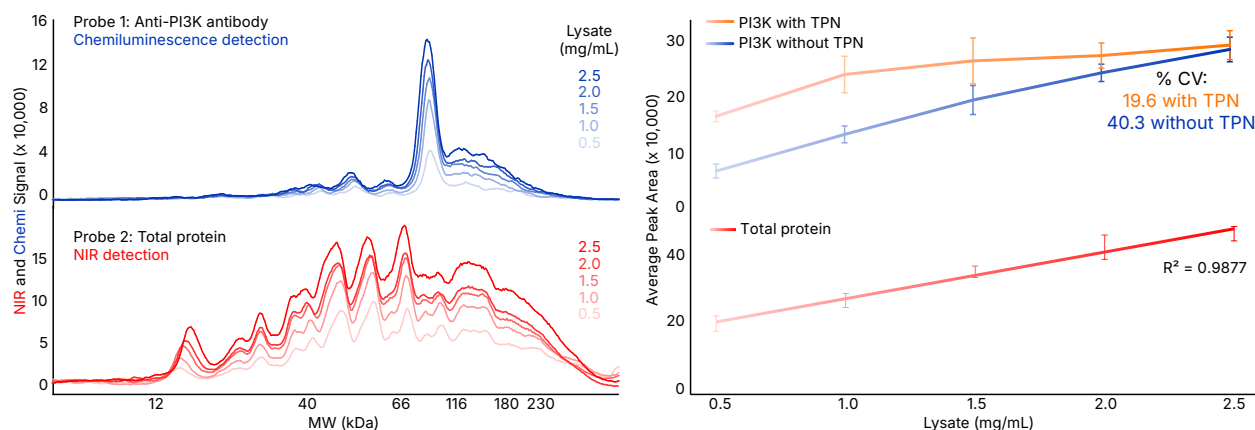


FIGURE 1. Simple Western analysis of PI3K expression (blue) in human cerebellum whole tissue lysate using the 12–230 kDa Fluorescence Separation Module with RePlex and NIR total protein detection (red) for reliable TPN (orange).

The protocol described here has been tested on whole-cell lysates, brain tissue, and muscle-skeletal tissue homogenates to measure low abundance targets. This protocol guides your TPN assays with high concentration samples to measure low abundance targets, including tips for setting up your assay and analyzing the data.

Materials and Methods

TPN using RePlex and NIR detection is performed on the **Jess System** using a **12-230 kDa** or **66-440 kDa** Fluorescence Separation Module, **RePlex Module** (RP-001), **Total Protein Detection Module** (DM-TP01) and the **Streptavidin-NIR, 1.6 mL Conjugate** (043-868).

Antibody Preparation

You may visit the **Simple Western Antibody Database** to find validated antibodies or validate your own. If you validate a new primary antibody, determine the saturating concentration. For proper normalization, the primary antibody should have a linear response to lysate titration that overlaps with the linear total protein detection range. Refer to the **RePlex Method Development Guide** for more information. Evaluating additional antibodies for the same target may be necessary, as antibodies can bind targets differently and produce titration slopes that may skew TPN.

Dilute the primary antibody to its saturating concentration in Antibody Diluent 2 and place the primary antibody and ready-to-use secondary antibody on ice. *Note: When using the Anti-Goat Detection Module, use Milk-Free Antibody Diluent.*

Sample Preparation

Denature the samples under reducing conditions in 1X Master Mix for 5 minutes at 95 °C (common denaturation setting for most targets) or 10 minutes at 70 °C (refer to our **protocol** for optimizing sample denaturation conditions).

Loading the Jess Plate

Load 8 µL of Streptavidin-NIR in row F (FIGURE 2). If you are not using chemiluminescence detection in Probe 1, fill row J with Wash Buffer using 170 µL per well. *Note: We recommend chemiluminescence detection for low abundance targets.*

Setting Up Your Assay and Creating a Protocol File

Optimize your assay conditions by performing a titration series of the sample to identify the linear total protein detection range.

This protocol uses NIR detection in a custom manner that is not currently standard protocol with Compass for Simple Western software. We recommend creating a protocol like the one described below and importing the protocol file each time you perform this assay.

