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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.

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. 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.

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).

big-techne[°] // Global Developer, Manufacturer, and Supplier of High-Quality Reagents, Analytical Instruments, and Precision Diagnostics. INCLUDES R&D Systems[®] Novus Biologicals[®] Tocris Bioscience[®] ProteinSimple[®] ACD[®] ExosomeDx[®] Asuragen⁹ Lunaphore[®] 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 μ L 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 μ L 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.



Biotinylated Ladder, 5 μL Prepared Samples, 3 μL
Antibody Diluent (2 or Milk-Free), 10 μL Total Protein Labeling Reagent, 10 μL
Antibody Diluent (2 or Milk-Free), 10 μL
Antibody Diluent (2 or Milk-Free), 10 μL
Antibody Diluent (2 or Milk-Free), 10 μL
Primary Antibody for Probe 1, 10 μL
Streptavidin-HRP or NIR, 10 μL
Streptavidin-NIR, 8 μL
Wash Buffer
500 μL/compartment
2.5 mL/row
Luminol-Peroxide Mix, 170 μL/compartment
RePlex[™] Reagent Mix, 300 μL/compartment

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.

📰 Protocol 🔚 History 耳 Note	s		
	Probe 1	Probe 2	
> Separation Matrix			
> Stacking Matrix			
> Sample			
Separation Time (min)	25.0		
> Separation Voltage (volts)	375		
> RePlex Purge Time (min)		30.0	
> Biotin Labeling Time (min)	30.0		
> Antibody Diluent Time (mi	5.0		
> Primary Antibody Time (m	30.0		
> Secondary Antibody Time	30.0		
> Total Protein HRP Time (m		30.0	
✓ Detection			
Well Row	J1	J1	
Detection Profile (Chen	RDR	1 Exposure	
Detection Profile (NIR)	None	None 🛄	
Detection Profile (IR)	None	None	



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.

Standards	Peak Fit	
Images Normalization ✓ Peak Names Standard Curves Loading Controls Peak Fit Lane Contrast Signal to Noise Advanced	Analysis Groups fit fit 2 2→ Add Remove Apply Default: fit fit Apply Override: Apply To Group Probe 1 fit Probe 2 fit 2 3→ Add Remove	Analysis Groups: fit 2 Range Minimum 0.1 Maximum 375.0 View Analysis Trul Baseline Baseline Baseline Type Threshold 0.1 Window 100.0 Stiffness 0.5 Peak Find Threshold 2.0 Width 1.0 Area Calculation Dropped Lines
Import Expo	ort	OK Cancel Apply

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:

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



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

Data Export

- 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.

File Edit View Instrument Wi	Nama
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"Human Skeletal Muscle Whole Tissue Lysate (NB820-5925)" "1.5 mg/mL" "Rb anti-PI3K mAb; CST3011" "1:200" "anti-Rabbit Secondary HRP Conjugate RTU" " "
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"Human Skeletal Muscle Whole Tissue Lysate (NB820-5925)" "1.5 mg/mL" "Rb anti-PI3K mAb; CST3011" "1:200" "anti-Rabbit Secondary HRP Conjugate RTU" "
"Human Skeletal Muscle Whole Tissue Lysate (NB820-5925)" "1 mg/mL" "Rb anti-PI3K mAb; CST3011" "1:200" "anti-Rabbit Secondary HRP Conjugate RTU" "
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"Human Skeletal Muscle Whole Tissue Lysate (NB820-5925)" "1 mg/mL" "Primary Antibody" "" " "" P2:11 279981.967 1734978.564 2
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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**

