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a bio-techne® brand

Simple Western
蛋白归一化

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2020-04-17

为什么归一化对于一个可靠的免疫定量实验很重要？

- **控制实验变异系数**
  - 最小化变异系数(实验设计)
    - 生物学重复
    - 人员/移液误差
    - 技术限制 (如 western blot 的转膜)
  - 校正误差(归一化)
    - 通过校正不同泳道间的误差，降低重复样本的变异系数

- **归一化** – 主要目标是降低泳道间差异，便于进行定量比较
  - "对所有目标蛋白丰度进行总蛋白归一化是减少计数误差，进行蛋白表达量相对比较的一种更可靠手段。"

- **无归一化**: 生物学差异是否由实验误差引起?

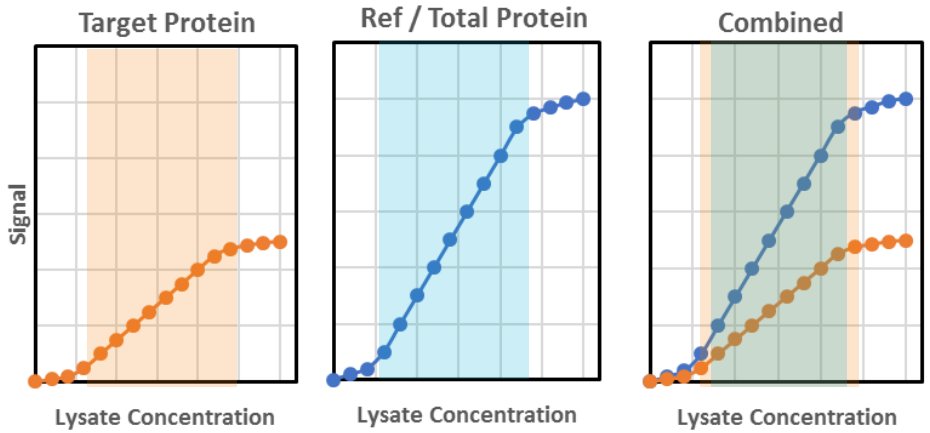
归一化方法各有优劣

- **归一化方法**
  - **内参蛋白**
    - 单个内参蛋白的信号 (GAPDH, Actin, Tubulin, etc.)
  - **总蛋白染色**
    - 所有蛋白的总信号强度
- **哪种方法好？**
  - 每种方法各有优劣
  - 不同验证过程
  - 选择哪种依赖于实验设计以及实验误差来源

	归一化选项	
	内参蛋白 管家蛋白	**总蛋白** 不可逆共价标记
优势	• 广泛应用和发表	• 越来越多研究推荐 • 不依赖于抗体，不受抗体和靶蛋白结合的动态范围影响 • 减少生物学误差的影响 • 直接在样品/毛细管中读出总蛋白信号
劣势	• 单一的参考点 • 对生物学误差更敏感 • 相比于目标蛋白表达量高 • 窄线性范围 • 受实验条件/实验处理的影响	• 标记有氨基酸依赖 • 氨基酸成分不一致导致样品标记不一致 • 可能影响下游免疫检测（抗体识别）
注意事项	• 验证在实验组和对照组中内参蛋白稳定表达 • 验证检测线性范围，避免信号饱和	• 验证检测线性范围，避免信号饱和