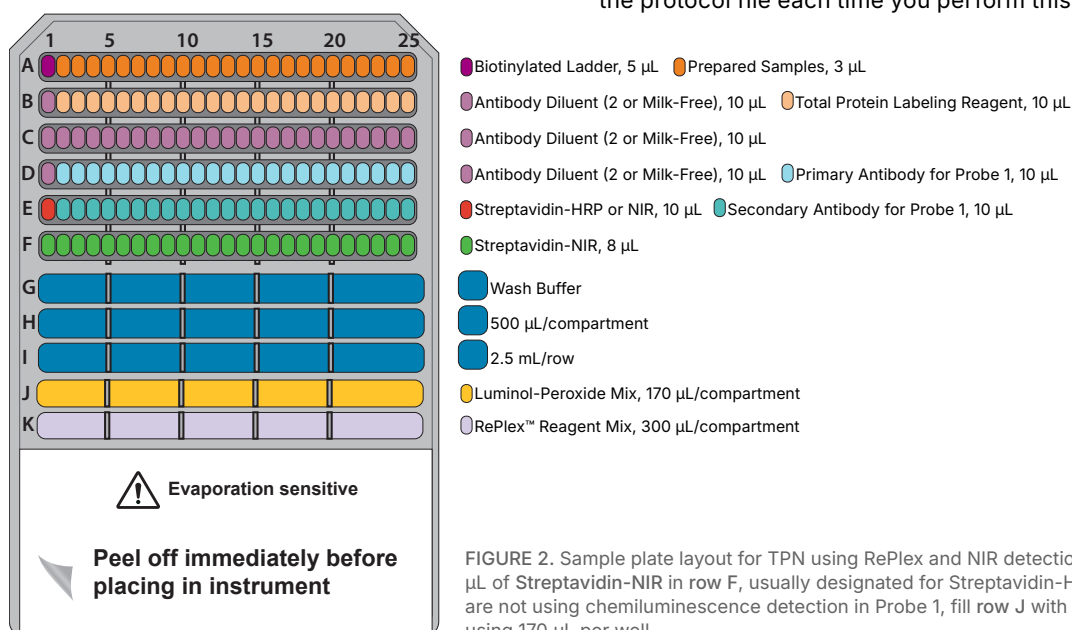


FIGURE 2. Sample plate layout for TPN using RePlex and NIR detection. Load 8 µL of Streptavidin-NIR in row F, usually designated for Streptavidin-HRP. If you are not using chemiluminescence detection in Probe 1, fill row J with Wash Buffer using 170 µL per well.

1. Open a new RePlex run with Total Protein Assay.
2. In the Protocol tab, under Probe 2, click on RDR in the Detection Profile (Chemi) row and click the option (...) button (FIGURE 3).
3. Unselect RePlex Dynamic Range, change the single exposure to 0.1 sec, then click OK (FIGURE 3).
4. Under Probe 2, click on None in the Detection Profile (NIR) row and click the option (...) button (FIGURE 4).
5. Add NIR exposures by clicking the Add button for a total of 6 times (FIGURE 4).
6. Set the NIR exposures as shown (FIGURE 4).
7. Click OK (FIGURE 4). You are ready to run TPN using RePlex and NIR detection!

FIGURE 3. Change the RePlex Dynamic Range (RDR) to 0.1 sec exposure.