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4	сно-	SLys 1.5 n	ng/mL		Rb anti-S1	1:20	anti-Rabb	it Seconda	P1:4	1082824		403620	113	560485			1.4	790073
5	сно-	S Lys 1 mg	/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:5	711601		482863	135	411746			1.9	794667
6	CHO-	S Lys 0.5 n	ng/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:6	364560		605242	169	211628			3.1	654373
7	сно-	SLys 2.5 n	ng/mL		Rb anti-S	1:20	anti-Rabb	it Seconda	P1:7	1557405		453286	127	721852			1.1	793535
8	CHO-	S Lys 2 mg	/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:8	1234903		390677	109	687496			1.3	900533
10	сно-	Sive 1 me	/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:9	737429		374538	120	428974			1.0	796431
11	сно-	S Lvs 0.5 n	ne/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:11	396612		500328	140	225780			3.5	788677
12	сно-	S Lys 2.5 n	ng/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:12	1412342		438281	123	615674			1.1	685234
13	сн <mark>о</mark> -	S Lys 2 mg	/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:13	1052563		390644	109	592035			1.3	769747
14	CHO-	S Lys 1.5 n	ng/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:14	969931		346909	97	522294			1.5	774032
15	сно-	S Lys 1 mg	/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:15	666028		410635	115	368076			1.9	688474
16	сно-	SLys 0.5 n	ng/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:16	352593	-	568676	159	233104			3.0	707579
17	сно-	SLys 2.5 n	ng/mL /ml		Rb anti-S	1:20	anti-Kabb	it Seconda	P1:17	1485/40		460498	129	611684			1.1	6/0223
19	сно-	Sivs 15 n	ne/ml		Rb anti-Si	1.20	anti-Rabb	it Seconda	P1-10	1096612		468308	131	518890			1.2	745646
20	сно-	SLvs 1 me	/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:20	861268		405754	114	370392			1.9	690179
21	сно-	S Lys 0.5 n	ng/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:21	510694		368869	103	227938			2.8	646868
22	сн <mark>о</mark> -	S Lys 2.5 n	ng/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:22	1749094		384773	108	770357			1.2	890630
23	CHO-	S Lys 2 mg	/mL		Rb anti-Si	1:20	anti-Rabb	it Seconda	P1:23	1496075		388264	109	583840			1.3	771691
24	сно-	SLys 1.5 n	ng/mL		Rb anti-ST	1:20	anti-Rabb	it Seconda	P1:24	898839		396386	111	472532			1.7	809684
25	CHO-	SLys 1 mg	/mL		Rb anti-S	1:20	anti-Rabb	it Seconda	P1:25	675320	7777475	343035	96	369592			2.2	828997
20	сно-	Sive 2 me	/mL		Primary A	ntibody			P2:2 P2:3	426471	6649473	426471	100					-
28	сно-	SLvs 1.5 n	ne/mL		Primary A	ntibody			P2:4	403620	5202219	403620	113					-
29	сно-	S Lys 1 mg	/mL		Primary A	ntibody			P2:5	482863	3799587	482863	135					
30	сн <mark>о</mark> -	S Lys 0.5 n	ng/mL		Primary A	ntibody			P2:6	605242	2371588	605242	169					
31	сн <mark>о</mark> -	S Lys 2.5 n	ng/mL		Primary A	ntibody			P2:7	453286	6670738	453286	127					
32	сно-	S Lys 2 mg	/mL		Primary A	ntibody			P2:8	390677	5598387	390677	109					
33	сно-	SLys 1.5 n	ng/mL		Primary A	ntibody			P2:9	430503	4706049	430503	120					-
35	сн0- сно	SLVS 1 mg	/mL ng/ml		Primary A	ntibody			P2:10	5/4538	2099219	5/4538	105					
36	сно-	S Lys 0.5 n	ng/mL		Primary A	ntibody			P2:12	438281	6588768	438281	140					
37	сно-	S Lys 2 mg	/mL		Primary A	ntibody			P2:13	390644	5640162	390644	109					-
38	сно-	SLys 1.5 n	ng/mL		Primary A	ntibody			P2:14	346909	4948212	346909	97					
39	сн <mark>о</mark> -	S Lys 1 mg	/mL		Primary A	ntibody			P2:15	410635	3920500	410635	115					
40	сно-	S Lys 0.5 n	ng/mL		Primary A	ntibody			P2:16	568676	2415837	568676	159					
41	CHO-	SLys 2.5 n	ng/mL		Primary A	ntibody			P2:17	460498	6692680	460498	129					
42	CHO-	S Lys 2 mg	/mL		Primary A	ntibody			P2:18	433083	5102104	433083	121					
44	CHO-	SLvs 1 me	/mL		Primary A	ntibody			P2:20	405754	3935430	405754	151					
45	сно-	S Lys 0.5 n	ng/mL		Primary A	ntibody			P2:21	368869	2584008	368869	103					
46	сно-	S Lys 2.5 n	ng/mL		Primary A	ntibody			P2:22	384773	6342887	384773	108					
47	сно-	S Lys 2 mg	/mL		Primary A	ntibody			P2:23	388264	5548074	388264	109					
48	сн <mark>о</mark> -	S Lys 1.5 n	ng/mL		Primary A	ntibody			P2:24	396386	4279649	396386	111					
49	сно-	S Lys 1 mg	/mL		Primary A	ntibody			P2:25	343035	3269350	343035	96					
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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.