两种归一化方法对线性区间有要求

对于目的蛋白信号，无论是内参蛋白归一化还是总蛋白归一化，都需要选择裂解液浓度落在线性范围中间。



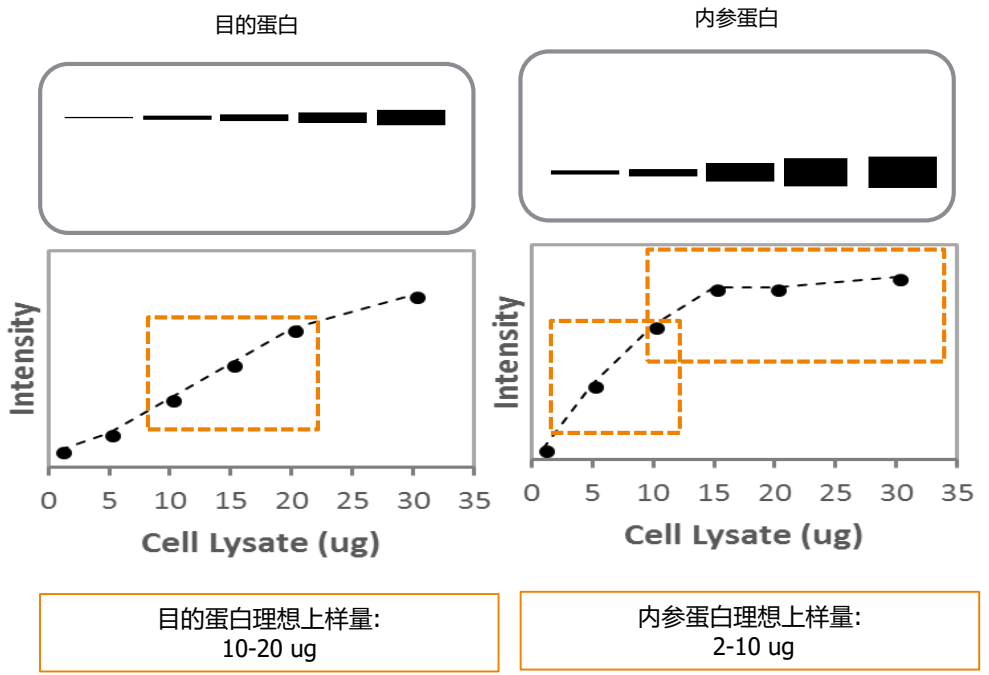
Simple Western 总蛋白分析和目的蛋白归一化流程：

内参蛋白归一化

内参蛋白经常超出线性范围

– 内参蛋白一般高表达，因此经常因为低估蛋白丰度而超出线性范围

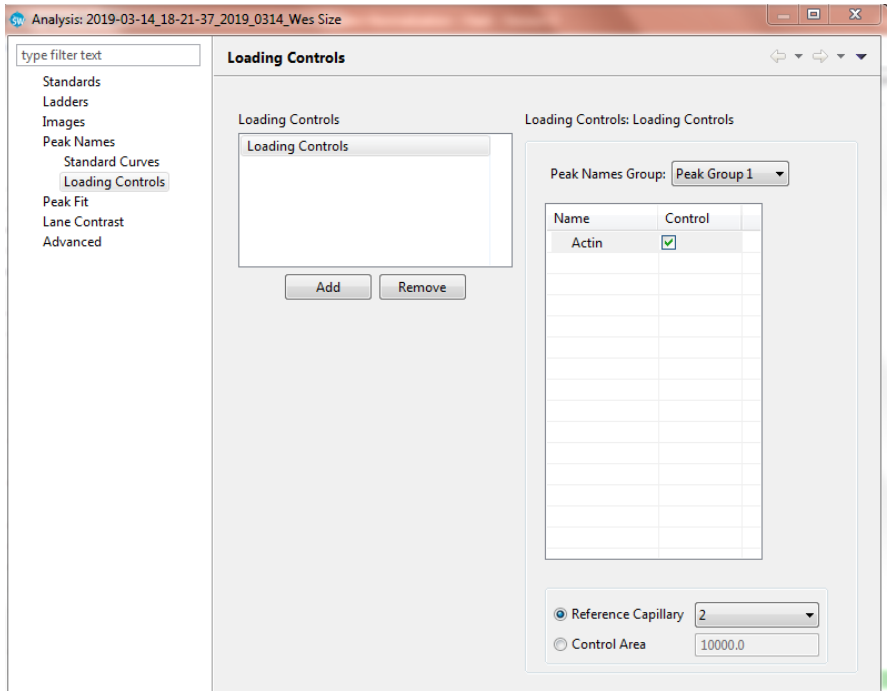
– 确保内参蛋白稳定表达且在线性范围内



管家蛋白(HKP) 信号可能跟目的蛋白线性范围错开

Compass for SW 软件简化内参蛋白归一化流程

- 命名目标蛋白和内参蛋白
- 选择内参蛋白 (Edit > Analysis > Loading Controls)