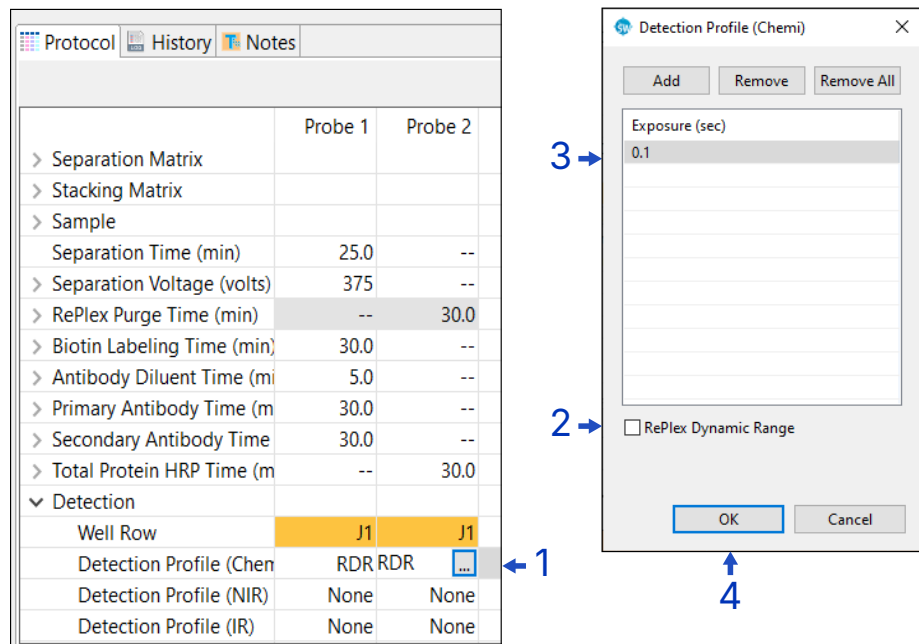
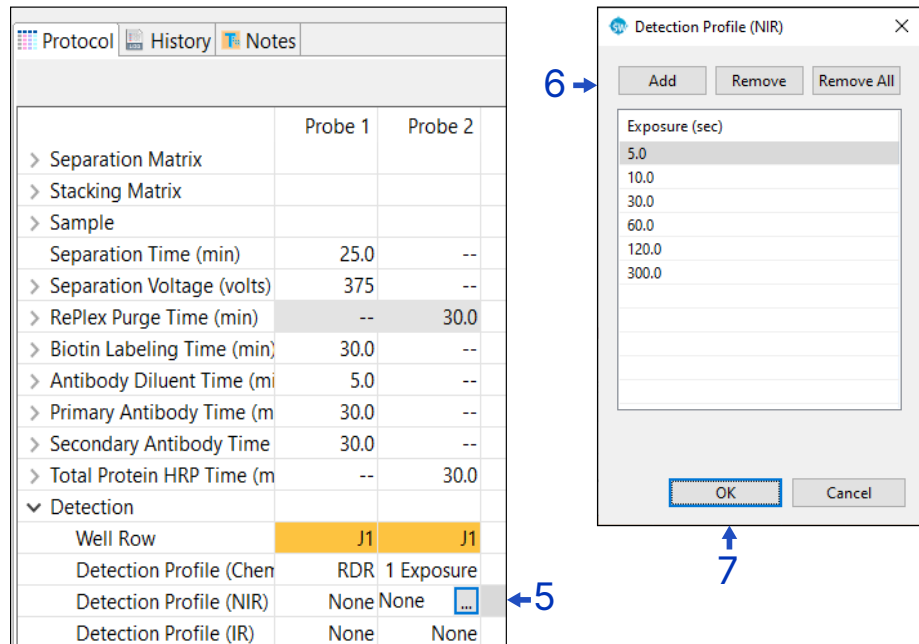


FIGURE 4. Add NIR exposures to Probe 2 of RePlex as shown.



Data Analysis

TPN using RePlex and NIR Detection Settings

The user defines the settings of Probe 1 of RePlex for the target of interest. The settings of Probe 2 for TPN using RePlex and NIR detection are below. The user should calculate the total protein area of two ranges to determine the impact on the normalization of the specific target. The user defines the optimal range for the target of interest, provided by the 12-230 and 66-440 kDa Separation Modules (0.1 kDa to 375 kDa and 500 kDa, respectively).

Review all capillaries to ensure that baseline points are present and anchored to the left, flat side of the capillary. Manual adjustment may be required (see next page).

