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SIMPLE WESTERN SIZE 方法优化

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4/17/2020

SIMPLE WESTERN SIZE 方法优化

如何选择初始实验条件

SIMPLE WESTERN SIZE 检测流程

1) 样本制备-提取蛋白
2) 测定蛋白浓度(BCA法)
3) 选择合适的分离试剂盒(2-40kDa, 12-230kDa 或 66-440kDa) 和分析类型(Wes免疫分析或总蛋白检测，Jess化学发光、荧光、总蛋白归一化)
4) 设计板布局
5) 准备样品、抗体和其他试剂
6) 加样
7) 放入毛细管卡盒、样品板，点击运行

样品制备影响实验结果

- 根据样品类型和目的蛋白属性选择、调整样品制备条件:
  如蛋白提取方法、变性方法的优化

- 不同的裂解液，参照裂解液兼容性表

Simple Western Size Assay Buffer Compatibility (Wes, Sally Sue, and Peggy Sue)

proteinsimple
a bio-techne brand

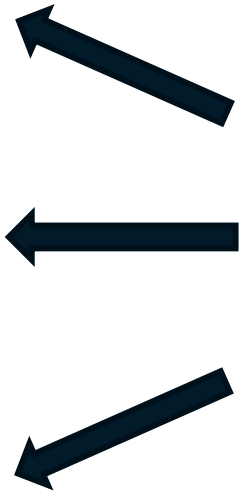
Lysis Buffer Compatibility Table

LYSIS BUFFER	USAGE	VENDOR & CATALOG	RANGE TESTED	CHEMI SIGNAL	CHEMI RESOLUTION	FLUORESCENT STANDARDS/ MW SIZING	RECOMMENDATIONS

- 参照文献里报道的目的蛋白在传统 WB 上的处理条件

最重要的三点

- **一抗**
  - 一个 **好的一抗** 做好免疫学实验的前提
- **样品浓度**
  - **信号** 与 **样品浓度** 应该有 **线性** 关系
- **抗体稀释度**
  - 定性实验无需过多关注
  - 定量实验需要优化抗体稀释度
  - 优化好的抗体浓度应该是:
    - **足够高到饱和**
    - **背景足够低**



所有利用抗体定量的检测技术都会涉及这三点

一抗的选择对于一个好的免疫测定试验至关重要

- Simple Western 验证过的抗体
  - ProteinSimple 抗体数据库
  - 文献（www.proteinsimple.com/citations.html）
- 传统 Western 用过的抗体
  - 与传统 Western 选择的抗体标准类似
    - 稀释度越高越好（说明抗体效价高）
    - 应用越广泛越好（说明抗原表位容易接近）
- 筛选一种以上抗体
  - 与传统Western和ELISA类似, **越多备选一抗越好**

一抗首选在SIMPLE WESTERN上验证过的抗体

http://www.proteinsimple.com/antibody/antibodies.html

Simple Western Antibody Database

The Simple Western Antibody Database is a user-interactive listing of antibodies that have been screened and tested in Simple Western Charge- and Size-based assays. This database is intended to provide assay general development guidance in identifying and selecting antibodies to test.

To bring you even more Simple Western-certified antibodies, we've partnered with our sister companies R&D Systems and Novus Biologicals so you don't have to do the work. You can find their antibodies listed below, or for more details visit their websites and search by Application (type "Simple Western"). We've given these antibodies the seal of approval they deserve after being certified in-house by our experienced scientists.

The performance of any antibody selected will need to be optimized for the test system being examined. We encourage users to contribute to the database by clicking the Submit New Antibody button and sharing specific information about the antibodies that have been tested. If you need help with or have questions about any of the information in this antibody database, please contact support@proteinsimple.com.

The database can be sorted by clicking on the column headings. In addition, keywords can be searched and identified from the database using the Search box.

ANTIBODIES (2311)

Search... Go > Reset Show All Submit New Antibody >

Page: << 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39
40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59
60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79
80 81 82 83 84 85 86 87 88 89 90 91 92 93 > >>

Protein Target ▲	Antibody Type	Antibody Vendor	Product Number	Protein Isoform	Antibody Species	Cell Model(s)	Separated by Size or Charge	Antibody Dilution	Apparent MW in kDa on Simple Western	Matrix
AGXT	Primary	Novus Biologicals	NBP1-89200	AGXT	Rabbit Polyclonal	Liver (left), HepG2 (right)	Size-Wes, Sally Sue/Peggy Sue	1:20	47	12-230kD
ARG1	Primary	Novus Biologicals	NBP2-14787	ARG1	Mouse Monoclonal	Liver	Size-Wes, Sally Sue/Peggy Sue	1:20	42	12-230kD
ARG1	Primary	Novus Biologicals	NBP1-87490	ARG1	Rabbit Polyclonal	Liver (left), HepG2 (right)	Size-Wes, Sally Sue/Peggy Sue	1:50	41	12-230kD

一抗的选择对于一个好的免疫测定试验至关重要

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  - 文献（www.proteinsimple.com/citations.html）
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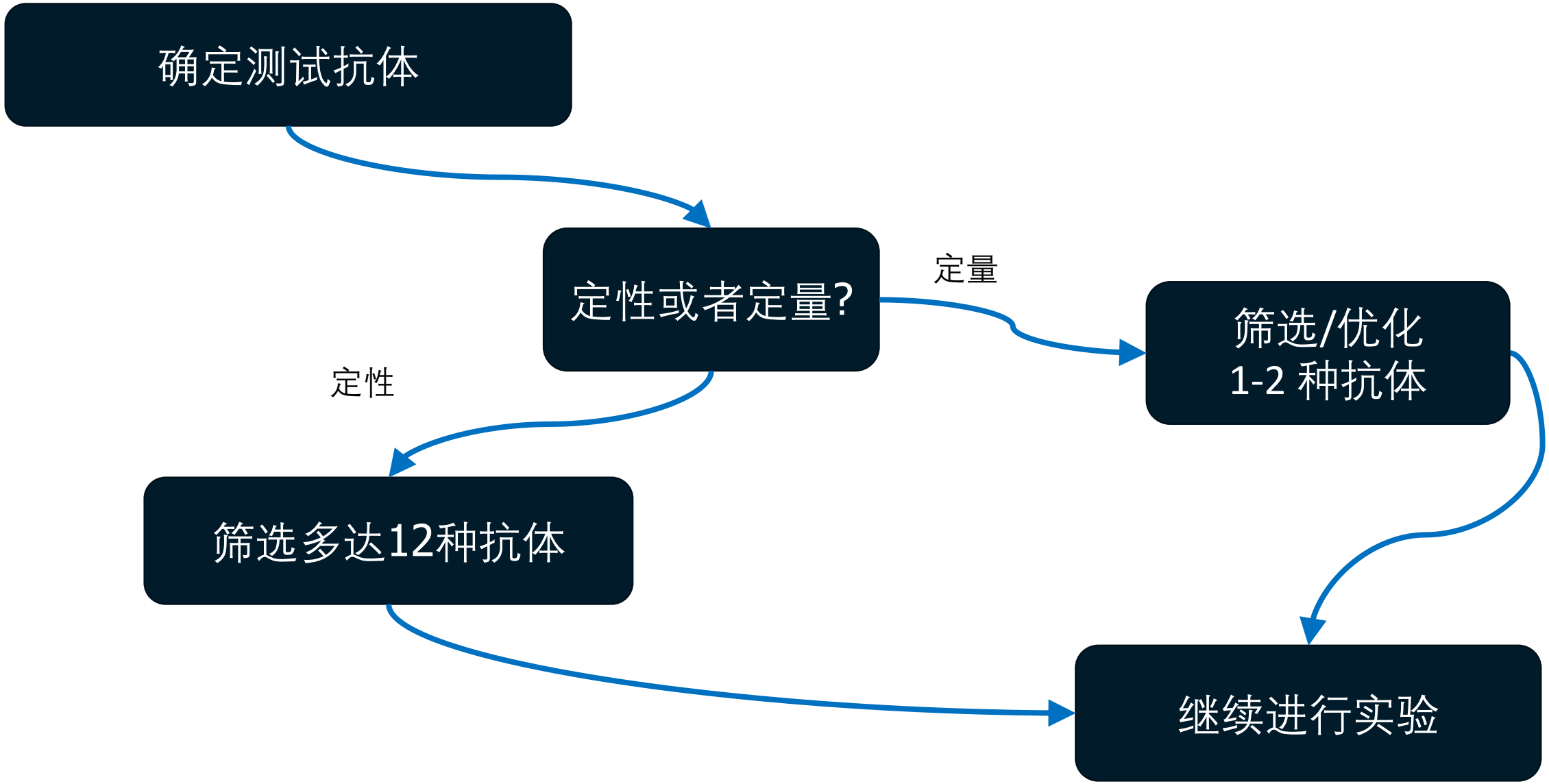
- 筛选一种以上抗体
  - 与传统Western和ELISA类似, **越多备选一抗越好**

一抗优化流程



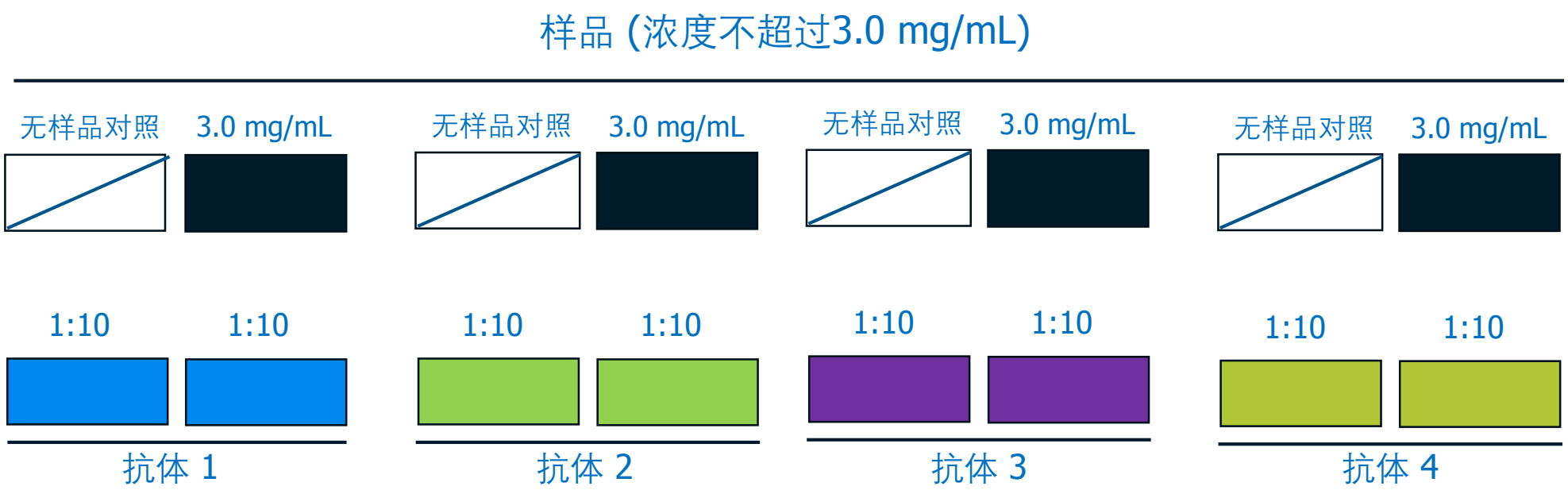
定性结果的实验优化

一般推荐简单的抗体筛选来优化定性实验

- 筛选新抗体时，尝试高的抗体浓度
    - **1:10**（不能高于1:5）
    - 如果做过传统 WB，测试 100倍 或者 20倍于传统 western blot 的浓度.