- 首先计算每个泳道的归一化因子，然后对每个信号的峰面积进行校正，确定校正面积

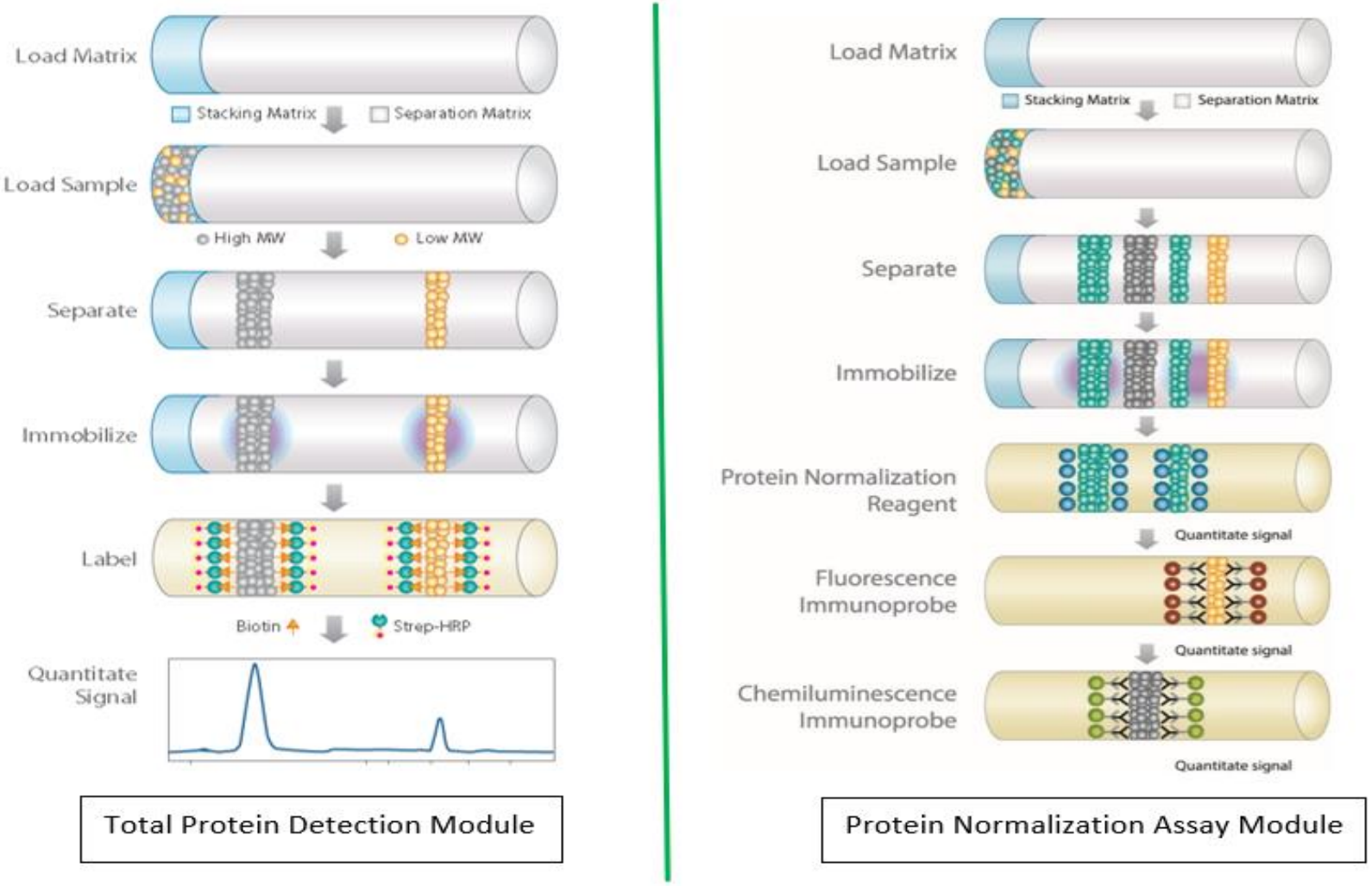
Simple Western
总蛋白归一化

Proteinsimple蛋白均一化的解决方案

Total Protein Detection Assay

Protein Normalization Assay Module

原理不同

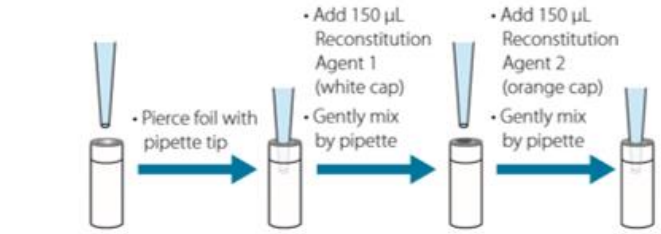


前者是利用Biotin标记的染料，借助SA-HRP进行化学发光检测，后者是利用TAMRA的荧光染料，借助荧光通道进行检测

试剂及适用的仪器类型不同

	Total Protein Detection Module	Protein Normalization Assay Module
分离试剂盒	相同 (毛细管和样品板都一样)	
检测试剂盒	DM-TP01	AM-PN01(包含毛细管，板子，Standard pack及均一化试剂) 之前的DM-TP02中的Protein Normalization Reagent (8 tubes) 货号已从043-825更新为043-824
仪器型号	Jess/Wes/Peggy sue/Sally sue	Jess

试剂制备方法不同



Biotin label reagent tube

Total Protein Detection

1、推荐上样浓度为 0.2 mg/ml，具体情况需要客户自行做样品稀释梯度，从而摸索检测的线性范围

2、检测分子量范围没有要求

3、需要设置阴性对照排除 endogenous biotinylated proteins 的影响 (No-label control)

- Prepare the Protein Normalization Reagent stock solution by adding 100 µL Protein Normalization Reconstitution Agent per tube. Thoroughly mix the reagent by pipetting 15 times.
- Use the following table to prepare the working solution of the reconstituted Protein Normalization Reagent stock solution. Thoroughly mix the working solution by pipetting 15 times.

PROTEIN MOLECULAR WEIGHT RANGE		
LYSATE CONCENTRATION	12–230 kDa	
	Stock Solution	Reconstitution Agent
0.2–1.2 mg/mL	50 µL	250 µL