Peak Fit Settings

1. Select Analysis in the Edit menu, then select Peak Fit (FIGURE 5).
2. In the Analysis Groups section, click Add to add a group called fit 2 (FIGURE 5).
3. Under Apply Override, click Add to add two groups (FIGURE 5). Assign the groups:

Probe 1/fit settings for specific target

Probe 2/fit 2 settings for NIR total protein area
4. For the fit 2 group, update the analysis parameters for Range, Baseline, and Peak Find (FIGURE 5).
5. Select Full View and select Apply (FIGURE 5). The Full View allows you to accurately identify the capillary's left (flat) end for proper baseline fitting.

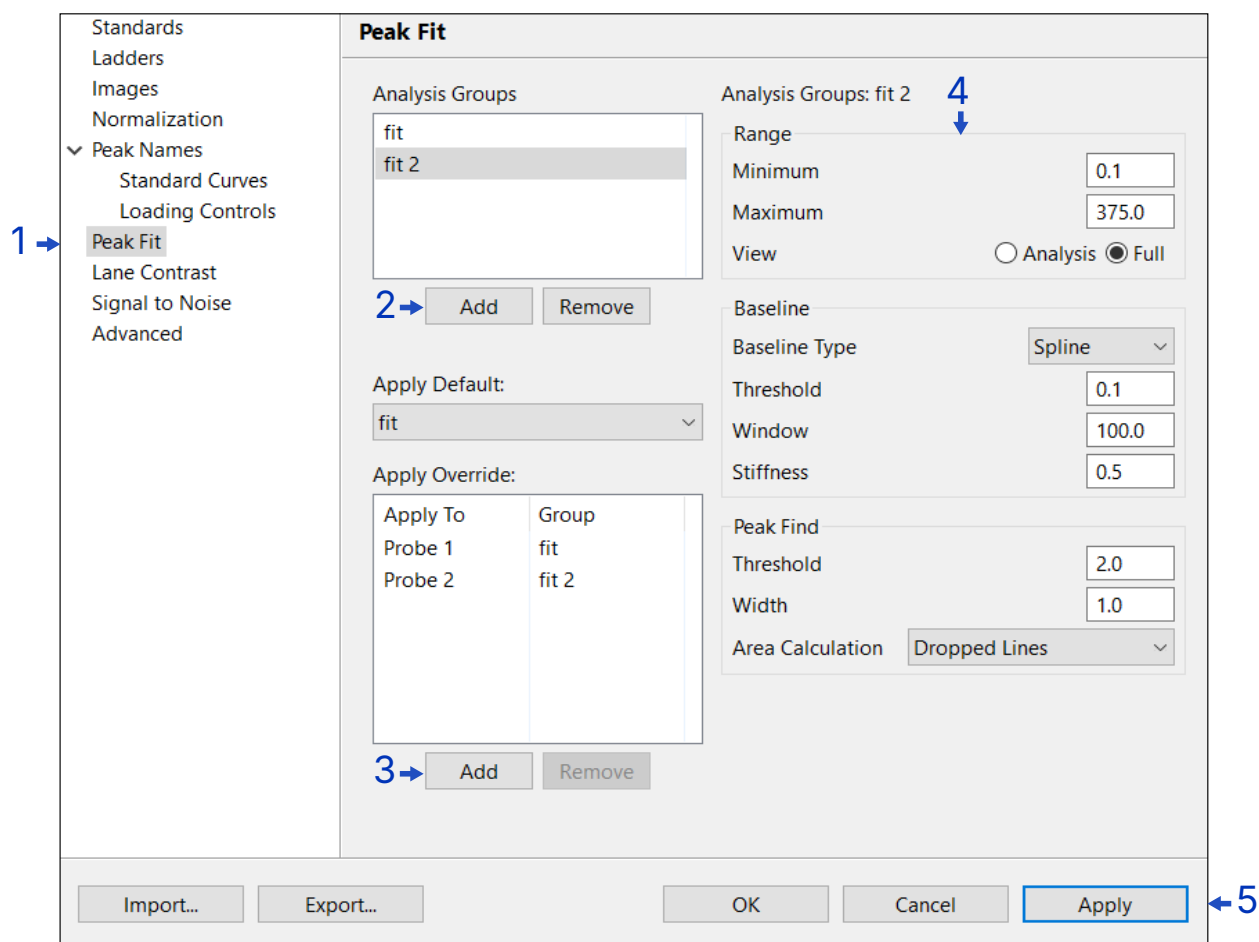


FIGURE 5. Peak fit settings for TPN using RePlex and NIR detection.