      例如:
      如果传统 WB 使用 1:2000 的抗体稀释度在 Simple Western 上尝试 1:20 (100x) or 1:100 (20x)的抗体浓度。

例如，可以在一次运行中筛选多种抗体

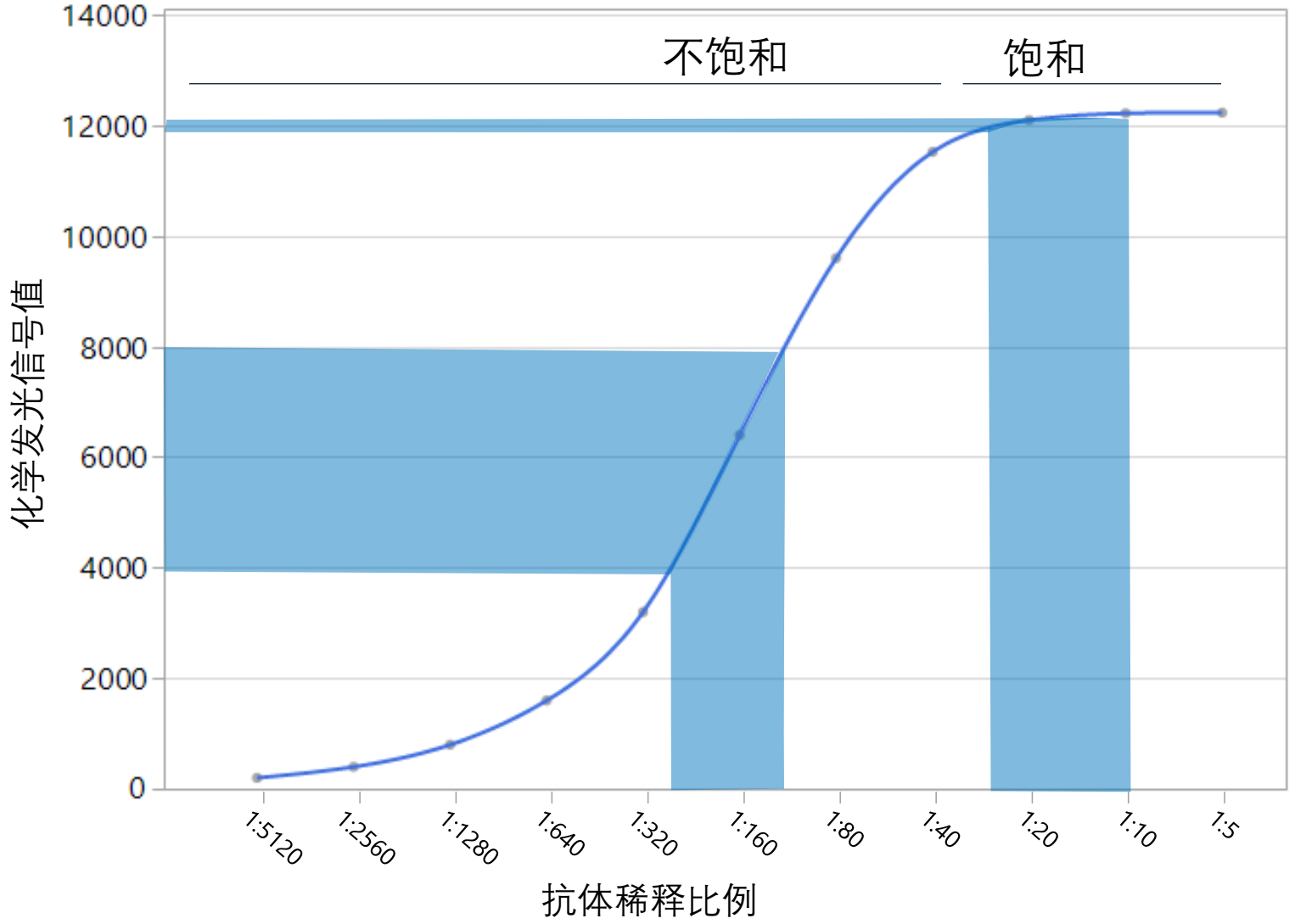


- 测试多种抗体 (最多12 种抗体)
- 使用高的样品浓度 (不超过 3 mg/mL)
- 无样品对照用于测试抗体背景

定量结果的实验优化

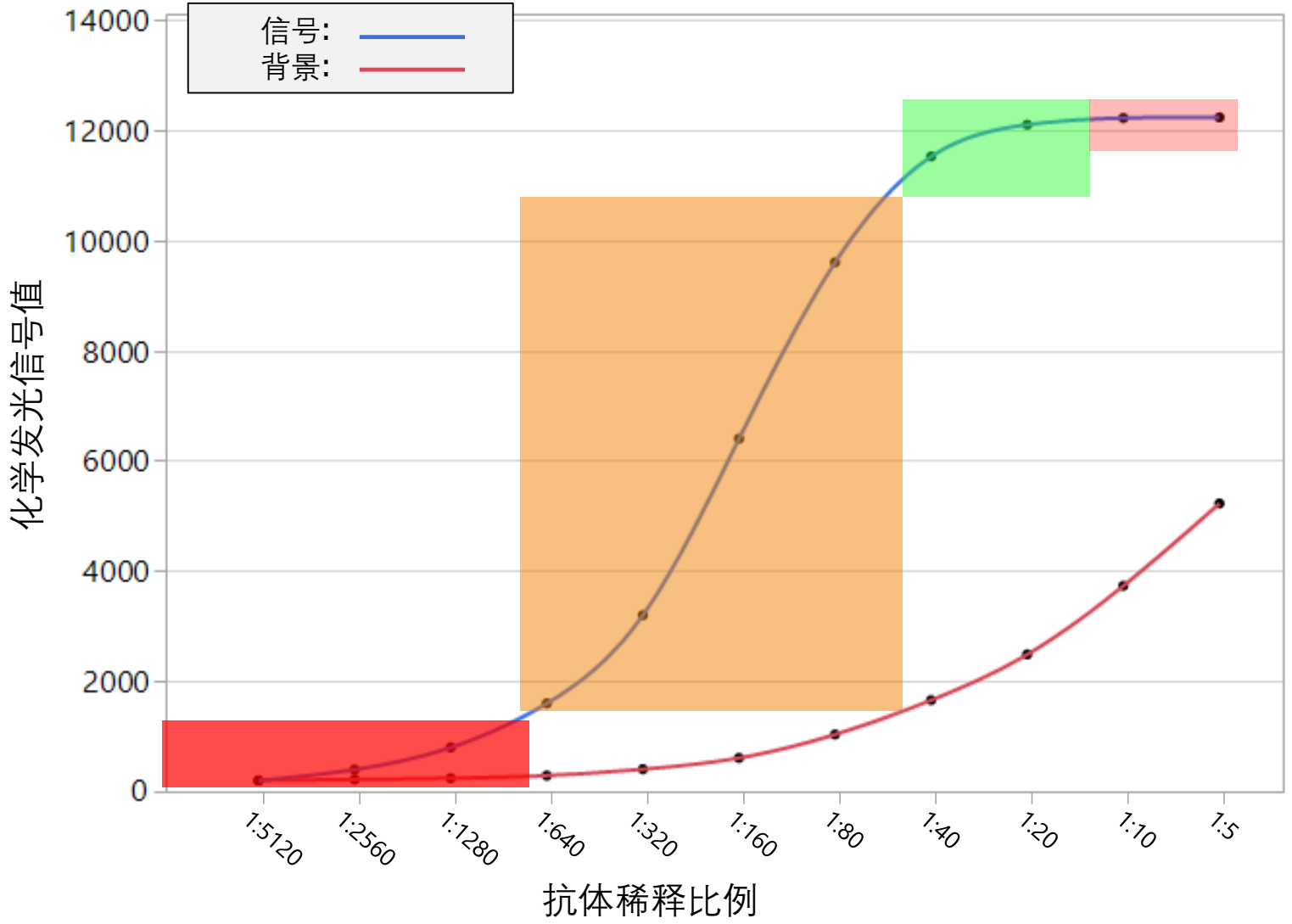
- 筛选抗体
- 抗体浓度/稀释比例的优化
- 样品浓度的优化

抗体不饱和的免疫测定会导致重复性不好



- 抗体不饱和，导致检测精确度不好
- 一旦您的抗体饱和，生物学重复的变异系数将大大降低

优化好的抗体浓度：刚饱和的抗体浓度



- 优化好的抗体浓度:
  - 足够高到饱和
  - 不能太高导致背景提高
- 抗体浓度避免落在低浓度区间 (红色区) 以及指数期（橙色区）
- 注意当抗体浓度太高，可能会导致高背景 (浅红色区)
- **平台期的前端是最理想的浓度 (绿色区)**

一抗 5 倍梯度稀释可帮助确定抗体是否饱和

- 对于大部分抗体，测试 **1:10, 1:50, 和 1:250** 三种稀释倍数
  - 或者 **100 µg/mL, 20 µg/mL 和 4 µg/mL** 三种不同抗体浓度

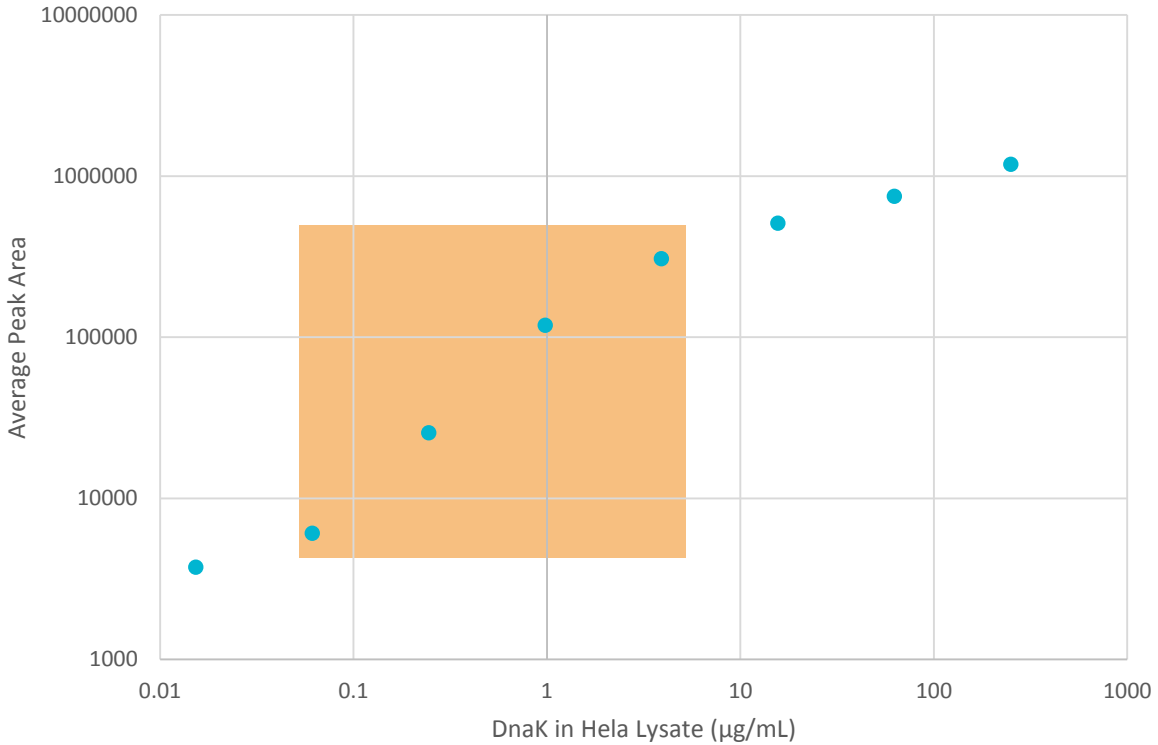
- 或者，如果您的抗体在传统 WB 上工作过，稀释倍数大于1000
  - **测试 100倍, 20倍, 4倍** 于传统 WB 的抗体浓度
    - 例如，在传统 WB 中稀释倍数为 1:2000, 在Simple Western 条件摸索中，尝试 1:20, 1:100, 和 1:500 三种抗体浓度