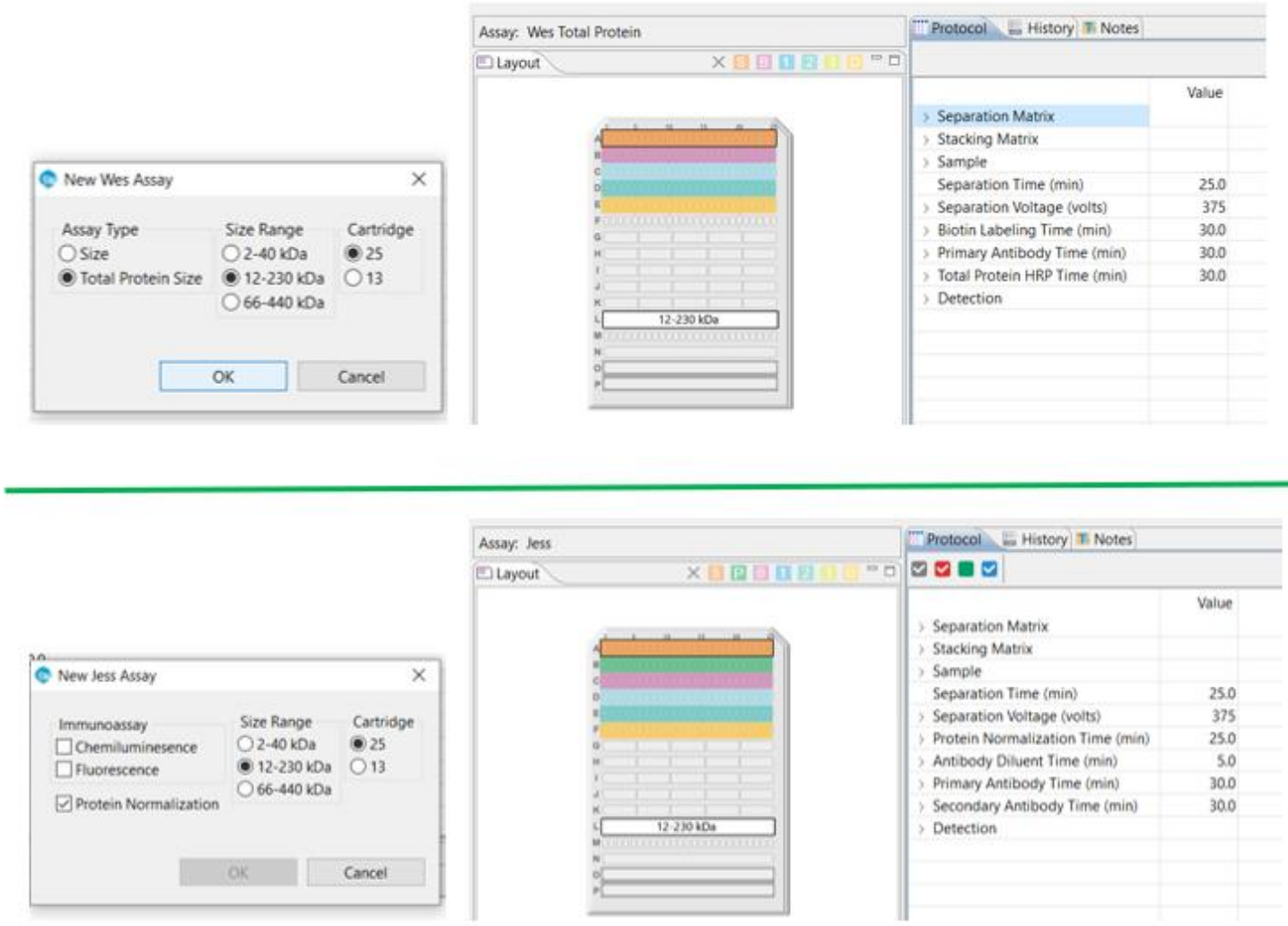
Protein Normalization

1、蛋白归一化试剂应现用现配

2、样品检测浓度在 0.2-1.2mg/ml

3、检测分子量范围为 12-230kD

运行程序不同



Protein Normalization不可以单独运行，必需跟化学发光或者荧光检测同时进行，而Total Protein Detection可以单独运行。也就是说如果您想同时做蛋白均一化和靶蛋白检测时，前者可以在同一根毛细管内实现，而后者可以在同一个Run的不同毛细管内进行

分析方法及结果展示不同

Total Protein Detection数据分析流程为：

1、先将capillary中的数值复制到excel中

2、选择一个样品(或者一根毛细管)的total protein area作为参比，其他样品均除以该值，得到每个样品的均一化系数

3、最后每个样品某个靶蛋白的峰面积乘以对应的均一化系数即可获得均一化之后的该蛋白相对表达量

4、设置无生物素标记对照，观察内源性生物素蛋白

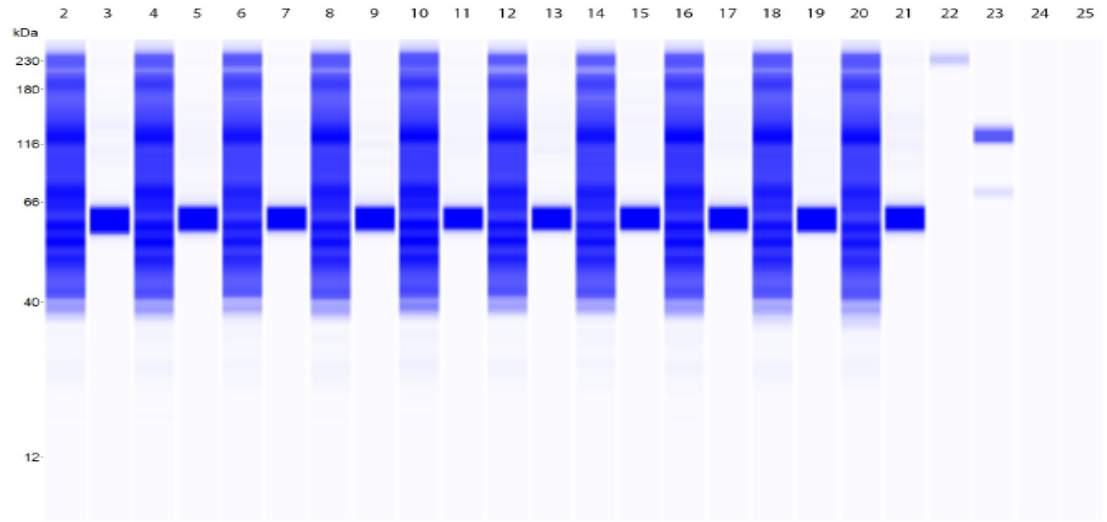


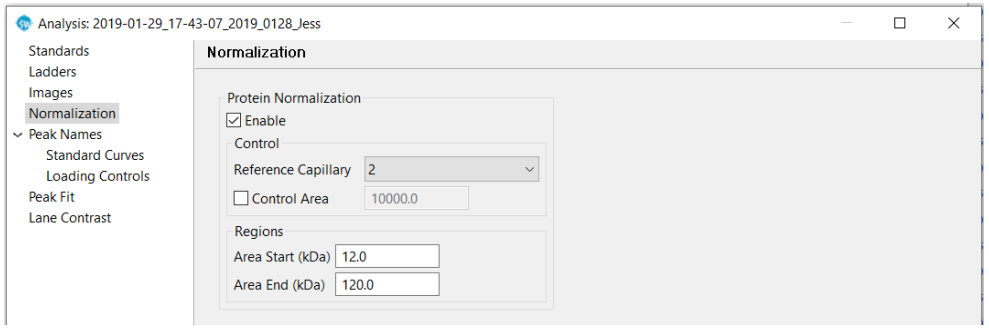
FIGURE 4. Example data set of a combined Total Protein and Immunoassay Wes run. HeLa lysate (0.2 mg/mL) was either examined with the Total Protein Assay (even-numbered lanes) or with an anti-AKT1 antibody (odd-numbered lanes). Controls (right) for the Total Protein Assay (lanes 22 and 23) show total protein labeling of the internal 230 kDa standard and detection of endogenous biotinylated proteins, respectively. Controls for primary antibody or secondary antibody-derived background are shown in lanes 24 and 25.