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1	Sample		Attrib	ute		Primary	Attribute	Secondar	Scdry Attr	Capilla	ry CHEMI Tot	NIR Total	TP Area	TPN (%)	STATS
2	CHO-SL	ys ve	2.5 mg	g/mL ml		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:2	1401088		357291	100	68279
4	CHO-SL	ys vs	1.5 mi	r/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:4	1082824		403620	113	56048
5	CHO-S L	ys	1 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	it Seconda	P1:5	711601		482863	135	41174
6	CHO-S L	ys	0.5 m	g/mL		Rb anti-S	T 1:20	anti-Rabb	it Seconda	P1:6	364560		605242	169	21162
7	CHO-S L	ys	2.5 mį	g/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:7	1557405		453286	127	72185
8	CHO-S L	ys	2 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:8	1234903		390677	109	68749
9	CHO-S L	ys	1.5 mg	g/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:9	1122603		430503	120	51771
10	CHO-S L	ys	1 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	it Seconda	P1:10	737429		374538	105	428974
12	CHO-ST	ys 	0.5 mg	g/mL g/ml		RD anti-s	1 1:20 T 1:20	anti-Kabb	it Seconda	P1:11 P1:12	1412242		420201	140	225/8
13	CHO-ST	γ.5 V 5	2.5 mg/r	mL		Rb anti-S	T 1.20	anti-Rabb	nt Seconda	P1-13	1052563		390644	109	59203
14	CHO-S L	γs	1.5 mg	g/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:14	969931		346909	97	52229
15	CHO-S L	γs	1 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:15	666028		410635	115	36807
16	CHO-S L	γs	0.5 mg	g/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:16	352593		568676	159	23310
17	CHO-S L	γs	2.5 mį	g/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:17	1485740		460498	129	61168
18	CHO-S L	γs	2 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:18	1168419		433083	121	61500
19	CHO-S L	γs	1.5 m	g/mL		Rb anti-S	1:20	anti-Rabb	oit Seconda	P1:19	1096612		468308	131	518890
20	CHO-SL	ys ve	0.5 m	mL z/ml		Rb anti-S	T 1:20	anti-Rabb	ait Seconda	P1:20	510694		405/54	103	22793
22	CHO-ST	ys vs	2.5 m	e/mL		Rb anti-S	T 1.20	anti-Rabi	nit Seconda	P1.21	1749094		384773	103	77035
23	CHO-S L	γs	2 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:23	1496075		388264	109	583840
24	CHO-S L	ys	1.5 mg	g/mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:24	898839		396386	111	47253
25	CHO-S L	ys	1 mg/r	mL		Rb anti-S	T 1:20	anti-Rabb	oit Seconda	P1:25	675320		343035	96	369593
26	CHO-S L	ys	2.5 mg	g/mL		Primary	Antibody			P2:2	357291	7333175	357291	100	
27	CHO-S L	ys	2 mg/r	mL		Primary	Antibody			P2:3	426471	6649473	426471	119	
28	CHO-S L	ys	1.5 mg	g/mL		Primary	Antibody			P2:4	403620	5202219	403620	113	
20	CHO-SL	γs	1 mg/r			Primary	Antibody			P2:5	482863	3799587	482863	155	
31	CHO-ST	ys ve	2.5 m	g/mL g/ml		Primary	Antibody			P2:0	453286	6670738	453286	105	
32	CHO-SL	vs	2 mg/r	mL		Primary	Antibody			P2:8	390677	5598387	390677	109	
33	CHO-S L	ys	1.5 m	g/mL		Primary	Antibody			P2:9	430503	4706049	430503	120	
34	CHO-S L	ys	1 mg/r	mL		Primary	Antibody			P2:10	374538	3949794	374538	105	
35	CHO-S L	ys	0.5 mg	g/mL		Primary	Antibody			P2:11	500328	2099319	500328	140	
36	CHO-S L	ys	2.5 mg	g/mL		Primary	Antibody			P2:12	438281	6588768	438281	123	
37	CHO-S L	ys	2 mg/r	mL		Primary	Antibody			P2:13	390644	5640162	390644	109	
38	CHO-SL	ys	1.5 mg	g/mL		Primary	Antibody			P2:14	346909	4948212	346909	97	
40	CHO-SL	ys ve	0.5 m	z/ml		Primary	Antibody			P2-15	568676	2415837	568676	115	
41	CHO-SI	yə VS	2.5 m	e/mL		Primary	Antibody			P2:17	460498	6692680	460498	129	
42	CHO-S L	ýs	2 mg/r	mL		Primary	Antibody			P2:18	433083	6040364	433083	121	
43	CHO-S L	ys	1.5 mj	g/mL		Primary	Antibody			P2:19	468308	5103104	468308	131	
44	CHO-S L	ys	1 mg/r	mL		Primary	Antibody			P2:20	405754	3935430	405754	114	
45	CHO-S L	ys	0.5 mj	g/mL		Primary	Antibody			P2:21	368869	2584008	368869	103	
46	CHO-SL	γs	2.5 m	g/mL		Primary	Antibody			P2:22	384773	6342887	384773	108	
47	CHO-SL	γs	2 mg/r	mL		Primary	Antibody			P2:23	388264	5548074	388264	109	
48	CHO-SL	ys 	1.5 mg	g/mL ml		Primary	Antibody			P2:24	396386	4279649	396386	111	
50	CHO-SL	γS	1 mg/1	n L		rimary	HIGDOOY			r 2:25	545035	5265550	545035	36	
		-	_			-	-	-	-		_			-	
				She	et 1	(+)									

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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