- 二抗也需要饱和

确定线性范围对于定量试验同样重要

当您的一抗饱和了:
- 您的样品浓度需要令您的目标蛋白浓度落在定量线性范围内
- 理想情况下, 落在橙色区间

动态范围 ~ 4.2 log



梯度稀释Hela 裂解液- 检测 DnaK

样品4倍梯度稀释能帮助确认样品的定量线性区间

- 内源性蛋白常用细胞/组织裂解液浓度:
  - 2.00 mg/mL
  - 0.50 mg/mL
  - 0.125 mg/mL

- 优化样品浓度之前，需要了解您研究的特殊需求

- 其它样品的摸索浓度：
  - 过表达/表达丰度高的细胞裂解液
    - 0.5 mg/mL
    - 0.1 mg/mL
    - 0.02 mg/mL
  - 重组蛋白，纯化后的蛋白
    - 10 µg/mL
    - 2.0 µg/mL
    - 0.4 µg/mL

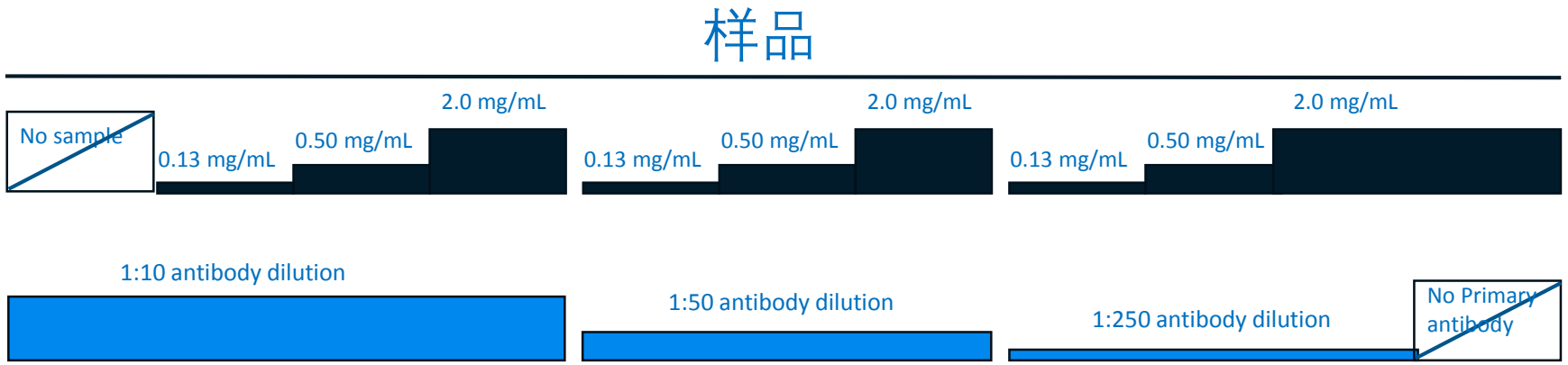
做过 WESTERN的样品浓度

- 比传统Western终浓度高 **3倍** 作为起始浓度

- 从该浓度开始，依次稀释 **5倍**
  - 例如传统 Western 上样浓度是 0.33mg/mL
  - Simple Western 相应浓度（/每孔）
    - 1mg/mL (3X)
    - 0.2mg/mL (0.6X)
    - 0.04mg/mL (0.12X)

把这些条件集中到一次运行中...

- 3 种抗体浓度 X 3 种样品浓度，共 9 根毛细管



- 两个关键阴性对照
  - 无一抗对照 (测试样品和二抗的交叉反应情况)
  - 无样品对照 (测试一抗背景)

- 共 11 根毛细管，25 根毛细管卡盒可优化 2 种样品和一抗

第一次实验原则

第一次实验 的目的是确定最佳的样本/抗体浓度，
为以后的实验做准备

对于每一个新抗体

1. 最少测试 3 种抗体稀释度

（最高浓度 1:5，一抗 5 倍梯度稀释可帮助确定抗体是否饱和）

2. 最少测试 3 种样品浓度

（样品4倍梯度稀释能帮助确认样品的定量线性区间）

3. 设定无样品对照

4. 设定阳性对照（阳性样本+阳性抗体）

5. 使用默认运行程序、参数

jove Journal of Visualized Experiments www.jove.com

Video Article

Use of Capillary Electrophoresis Immunoassay to Search for Potential Biomarkers of Amyotrophic Lateral Sclerosis in Human Platelets

Jessica M. Sage[1], LaSharice Hall[2], April McVey[3], Richard J. Barohn[3], Jeffrey M. Statland[3], Omar Jawdat[3], Mazen M. Dimachkie[3], Abdulbaki Agbas[1]

The most critical part of CEI is optimizing the assay conditions for each antibody purchased from different vendors, type of antibody (monoclonal *vs.* polyclonal), optimum protein concentrations, sample preparation, sample denaturation temperature, and electrophoresis voltage applied on the capillaries. We have developed a single-assay format optimization method for the CEI that should be implemented before any new assays, which will save time and resources.

Target protein: **total and phosphorylated** transactivation response DNA/RNA binding protein (TARDP). Due to its size (43 kDa), the acronym **TDP-43** will be used.

样品制备的优化

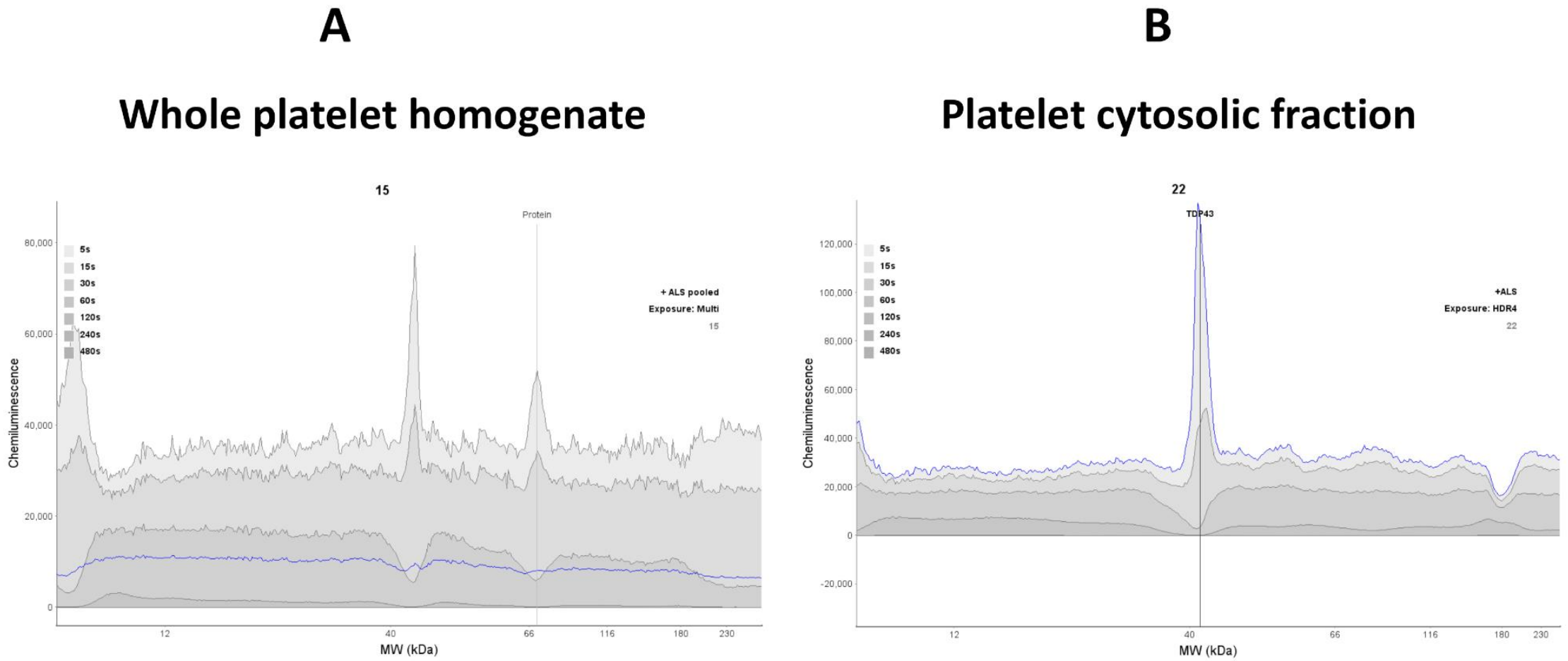


Figure 2: Signal clarity depends on the sample quality. (**A**) Whole platelet lysate homogenate interferes with anti-TDP-43 antibody binding; therefore, a noisy electropherogram was observed. (**B**) Platelet cytosolic fraction was obtained from subjecting the whole lysate to centrifugation(16,000 *x g* for 30 min). Most of the membranous proteins were removed; hence, anti-TDP-43 antibody binding to TDP-43 protein was enhanced(blue line trace).

样品浓度与抗体稀释比例的优化

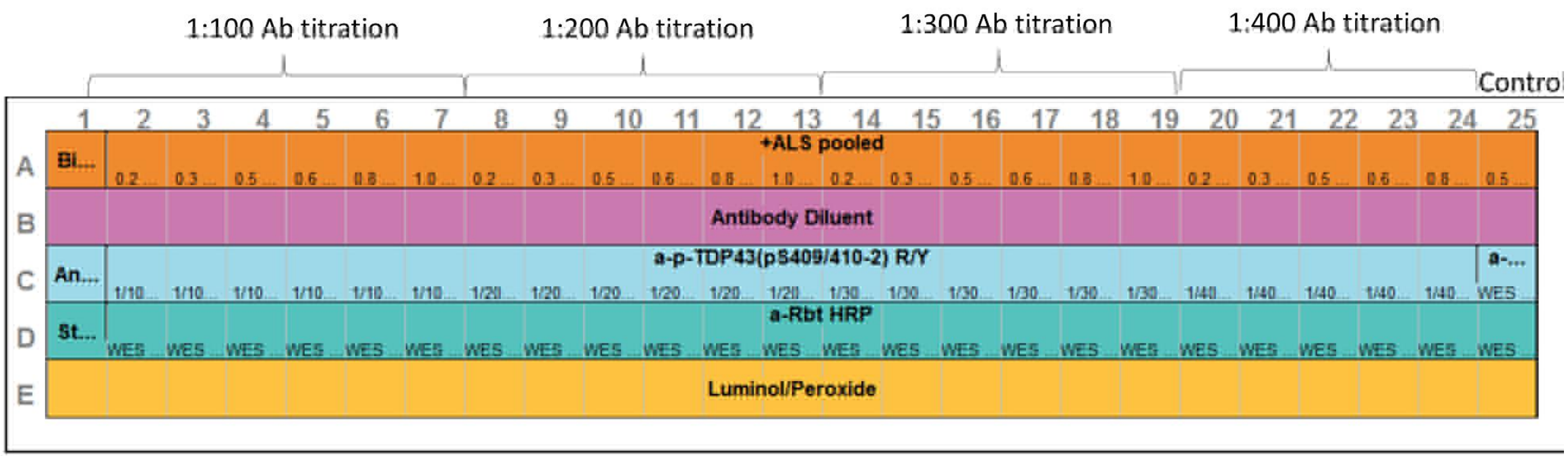


Figure 1: Assay layout. Both primary antibody and target protein sample optimization can be performed in one assay. Capillaries 2–7, 8–13, 14–19, and 20–24 represent various protein concentration and primary antibody range. Capillary 25 represents positive control. Anti-ERK antibody was used