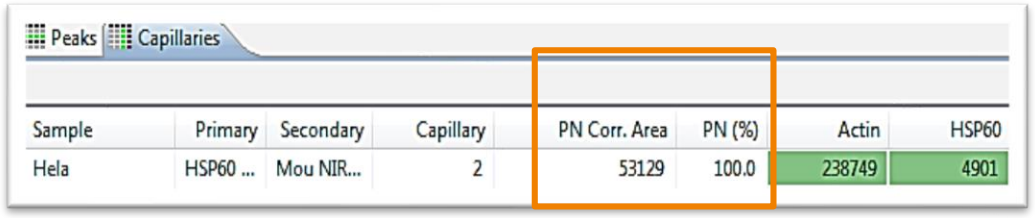
Protein Normalization数据分析流程为：

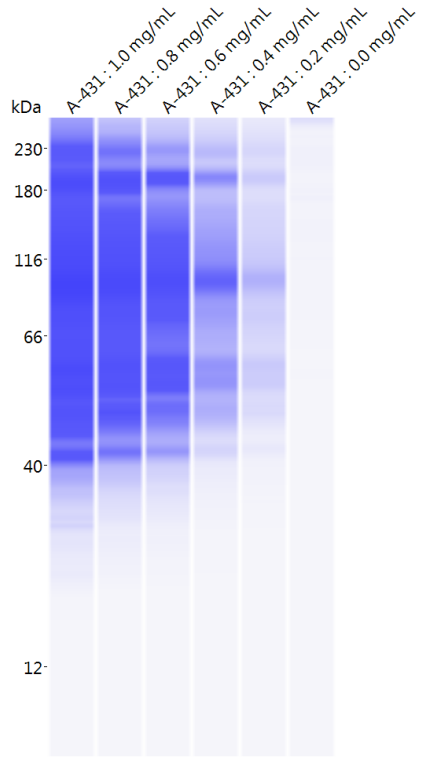
1、Compass工具栏可以自动选择PN模式，如下图蓝色圆圈

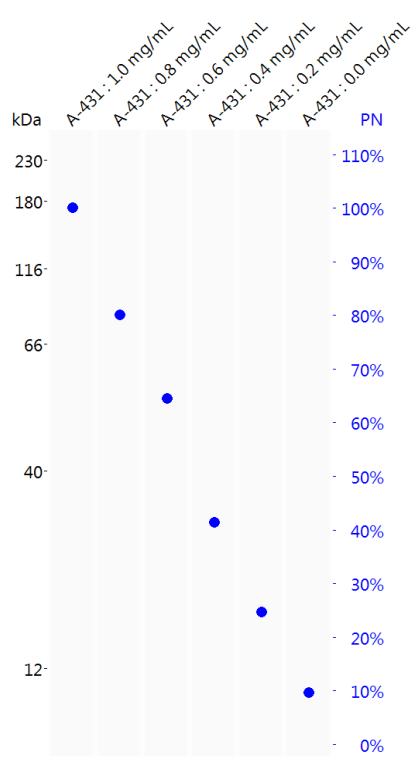
2、可在Edit>Analysis>Normalization选择那个毛细管作为参比，并选择计算面积的分子量区间