Optimize Baseline

Occasionally, the total protein peak fit settings do not add baseline points to the baseline, resulting in an unanchored baseline. If left unanchored, this will skew normalization data, overestimating the NIR total protein area. If this occurs, follow these steps:

1. Manually add baseline points to the left, flat side of the capillary to anchor down the baseline (FIGURE 6). With your mouse, hover over the left, flat region of the capillary, then right-click and select Add Baseline Point.
2. Repeat 4-5 times until you have anchored down the baseline.

In this example, the NIR total protein area decreased by approximately 10% after adding baseline points (FIGURE 6).

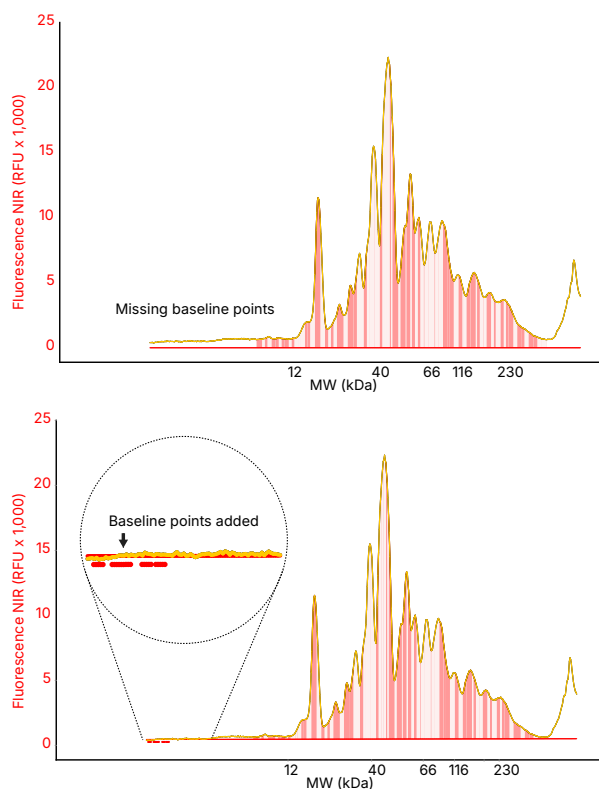


FIGURE 6. Example electropherograms of NIR total protein detection with missing baseline points (top) and added baseline points (bottom).

Conversely, removing baseline points that negatively influence the baseline may be necessary if baseline anchors are outside the left, flat end of the capillary. The example in FIGURE 7 shows replicates with baseline points negatively impacting the NIR total protein detection, resulting in an underestimation of the total area.

If this occurs, remove unwanted baseline points by right-clicking them with your cursor and selecting Remove Baseline Point. Removing unwanted baseline points may have minimal impact on the electropherograms visually, but the effect on total area is significant. In this example, the NIR total protein area increased by approximately 10% after removing baseline points (FIGURE 7).