优化结果

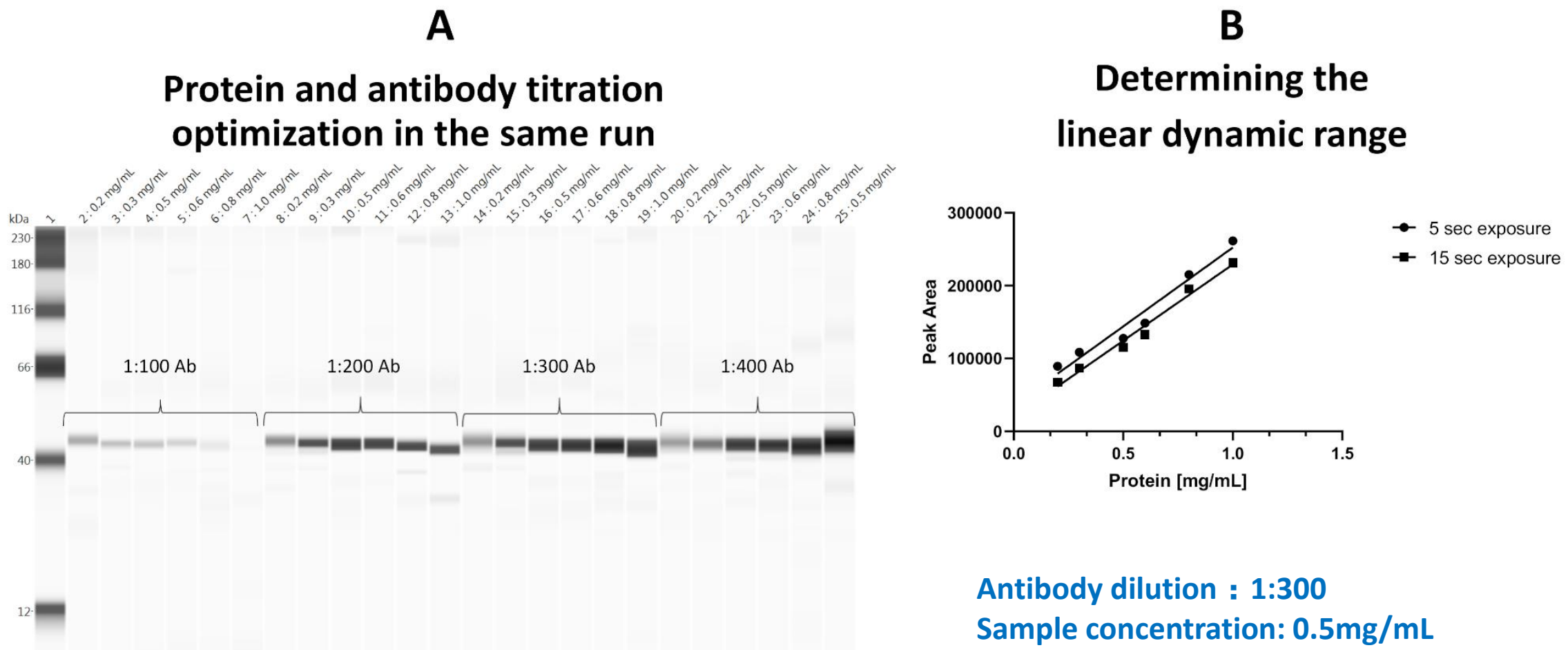


Figure 3: Linear dynamic range for platelet cytosol protein concentration. (**A**) Both protein concentrations and antibody titrations were optimized in one plate during the same run. (**B**) The linear working range (0.2–0.8 mg/mL) for protein was established. A 0.5 mg/mL protein load was labeled by a-ERK antibody as the positive control (capillary 25).

ALS患者血小板裂解上清液中TDP-43水平检测

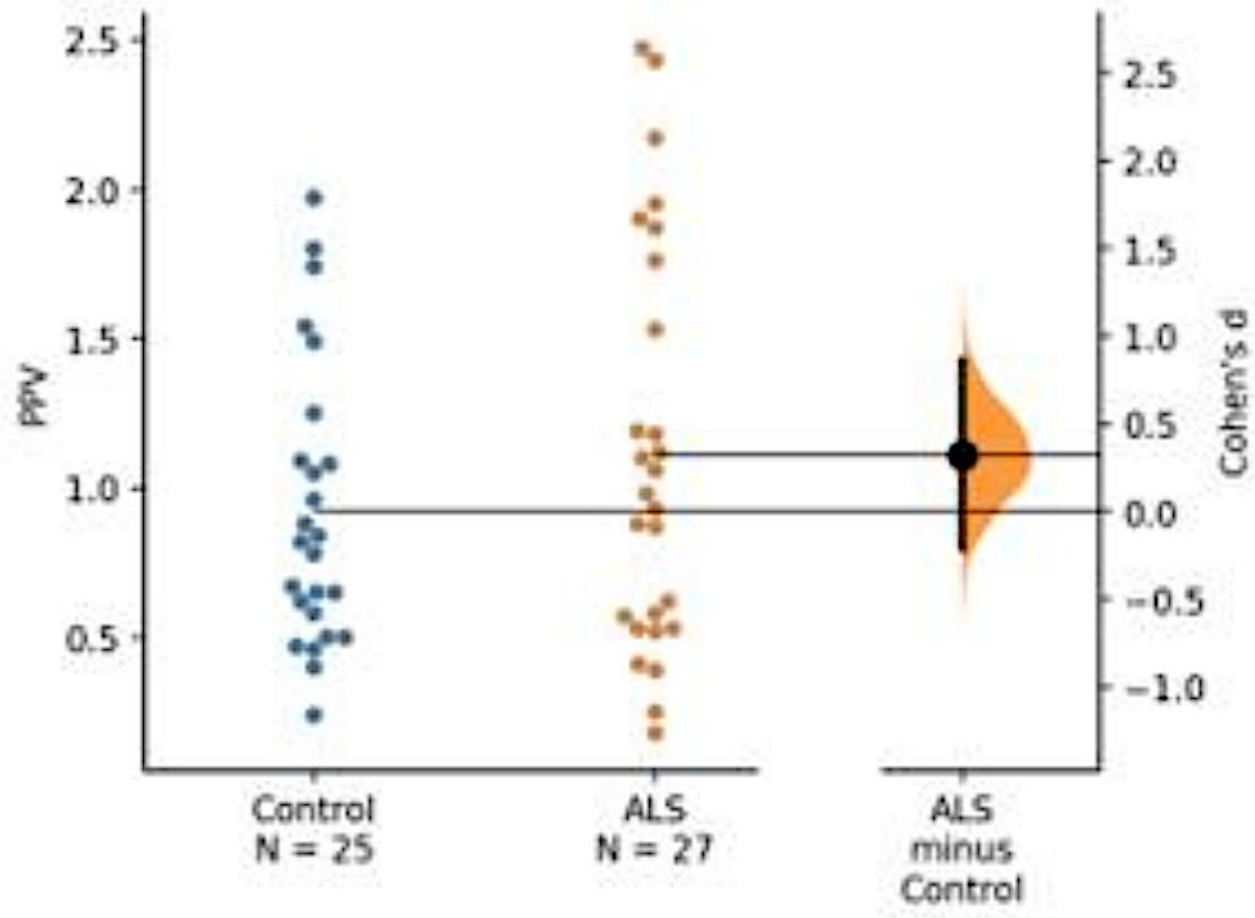
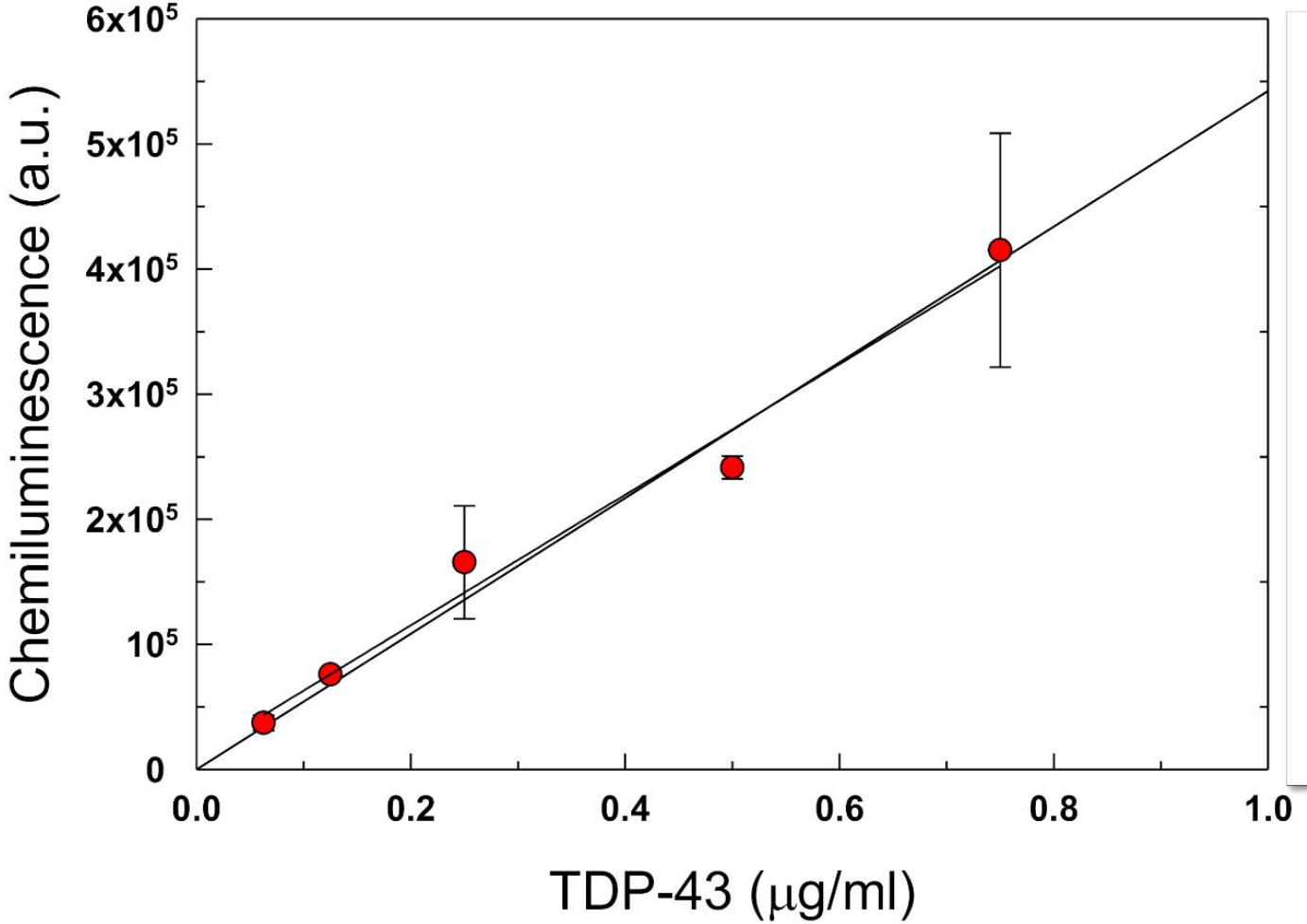
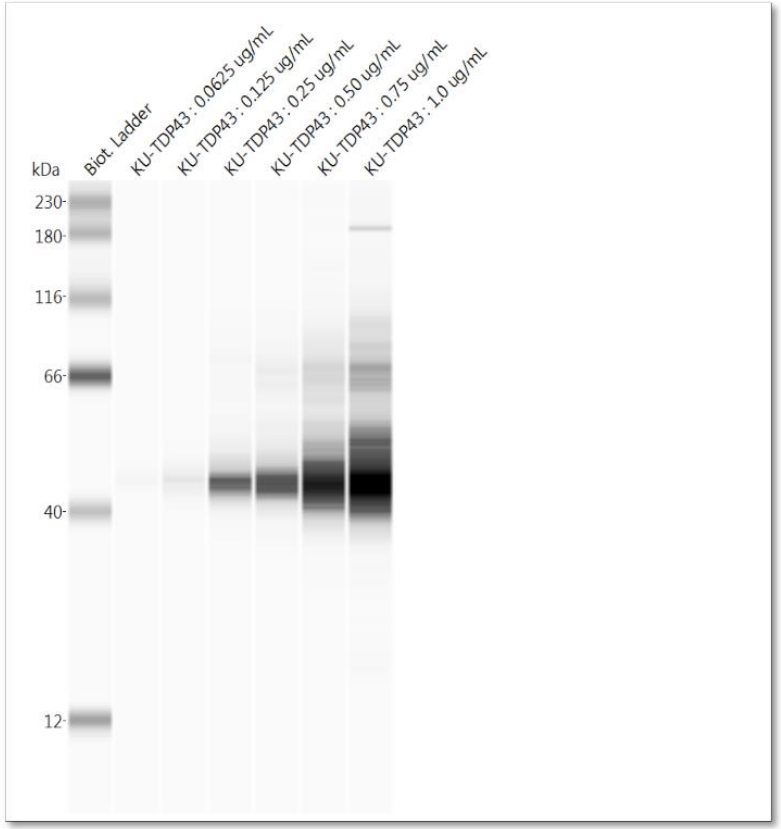