3、在Lane界面可以直观的看到均一化之后的结果，如下图有PN Corr. Area及PN%









补充页

总蛋白归一化对于免疫定量实验是可靠内参方案

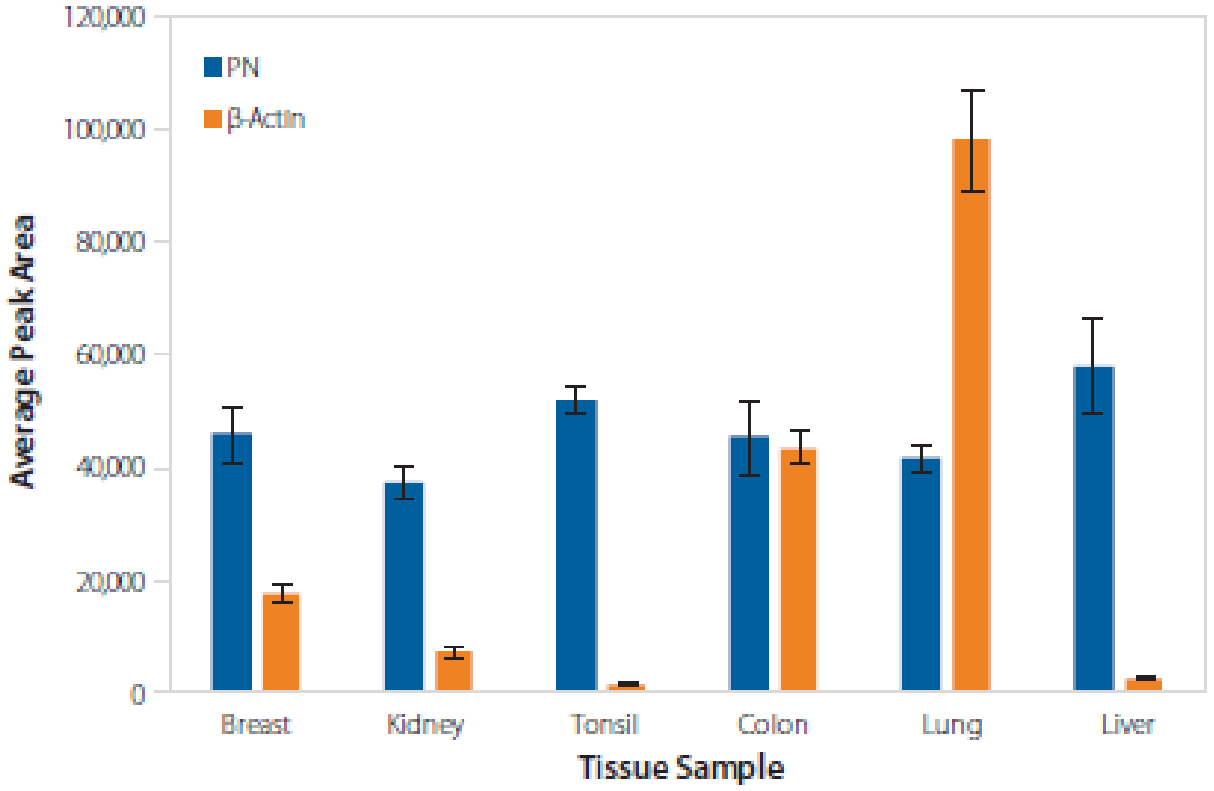
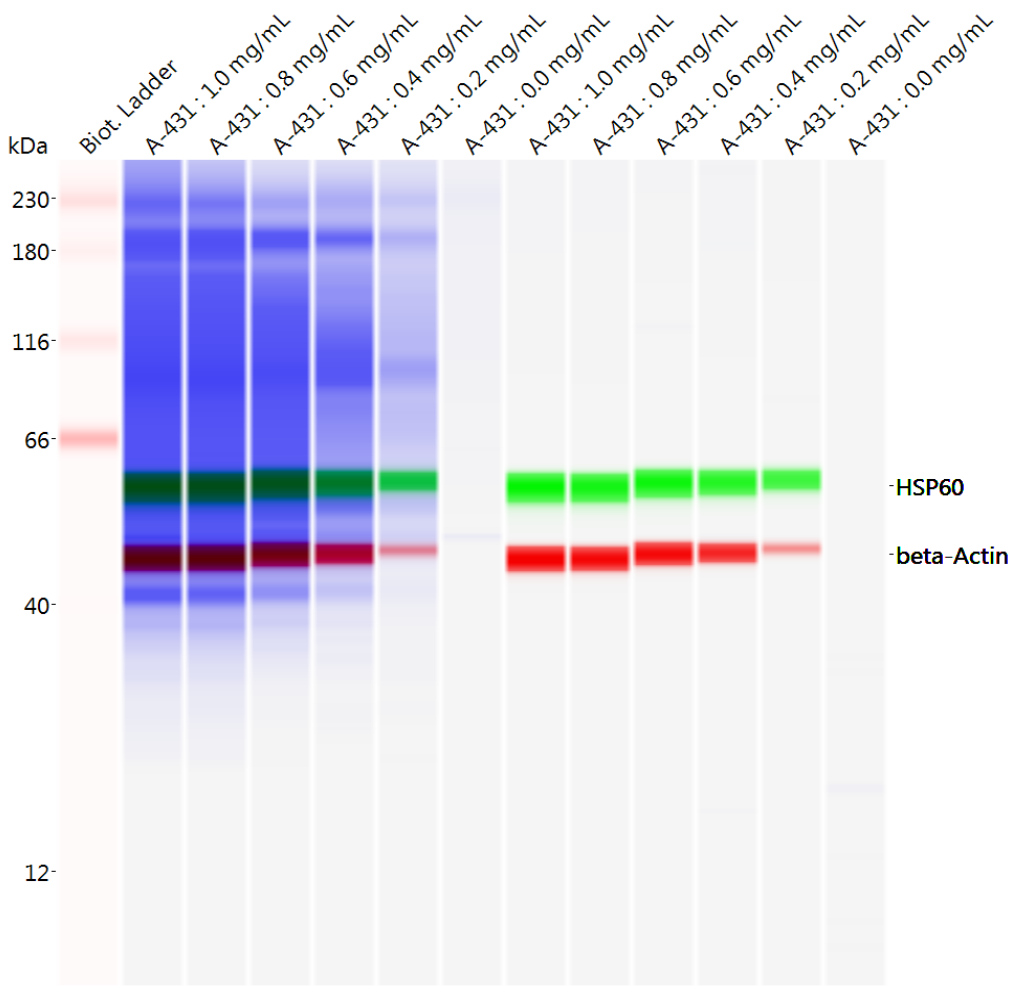
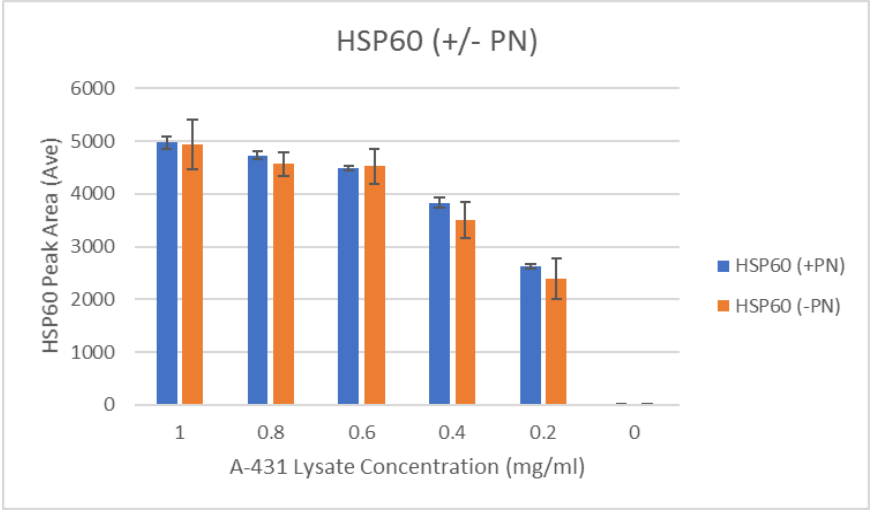
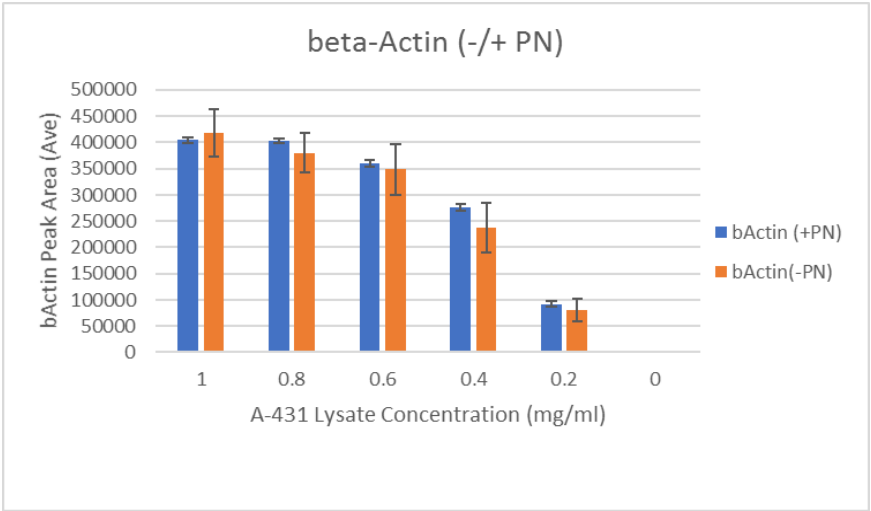