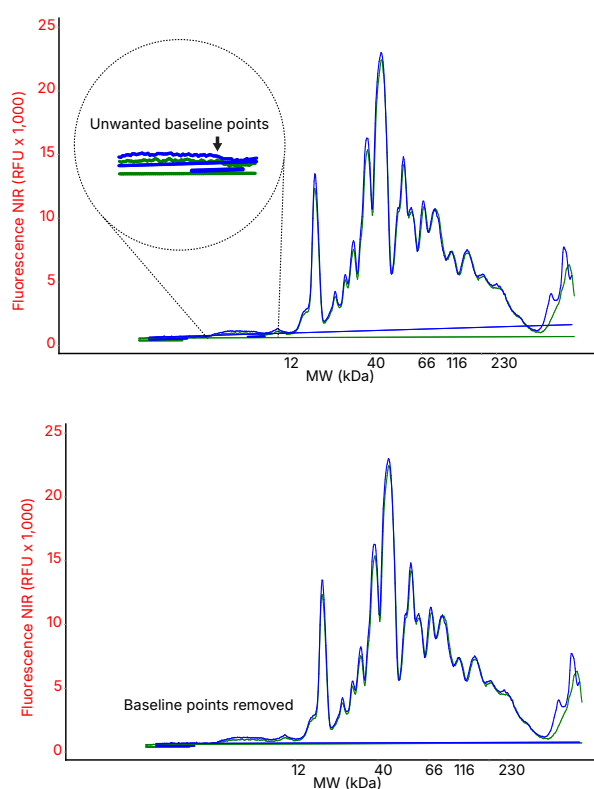


FIGURE 7. Example electropherograms of NIR total protein detection with unwanted baseline points (top) and unwanted baseline points removed (bottom).

Data Export

1. After the analysis of Probe 1 and Probe 2 is complete, export the data by selecting Export Table... in the File menu (FIGURE 8).
2. Double-click on the exported data folder and open Capillary Peaks Area.txt, right-click to Select All, and Copy (FIGURE 8).
3. The raw data can be pasted into the TPN using RePlex and NIR Detection Worksheet below to automate your data normalization (FIGURE 9). For more information, see the Normalization Calculation on the following page.

The figure illustrates the process of exporting capillary peaks area data from Compass to a text file. It is divided into three numbered steps:

- Step 1:** The 'File' menu is open, and 'Export Tables...' is selected. A blue arrow labeled '1' points to this option.
- Step 2:** A file explorer window shows a folder containing several exported files. 'Capillary Peaks Area.txt' is selected. A blue arrow labeled '2' points to this file.
- Step 3:** A Notepad window titled 'Capillary Peaks Area.txt - Notepad' displays the exported data. A blue arrow labeled '3' points to the top of the data table.

The data table in Notepad has the following columns: Sample, Attribute, Primary Attribute, Secondary, Scdry Attr, Capillary, CHEMI Total Area, NIR Total Area, TP Area, and TPN (%). The table contains 20 rows of data for various samples and their corresponding attributes and areas.

Sample	Attribute	Primary Attribute	Secondary	Scdry Attr	Capillary	CHEMI Total Area	NIR Total Area	TP Area	TPN (%)
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.25 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.25 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.5 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.25 mg/mL"	"Rb anti-PI3K mAb; CST3011"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"	"1:200"	"anti-Rabbit Secondary HRP Conjugate RTU"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2.5 mg/mL"	"Primary Antibody"	"P2:2 408528.84"	"3714091.083"	"4"	"6"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2 mg/mL"	"Primary Antibody"	"P2:3 407944.104"	"3236709.112"	"4"	"6"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1.5 mg/mL"	"Primary Antibody"	"P2:4 435718.263"	"3089749.746"	"4"	"6"
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"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.5 mg/mL"	"Primary Antibody"	"P2:6 465158.046"	"1291654.21"	"4"	"6"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.25 mg/mL"	"Primary Antibody"	"P2:7 452923.07"	"826222.86"	"4"	"6"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2.5 mg/mL"	"Primary Antibody"	"P2:8 371519.225"	"3481686.783"	"3"	"4"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"2 mg/mL"	"Primary Antibody"	"P2:9 416921.148"	"2934647.271"	"4"	"6"
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"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"1 mg/mL"	"Primary Antibody"	"P2:11 279981.967"	"1734978.564"	"2"	"3"
"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"Human Skeletal Muscle whole Tissue Lysate (NB820-59253)"	"0.5 mg/mL"	"Primary Antibody"	"P2:12 363442.302"	"1220011.86"	"3"	"4"

FIGURE 8. Export the capillary peaks area table from Compass for Simple Western Software.

Normalization Calculations

1. Open the TPN using RePlex and NIR Detection Worksheet, click cell A1 (yellow), and Paste the data into the worksheet (FIGURE 9).
2. The worksheet will automatically normalize your data (green) to Control Reference Capillary 2 using the NIR Total Area (orange) (FIGURE 9).