Figure 5: A representation of predictive phosphorylation value (PPV) of TDP-43. Absolute amount of phosphorylated TDP-43 and pan TDP-43 alone did not show much difference between the ALS and control groups. However, PPV indicated a slight increase in the ALS cohort, although there was no statistical difference between the two groups due to insufficient numbers of subjects (ALS = 25, control = 27). A low Cohen's d value between the means of ALS and control group showed a low effect size between the two groups due to small sample size (control = 25, ALS = 27).

TDP-43蛋白的绝对定量





SIMPLE WESTERN
高级条件优化

高级实验优化概览

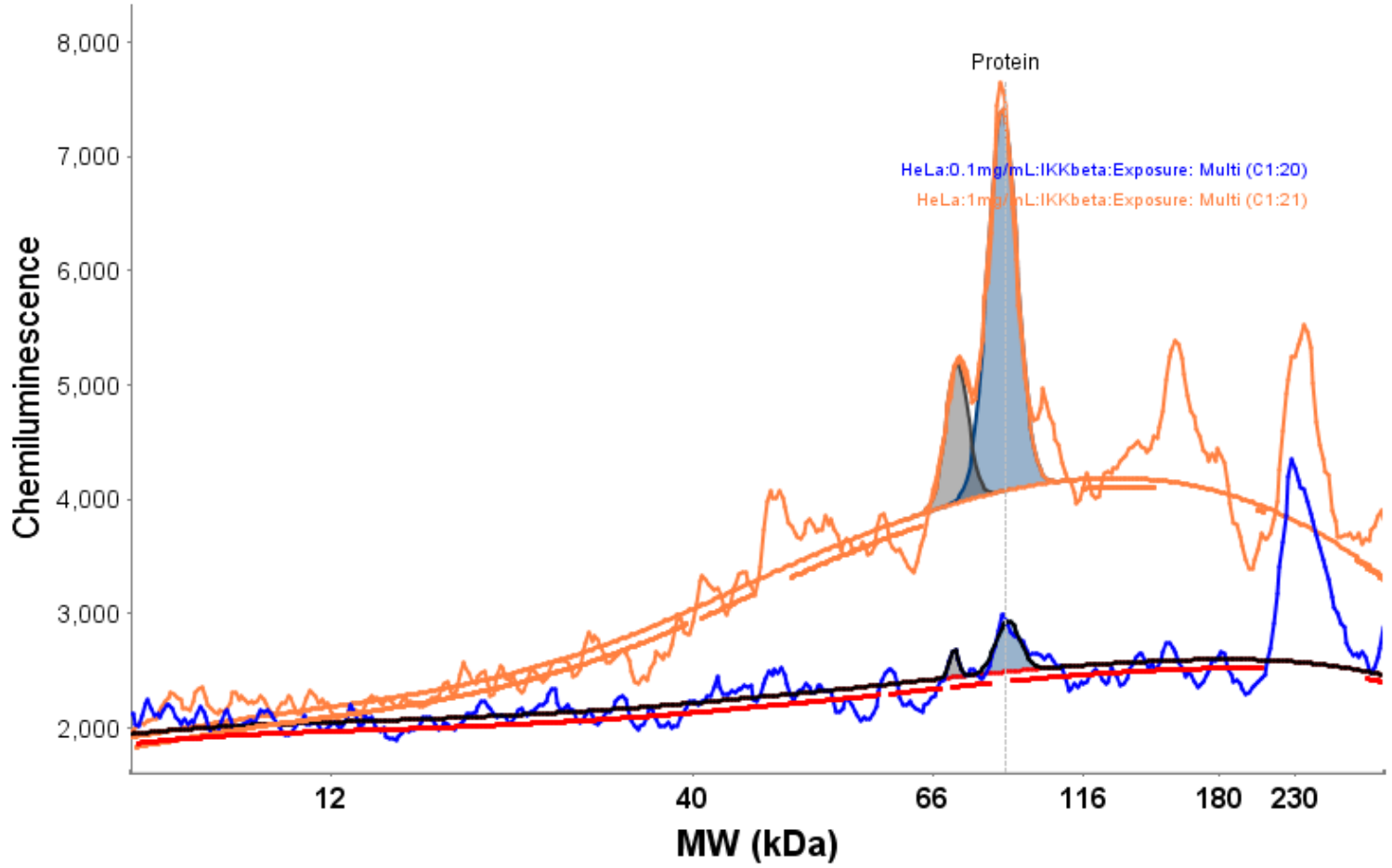
- 信号灵敏度
  - 样品因素
  - 抗体因素
  - 运行参数
  - 检测方法
- 免疫检测
  - 抗体条件
  - 样品条件
  - 共同实验对照
- 重现性优化
- 微调运行参数

1.3种因素可以调整以优化信号强度/灵敏度

- 样品因素:
  - 增加最终蛋白的浓度（注意背景、非特异性）
  - 考虑先进行样品免疫沉淀
- 抗体因素:
  - 增加抗体浓度
  - 考虑信号放大: 生物素化二抗+ strep-HRP
  - 考虑不同的抗体品牌
- 运行参数:
  - 增加样品上载时间
  - 增加抗体孵育时间

样品因素

增加样品浓度



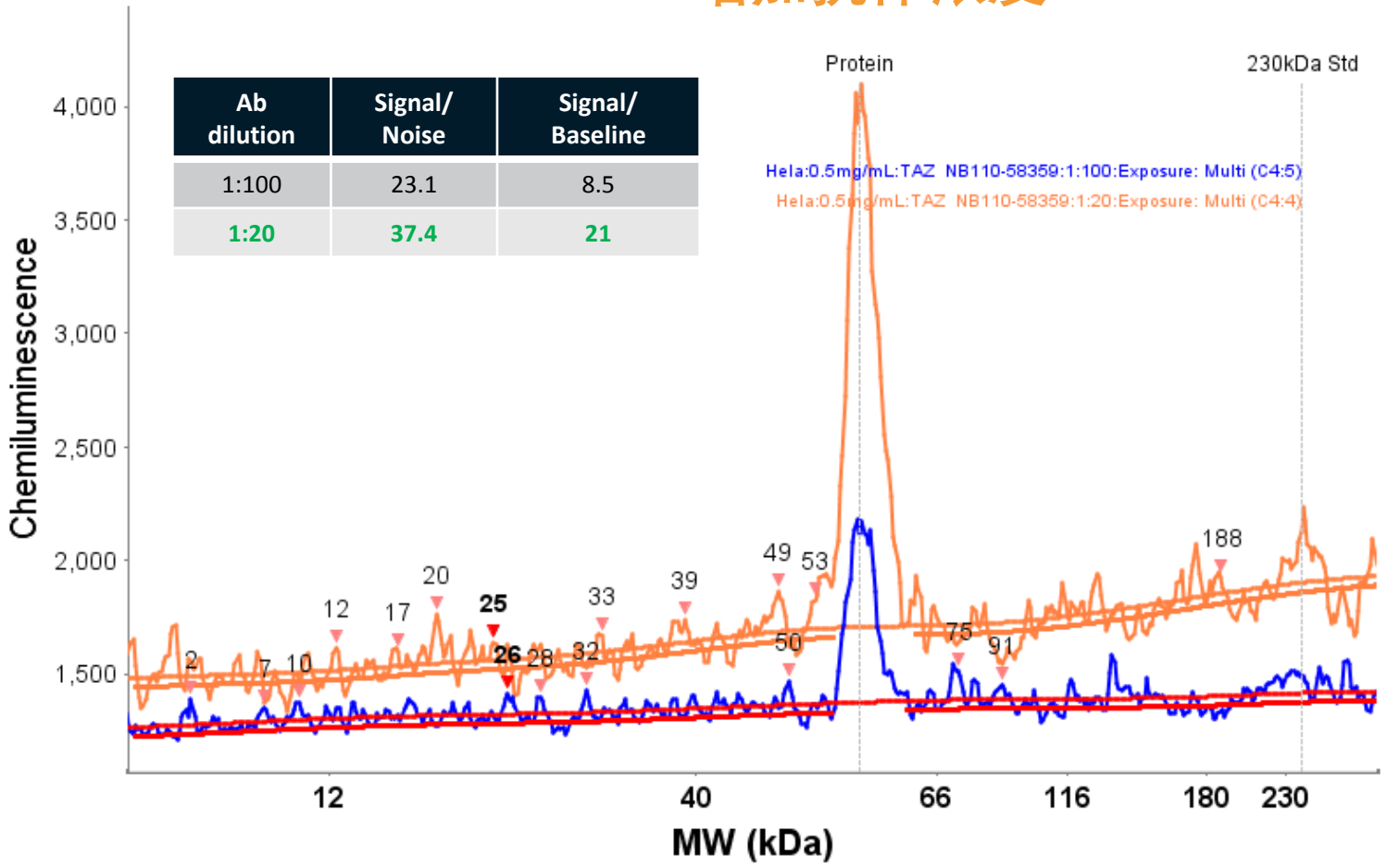
0.1mg/mL
1mg/mL

IKK beta in HeLa Lysate (anti IKK beta, Novus, NB100-56509, 1:12.5)

抗体因素

增加抗体浓度

Ab dilution	Signal/ Noise	Signal/ Baseline
1:100	23.1	8.5
1:20	37.4	21



抗体稀释倍数:

1:20

1:100

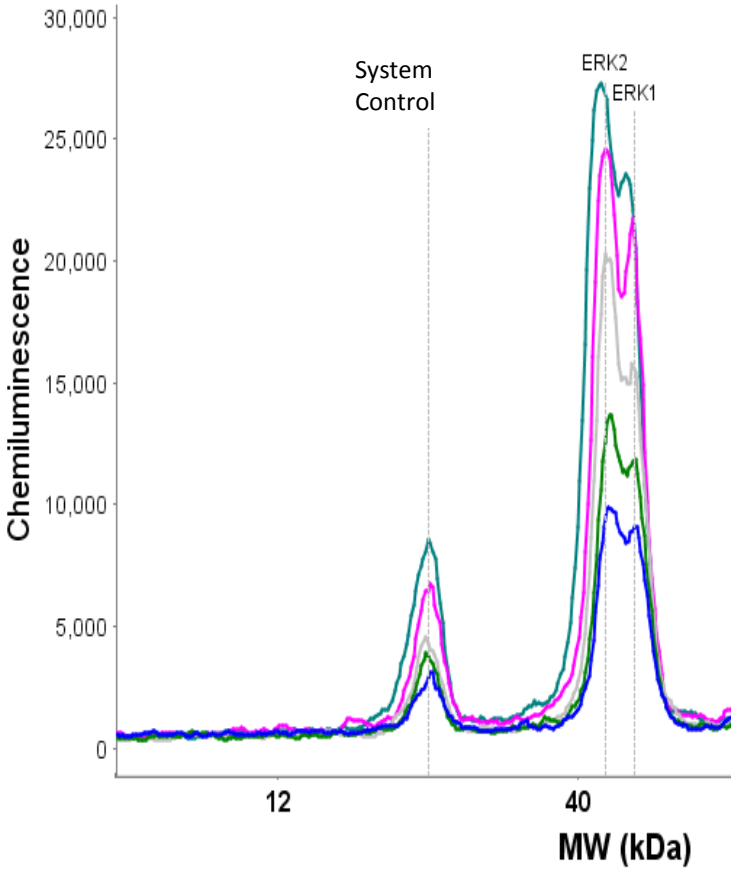
TAZ in 0.5mg/mL HeLa Lysate (anti TAZ, Novus, NB110-58359)

- 抗体浓度提高信号明显提高，背景信号略微增加
- 计算 Signal/Baseline, Signal/Noise 以确定数据是否改善

运行参数运行参数

增加样品上载时间

Graph View



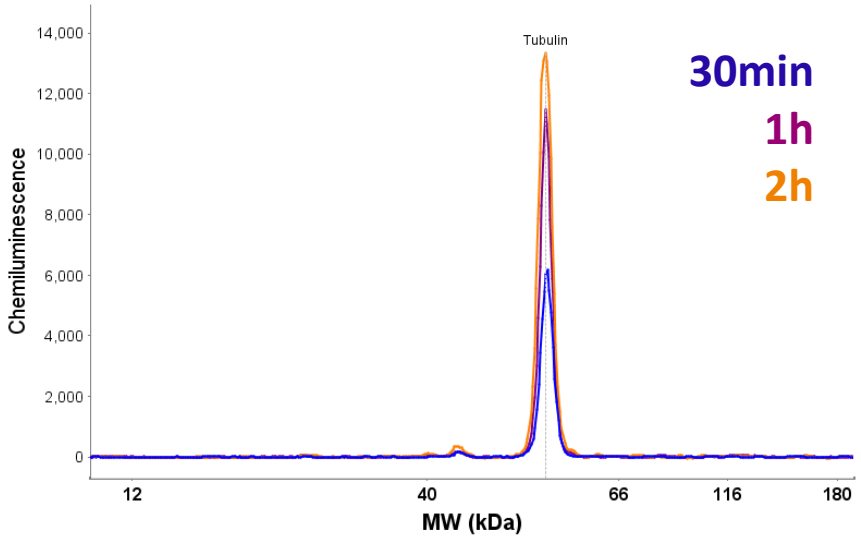
默认运行条件

Stacking (sec)	6	9	12	15	18
Sample (sec)	3.6	5.4	7.2	9	10.8
Separation time	40min				

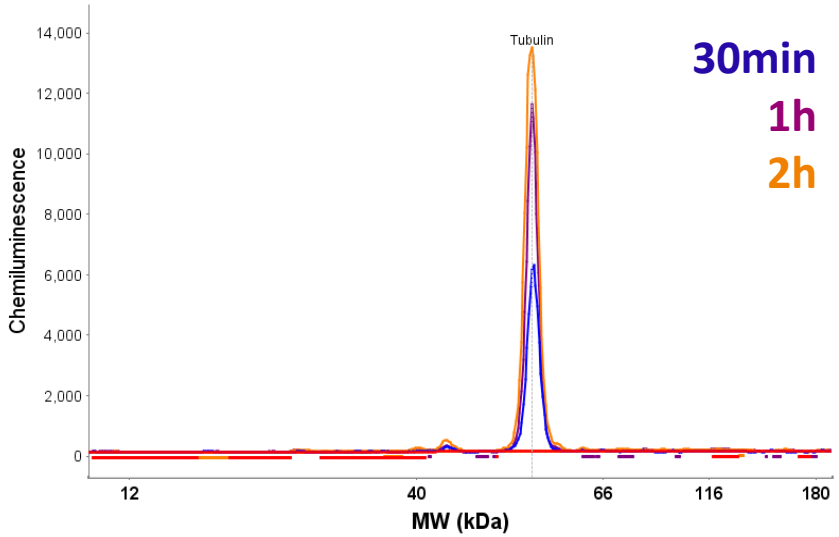
- 保持 stacking:sample 上样比（1.7:1 for 12-230kDa）
- 保持分离分辨率

运行参数

增加抗体孵育时间以增加抗体结合



背景信号扣除



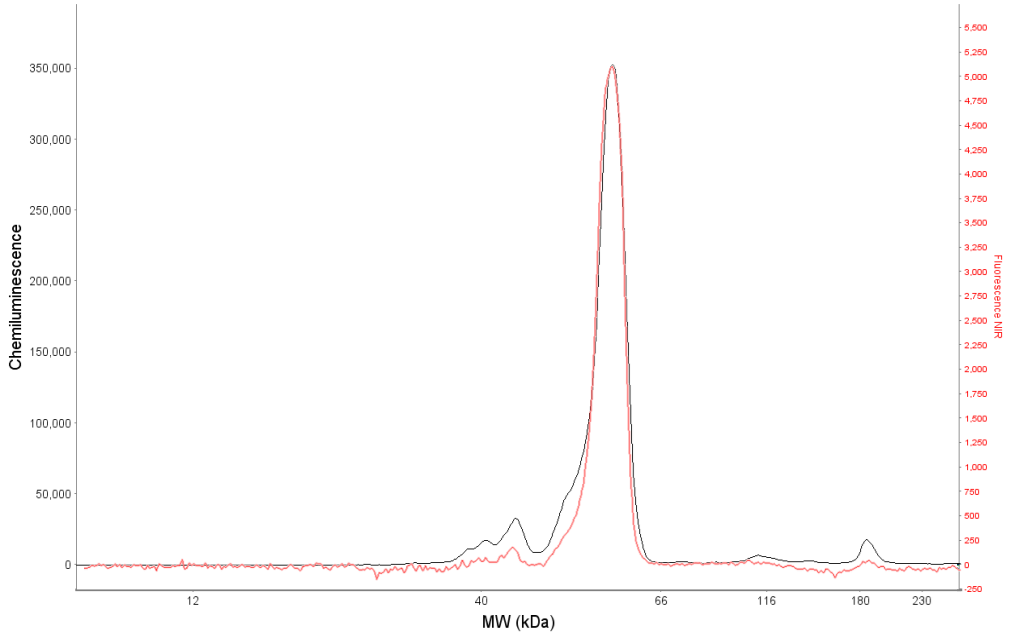
原始数据

NOTE: 注意背景信号是否也增加，本例中背景信号无明显增加

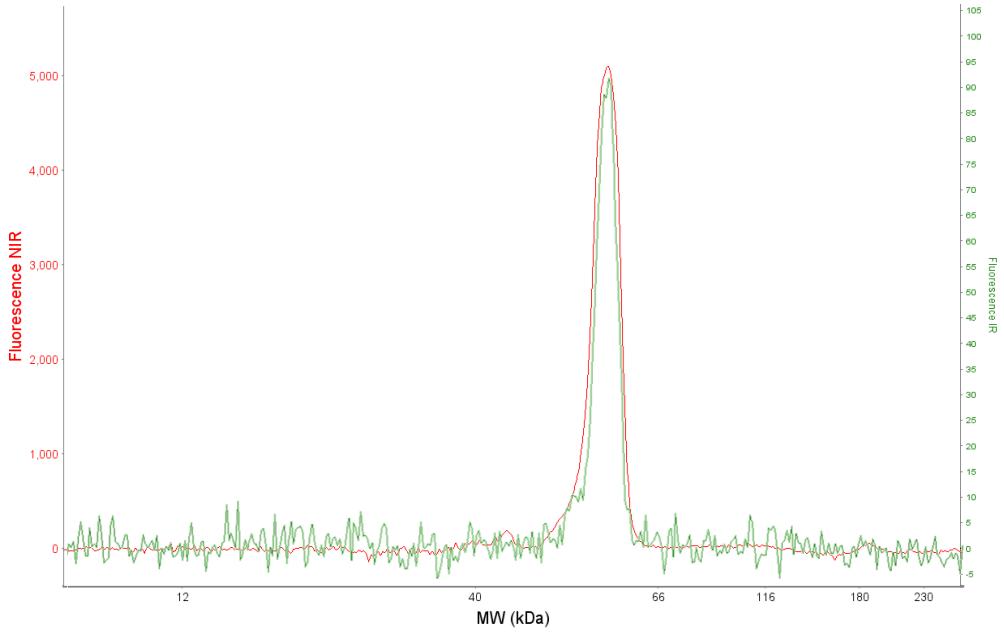
Tubulin (CS2105)

在 SIMPLE WESTERN 中化学发光相比于NIR 和 IR 具有更高灵敏度

化学发光 和 NIR



NIR 和 IR



样品	名称	峰高	峰面积	信噪比
A-431	Hsp60 Chemi	349873	5126616	21633
A-431	Hsp60 NIR	8009	93108	346
A-431	Hsp60 IR	93	1038	64

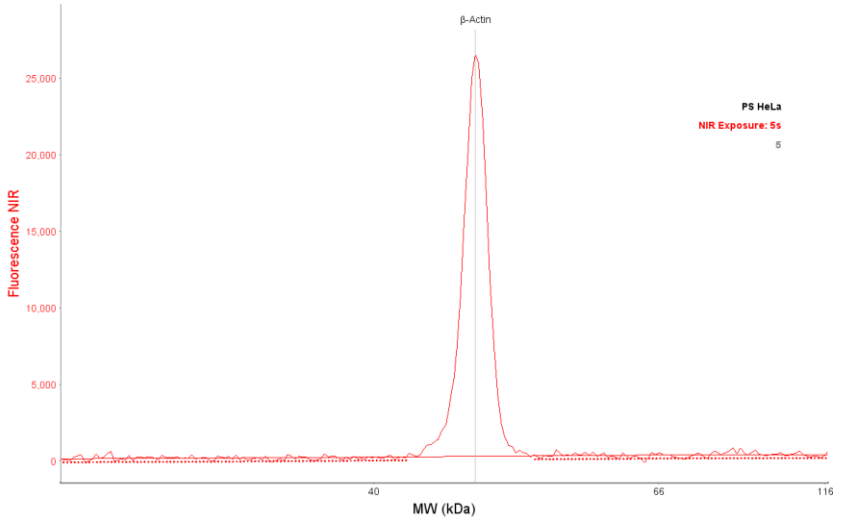
A431 裂解液 @ 1 µg/µL. Target = HSP60 (1:50). FL 曝光时间300sec.