FIGURE 6. Comparative total protein data (blue bars) and the expression of β-actin (orange bars) in six human whole tissue lysates (0.3 mg/mL) tested using Jess.

Simple Western 的蛋白归一化试剂对免疫检测无明显影响

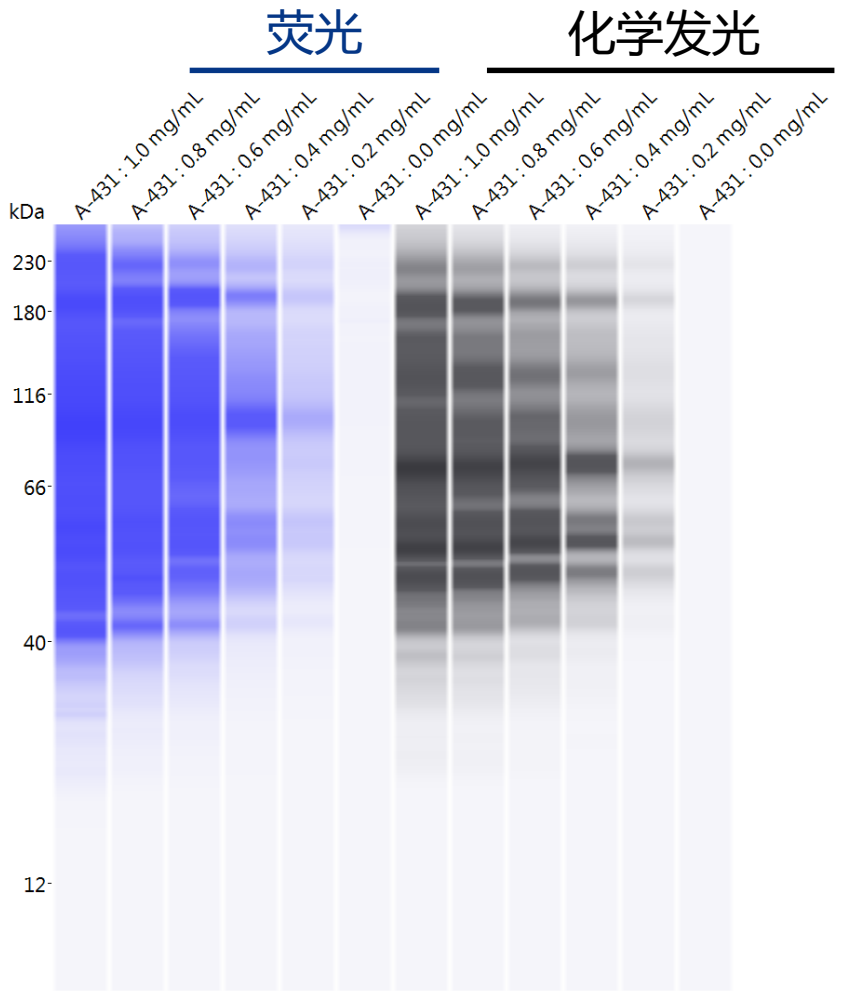


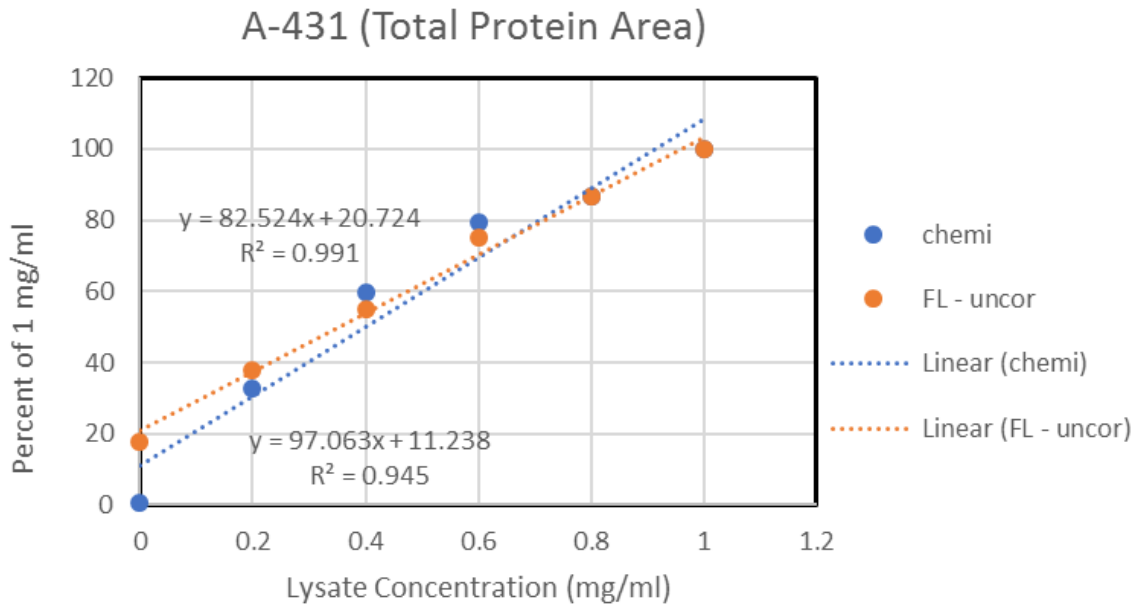




N=2

蛋白归一化信号和总蛋白检测趋势相近





附加资源

application note

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**).

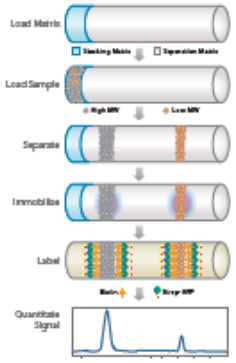


FIGURE 1. How the Total Protein Assay works.

how-to guide

Using the Simple Western Total Protein Assay to Normalize Immunoassay Data in the Same Run

Introduction

Housekeeping proteins such as GAPDH, tubulin, and others are typically used to normalize the target protein signal on traditional Western blots. But, many of these proteins aren't expressed as consistently as previously thought, making single target normalization less than ideal. Finding an unbiased way to normalize all the protein immobilized on a membrane or capillary is critical.

Scientific journals, like the Journal of Biological Chemistry, have recently updated their Western blot submission guidelines[1] and are starting to recommend the use of total protein normalization for better data quality that reflects expression changes more accurately. To analyze total protein using traditional Western blots, they ask that authors stain the blot membrane with Coomassie or Ponceau S and generate a normalizing value per lane.

In this guide, we'll show you how to use the Total Protein Detection Module (DM-TP01) with any immunoassay detection module of your choosing to get total protein and immunoassay data in the same run. Going this route gives you a high throughput and more accurate way of getting that normalization factor you'll need when submitting articles to journals, without adding any experimental time per run.

How the Simple Western Total Protein Assay works

The total protein assay shown in **Figure 1** is an in-capillary labeling technique where proteins are separated by molecular weight, immobilized in the capillary, and then labeled with biotin before blocking. Next, the biotin-labeled proteins in the capillary are bound by HRP-conjugated streptavidin (SA-HRP) for detection in a chemiluminescent reaction.[2]

In Compass for Simple Western, you can select the Total Protein Assay from the assay menu. This assay has optimized incubation times for the Total Protein Detection Module reagents. Check out the product insert and the Total Protein Assay Plate Layout included with the Total Protein Detection Module (DM-TP01) for more information.

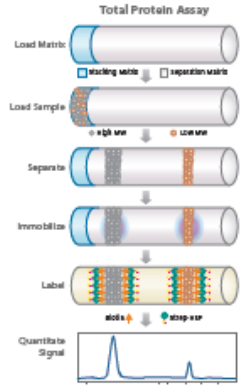


FIGURE 1. Proteins are separated and then UV-captured in the capillary the same way as the immunoassay. Next, captured proteins are exposed to the biotin labeling reagent, which allows for recognition by streptavidin-HRP.

Running the Total Protein assay and Immunoassay at the same time