The Normalization Ratio column (N) divides the NIR Total Area of the Control Reference Capillary 2 (I26) by the NIR Total Area of each subsequent capillary (I27:I49). The Corrected Area column (O) multiplies the target area value by the Normalization Ratio.



TPN using RePlex and NIR Detection Worksheet

Scan the QR Code to download or [Click Here](#)

Sample	Attribute	Primary	Attribute	Secondary	Scdry Attr	Capillary	CHEMI Tot	NIR Total	TP Area	TPN (%)	STATSb	Normalization Ratio	Corr. Area
1	CHO-S Lys 2.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:2	1401088		357291	100	682798	1.0	682798
2	CHO-S Lys 2 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:3	1136130		426471	119	682268	1.1	752419
3	CHO-S Lys 1.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:4	1082824		403620	113	560485	1.4	790073
4	CHO-S Lys 1 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:5	711601		482863	135	411746	1.9	794667
5	CHO-S Lys 0.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:6	364560		605242	169	211628	3.1	654373
6	CHO-S Lys 2.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:7	1557405		453286	127	721852	1.1	793535
7	CHO-S Lys 2 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:8	1234903		390677	109	687496	1.3	900533
8	CHO-S Lys 1.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:9	1122603		430503	120	517718	1.6	806732
9	CHO-S Lys 1 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:10	737429		374538	105	428974	1.9	796431
10	CHO-S Lys 0.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:11	396612		500328	140	225780	3.5	788677
11	CHO-S Lys 2.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:12	1412342		438281	123	615674	1.1	685234
12	CHO-S Lys 2 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:13	1052563		390644	109	592035	1.3	769747
13	CHO-S Lys 1.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:14	969991		346909	97	522294	1.5	774032
14	CHO-S Lys 1 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:15	666028		410635	115	368076	1.9	688474
15	CHO-S Lys 0.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:16	352593		568676	159	233104	3.0	707579
16	CHO-S Lys 2.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:17	1485740		460498	129	611684	1.1	670223
17	CHO-S Lys 2 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:18	1168419		433083	121	615006	1.2	746635
18	CHO-S Lys 1.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:19	1096612		468308	131	518890	1.4	745466
19	CHO-S Lys 1 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:20	861268		405754	114	370392	1.9	690179
20	CHO-S Lys 0.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:21	510694		368869	103	227938	2.8	646868
21	CHO-S Lys 2.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:22	1749094		384773	108	770357	1.2	806300
22	CHO-S Lys 2 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:23	1496075		388264	109	583840	1.3	771691
23	CHO-S Lys 1.5 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:24	898839		396386	111	472532	1.7	809684
24	CHO-S Lys 1 mg/mL	Rb anti-ST	1:20	anti-Rabbit	Seconde	P1:25	675320		343035	96	369592	2.2	828997
25	CHO-S Lys 2.5 mg/mL	Primary Antibody				P2:2	357291	7333175	357291	100			
26	CHO-S Lys 2 mg/mL	Primary Antibody				P2:3	426471	6649473	426471	119			
27	CHO-S Lys 1.5 mg/mL	Primary Antibody				P2:4	403620	5202219	403620	113			
28	CHO-S Lys 1 mg/mL	Primary Antibody				P2:5	482863	3799587	482863	135			
29	CHO-S Lys 0.5 mg/mL	Primary Antibody				P2:6	605242	2371588	605242	169			
30	CHO-S Lys 2.5 mg/mL	Primary Antibody				P2:7	453286	6670738	453286	127			
31	CHO-S Lys 2 mg/mL	Primary Antibody				P2:8	390677	5598387	390677	109			
32	CHO-S Lys 1.5 mg/mL	Primary Antibody				P2:9	430503	4760649	430503	120			
33	CHO-S Lys 1 mg/mL	Primary Antibody				P2:10	374538	3949794	374538	105			
34	CHO-S Lys 0.5 mg/mL	Primary Antibody				P2:11	500328	2099319	500328	140			
35	CHO-S Lys 2.5 mg/mL	Primary Antibody				P2:12	438281	6588768	438281	123			
36	CHO-S Lys 2 mg/mL	Primary Antibody				P2:13	390644	5640162	390644	109			
37	CHO-S Lys 1.5 mg/mL	Primary Antibody				P2:14	346909	4948212	346909	97			
38	CHO-S Lys 1 mg/mL	Primary Antibody				P2:15	410635	3920500	410635	115			
39	CHO-S Lys 0.5 mg/mL	Primary Antibody				P2:16	568676	2415837	568676	159			
40	CHO-S Lys 2.5 mg/mL	Primary Antibody				P2:17	460498	6692680	460498	129			
41	CHO-S Lys 2 mg/mL	Primary Antibody				P2:18	433083	6040364	433083	121			
42	CHO-S Lys 1.5 mg/mL	Primary Antibody				P2:19	468308	5103104	468308	131			
43	CHO-S Lys 1 mg/mL	Primary Antibody				P2:20	405754	3935430	405754	114			
44	CHO-S Lys 0.5 mg/mL	Primary Antibody				P2:21	368869	2584008	368869	103			
45	CHO-S Lys 2.5 mg/mL	Primary Antibody				P2:22	384773	6342887	384773	108			
46	CHO-S Lys 2 mg/mL	Primary Antibody				P2:23	388264	5548074	388264	109			
47	CHO-S Lys 1.5 mg/mL	Primary Antibody				P2:24	396386	4279649	396386	111			
48	CHO-S Lys 1 mg/mL	Primary Antibody				P2:25	343035	3269350	343035	96			