2.免疫检测优化--结果依赖于抗体质量

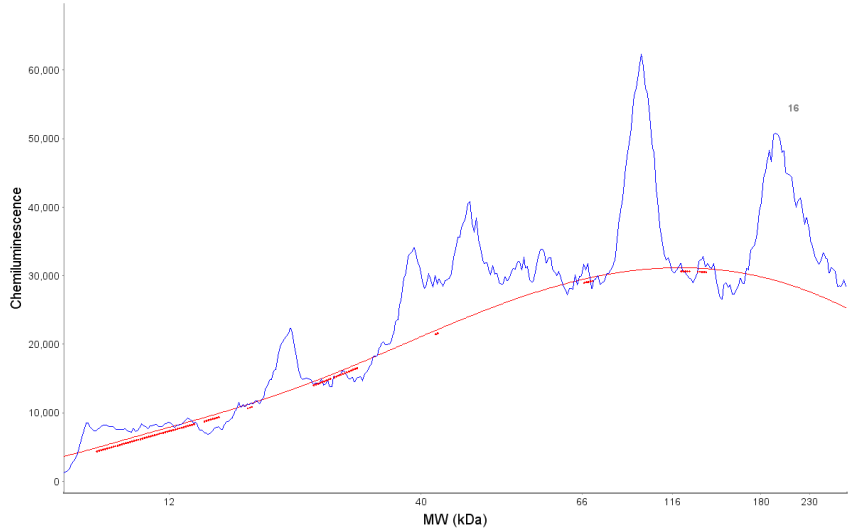
- 一个好的一抗:
  1. 高信噪比
  2. 低背景
  3. 条带符合预期

- 一个不好的一抗:
  1. 高背景
  2. 非特异条带

- 如果背景高:
  1. 测试新的一抗
  2. 测试不同的封闭液

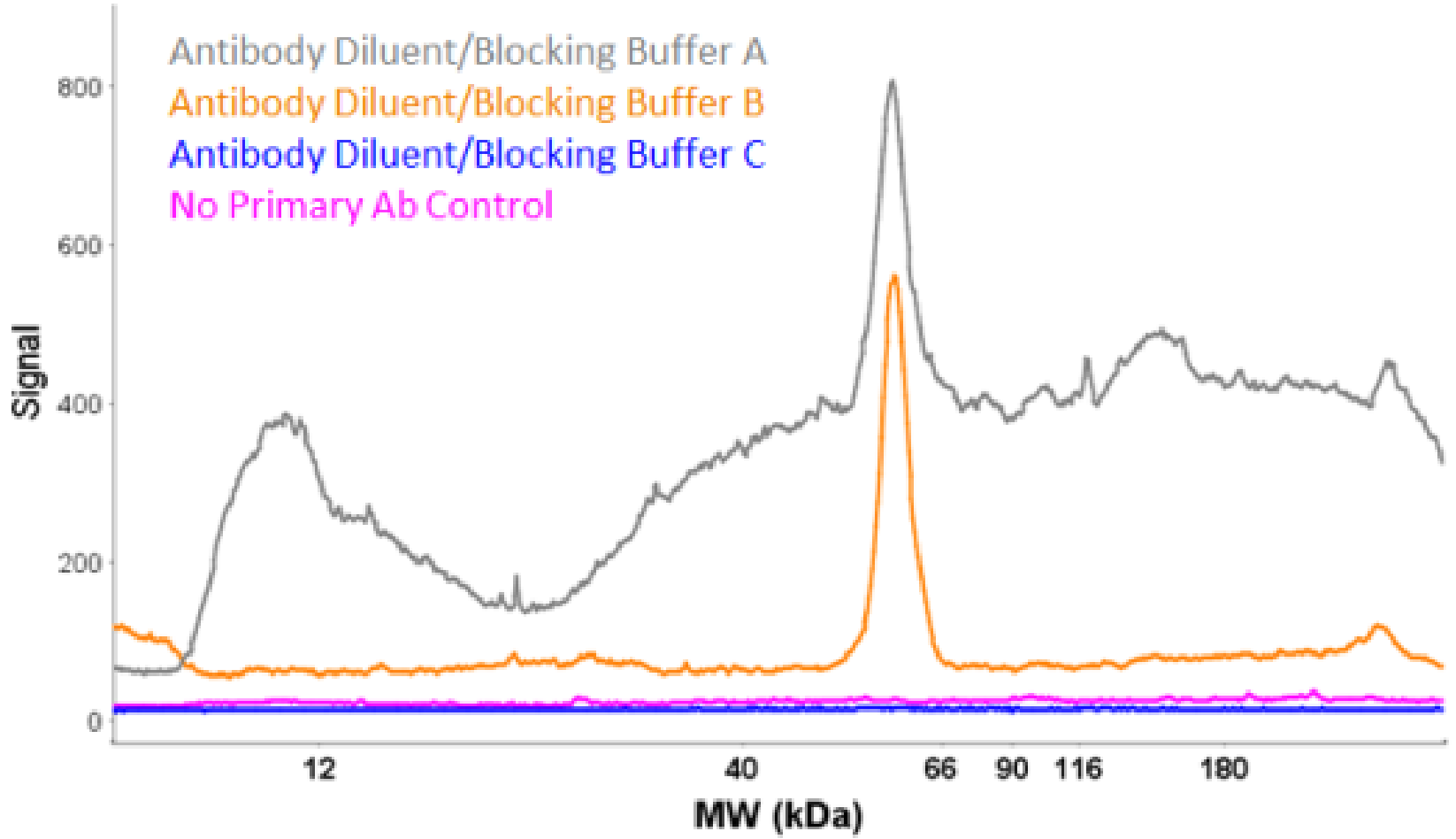


背景 = ~300



背景 = ~30,000

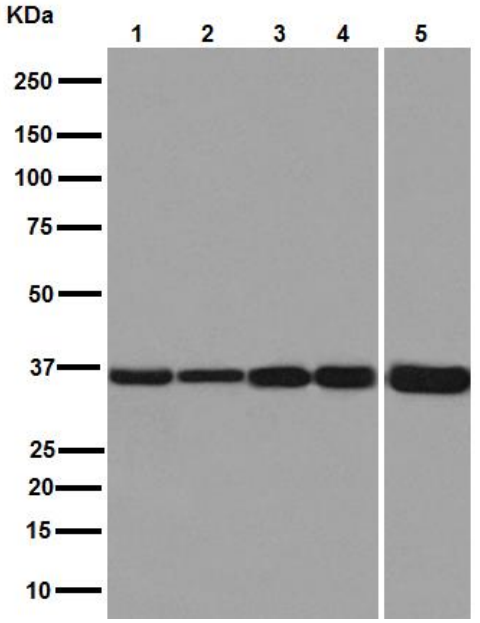
不同的封闭液效果不同



3.重现性优化--变异系数来源于多方面

- 移液误差 (小体积!)
- 不同人员
- 稀释方式
  - 梯度稀释 vs. 直接稀释
- 裂解液的蛋白定量
- 样品间的生物学重复
- 抗体不饱和
- 试剂&耗材
  - 不同批次
  - 保存条件
- 定量方法

Abcam GAPDH 抗体 [EPR16891]



Lane 1 : Mouse kidney lysates
Lane 2 : Mouse spleen lysates
Lane 3 : RAW 264.7 (Mouse macrophage cells transformed with Abelson murine leukemia virus) whole cell lysates
Lane 4 : PC-12 (Rat adrenal gland pheochromocytoma) whole cell lysates
Lane 5 : Rat brain lysates

Lysates/proteins at 10 µg per lane.

蛋白归一化可改善重现性

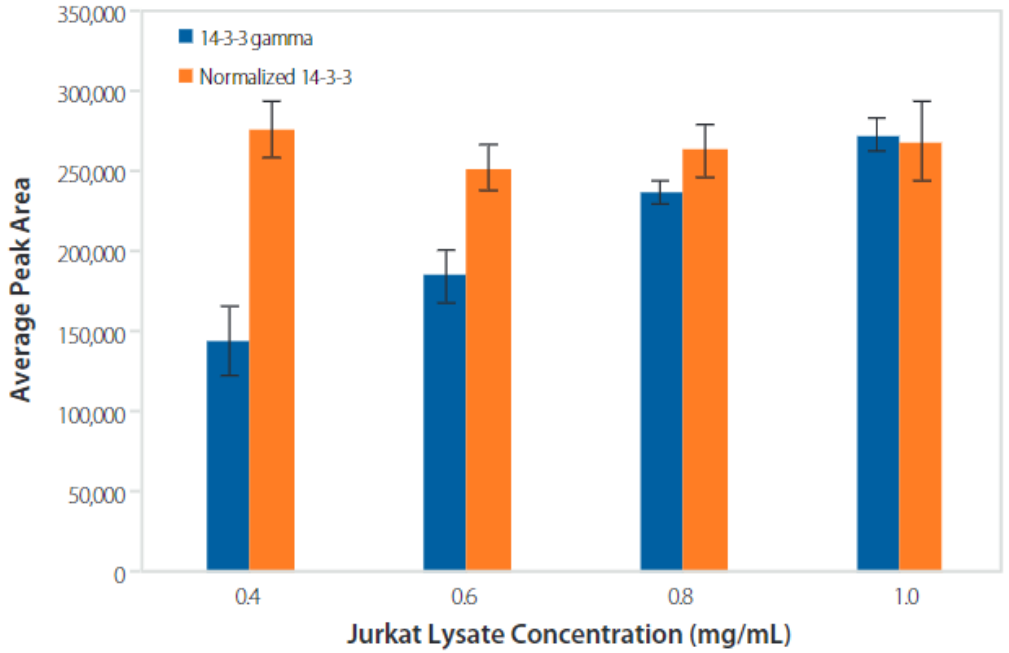


FIGURE 5. Comparative data showing 14-3-3 gamma protein expression (blue bars) and the normalized expression (orange bars) in various concentrations of Jurkat cell lysate. Targets were detected by the NIR channel on Jess.