To get total protein and immunoassay data on the same set of samples, the two assays would need to be run in separate capillaries. If they're done in separate runs on Wes™, that would be ~6 hours of total run time as each

technical note

Better Housekeeping: Protein Normalization on Jess

Introduction

Quantitative interpretation of Western blot data is often a challenge due to improper standardization. For researchers trying to perform comparative analyses of protein expression between biological samples that vary in complexity, disease state or applied external stimuli, an approach that reproducibly measures legitimate variance is especially important.

"Housekeeping" proteins (HKPs), also known as loading controls, are commonly used for protein normalization and to rule out technical variations in Western blot data. This approach compares the relative expression of a target protein to that of an unrelated, thought-to-be ubiquitously and constitutively expressed loading control—most often β-actin, glyceraldehyde-3-phosphate dehydrogenase, or β-tubulin, among others. However, more and more published work points to HKPs as an unreliable choice for normalization since their expression, too, can be influenced by various factors such as experimental treatment and growth conditions, stress, cell cycle phase, proliferation status, age or sex of biological source and pathological state[1-3]. Therefore, assuming a comparable expression of HKPs between your samples for normalization purposes may lead you to make inaccurate hypotheses and conclusions about the target(s) you are investigating.

Instead, normalizing target protein abundance to the overall amount of protein present in a sample is a more accurate means for eliminating technical errors and determining fold-change in protein expression. This method, for which total protein stains and stain-free products are commercially available, is antibody-independent and minimizes the impact of varying expression of a loading control. On Jess, it's a simple-to-perform added step in a Simple Western size assay. In this technical note, we'll walk you through the protocol and show you that protein normalization on Jess is indeed superior to the HKP approach.

How Does Protein Normalization on Jess Work?

Jess gives you an easy way to see if your samples contain a consistent protein load—just load the proprietary in-capillary protein normalization reagent into the assay plate and she'll take care of the rest. The fluorescent reagent binds to proteins immobilized in the same capillary as your immunoassay via primary and secondary amine interactions. The result? You can quickly see if your samples contain a consistent protein load, identify experimental setup and user errors and effectively normalize expression of your target protein to get accurate and consistent data, giving you the confidence you need in your results.