FIGURE 9. Paste the data in cell A1 of the TPN using RePlex and NIR Detection Worksheet.

TPN using RePlex and NIR detection data should be normalized using Capillary 2 as the Reference Capillary to establish a Normalization Ratio using the NIR Total Area (orange). The Normalization Ratio

formula is in FIGURE 10. Capillary 2 will always have a Normalization Ratio of 1. Simply multiply the target signal for each capillary by that capillary's normalization ratio.

Normalization Ratio Determination

Capillary	Norm. Ratio
P2:2	Cap2 NIR Total Area/Cap2 NIR Total Area
P2:3	Cap2 NIR Total Area/Cap3 NIR Total Area
P2:4	Cap2 NIR Total Area/Cap4 NIR Total Area
P2:5	Cap2 NIR Total Area/Cap5 NIR Total Area
P2:6	Cap2 NIR Total Area/Cap6 NIR Total Area
P2:7	Cap2 NIR Total Area/Cap7 NIR Total Area
P2:8	Cap2 NIR Total Area/Cap8 NIR Total Area
P2:9	Cap2 NIR Total Area/Cap9 NIR Total Area
P2:10	Cap2 NIR Total Area/Cap10 NIR Total Area
P2:11	Cap2 NIR Total Area/Cap11 NIR Total Area
P2:12	Cap2 NIR Total Area/Cap12 NIR Total Area
P2:13	Cap2 NIR Total Area/Cap13 NIR Total Area
P2:14	Cap2 NIR Total Area/Cap14 NIR Total Area
P2:15	Cap2 NIR Total Area/Cap15 NIR Total Area
P2:16	Cap2 NIR Total Area/Cap16 NIR Total Area
P2:17	Cap2 NIR Total Area/Cap17 NIR Total Area
P2:18	Cap2 NIR Total Area/Cap18 NIR Total Area
P2:19	Cap2 NIR Total Area/Cap19 NIR Total Area
P2:20	Cap2 NIR Total Area/Cap20 NIR Total Area
P2:21	Cap2 NIR Total Area/Cap21 NIR Total Area
P2:22	Cap2 NIR Total Area/Cap22 NIR Total Area
P2:23	Cap2 NIR Total Area/Cap23 NIR Total Area
P2:24	Cap2 NIR Total Area/Cap24 NIR Total Area
P2:25	Cap2 NIR Total Area/Cap25 NIR Total Area

FIGURE 10. The TPN using RePlex and NIR Detection Worksheet will automatically calculate the Normalization Ratio (orange column).



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PL9-0075 Rev A

10/24

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