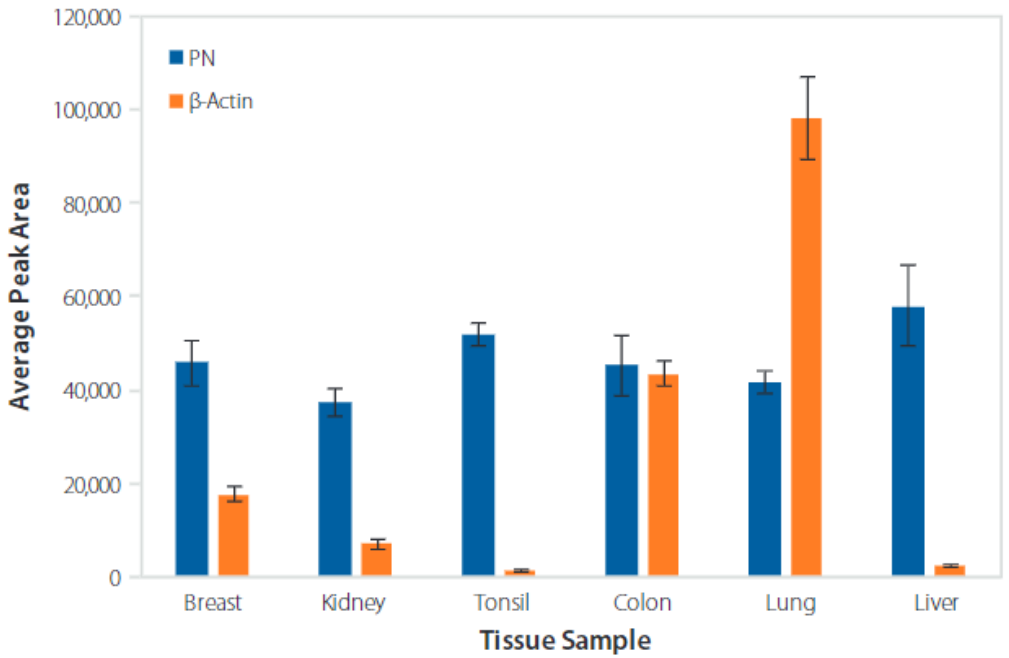


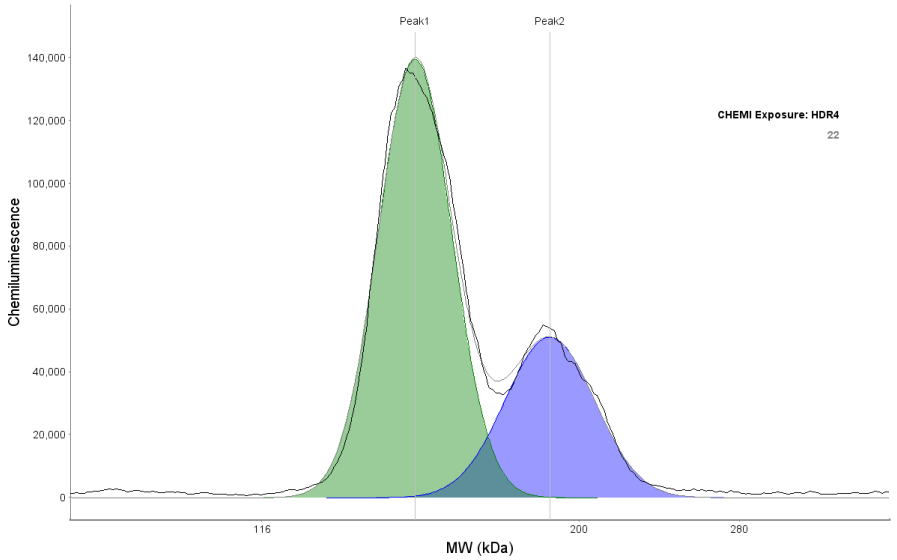
FIGURE 6. Comparative total protein data (blue bars) and the expression of β-actin (orange bars) in six human whole tissue lysates (0.3 mg/mL) tested using Jess.

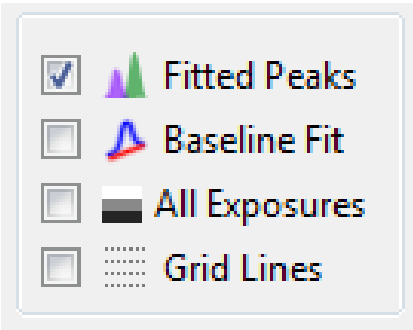
参考 application note: “Better Housekeeping: Protein Normalization on Jess”

峰拟合影响峰定量及重现性

- Compass for SW 软件默认用高斯拟合 Gaussian fits

1. 确保软件正确识别并拟合每个峰
2. 确保拟合峰和实际峰形吻合





SYSTEM CONTROL 在荧光 MASTER MIX 中

- System Control 蛋白可帮助减少实验误差

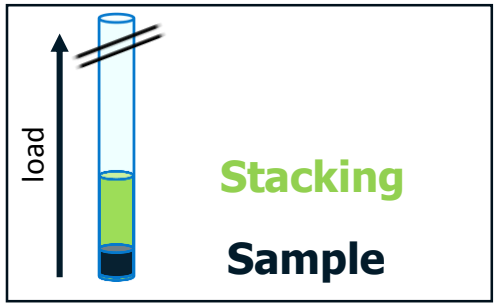
042-196 Buy Online	**10X System Control Primary Antibody-Rabbit** To be used with rabbit primary antibody to Antibody Diluent. Detects system controls. Match with Standard pack 1, 2, 3 or 4 (250 µl/vial)
042-191 Buy Online	**10X System Control Primary Antibody-Mouse** To be used with mouse primary antibody to Antibody Diluent. Detects system controls. Match with Standard pack 1, 2, 3 or 4 (250 µl/vial)

- 不同规格技术重复的 CV 水平

DESCRIPTION	TOTAL PROTEIN SPECIFICATION	CHEMILUMINESCENCE SPECIFICATION	FLUORESCENCE SPECIFICATION	PROTEIN NORMALIZATION SPECIFICATION
Sizing CV	<10%			
Intra-assay CV	<15%			<20%
Inter-assay CV	<20%			N/A

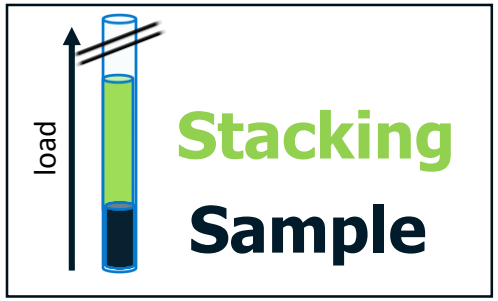
4.微调运行参数优化--调整样品和浓缩胶的上载量

默认上载



默认上载
样本和浓缩胶的上样时间固定

增加信号



同比例增加浓缩胶和样本
- 用于增加信号
- 保持 Stacking/Sample 比例不变

增加分辨率

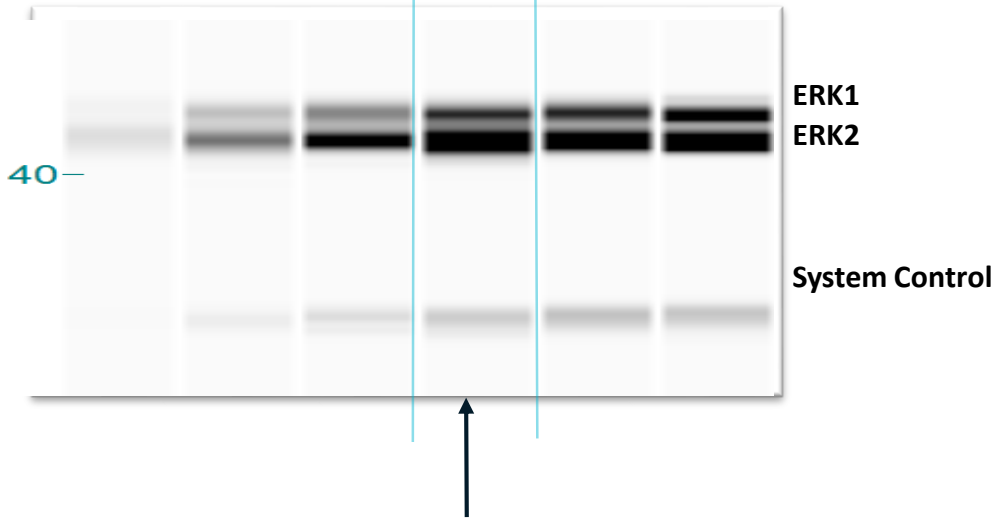
增加浓缩胶的上载量
- 改善峰的锐利度和分辨率
- 保持默认样品上样量

运行条件

参数	试剂盒	默认条件	范围	备注
Separation Time 分离时间	12-230 kDa	25 min at 375 V	25-31 min	增加分离时间能增加<75kDa 蛋白的分辨率，但是可能降低高分子量蛋白分辨率(PLCγ)
	66-440 kDa	275V, 50 min	50-55 min	增加分离时间，<200kD 蛋白分辨率增加，但可能降低高分子量（ > 200KD）蛋白分离分辨率
	2-40kDa	375V, 27 min	27-30 min	增加分离时间会导致1kDa 的荧光内参跑出胶
Sample Loading Time 样品上载时间	12-230 kDa	9 sec	3.6-12.6 sec	增加样品上载时间能增强峰信号，但降低分离分辨率
	66-440 kDa	8 sec	8-9 sec	
	2-40kDa	9 s	6-11 s	
Stacking Loading Time 浓缩胶上载时间	12-230 kDa	15 sec	6-21 sec	保持 stacking: sample 上载时间比为 **1.7:1**
	66-440 kDa	12 sec	12-14 sec	保持 stacking: sample 上载时间比为 **1.5:1**
	2-40kDa	12 sec	8-18 sec	保持 stacking: sample 上载时间比为 **1.33:1**

如何增强信号 -- 增加浓缩胶和样品上载时间

Stacking (sec)	6	9	12	15	18	21
Sample (sec)	3.6	5.4	7.2	9	10.8	12.6
Separation (min)	40	40	40	40	40	50

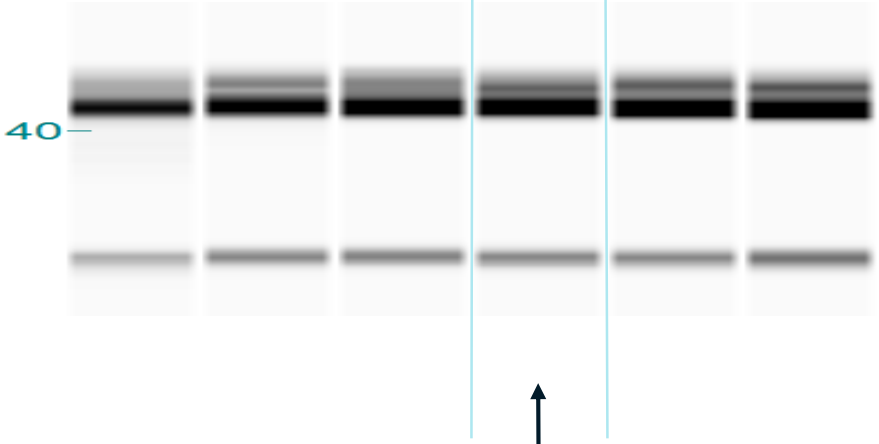


Notes:

- 生物素标记的Marker需要稀释，避免信号饱和
- 该 stacking/sample 上载比仅适用于12-230kDa 试剂盒

如何提高分辨率 -- 增加浓缩胶上载时间

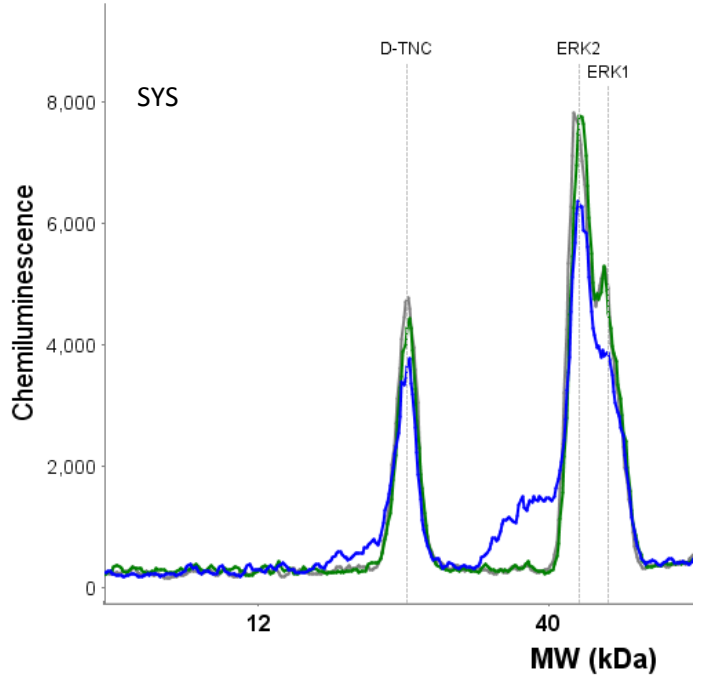
Stacking (sec)	6	9	12	15	18	21
Sample (sec)	9					
Separation time	40 min					



Default run condition

峰锐利度= 峰高 / 峰宽

Stacking (sec)	6	12	21
Sample (sec)		9	
Separation time	40 min		



注: 本数据仅适用于12-230kDa 试剂盒

proteinsimple

Simple Western Size Assay Development Guide

Table of Contents

THANK YOU