Accurate, Reproducible Western Blots and more

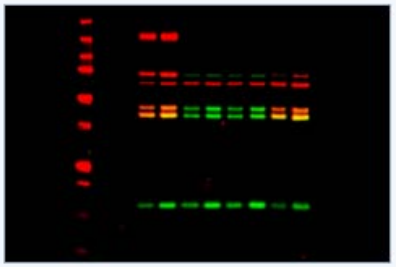
ODYSSEY® Classic Infrared Imaging System

吳美萱
騰達行儀器部

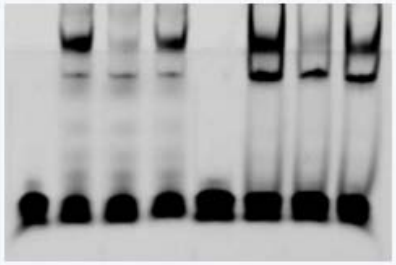
ODYSSEY® Classic Infrared Imaging System

Fluorescence direct detection: **Stable Signal**

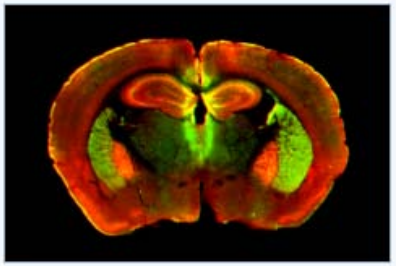
In-Cell Western™ Assay



NIR Fluorescent Western Blot



EMSA / Gel Shift Assay



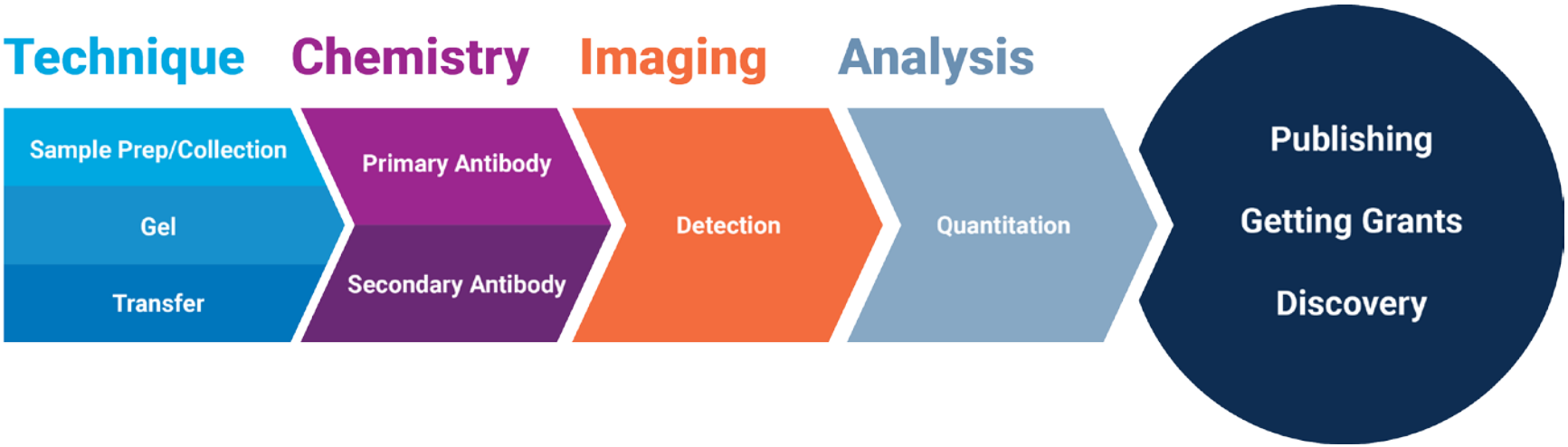
Tissue Section

ODYSSEY® Classic Infrared Imaging System

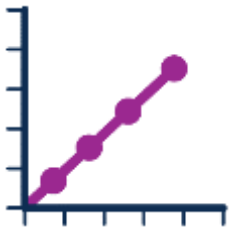
SPECIFICATIONS

- ☑ *Image Field Size*
  25 cm x 25 cm
- ☑ *Dynamic Range*
  Manual scan: 4 logs
- ☑ *Laser*
  680 & 780 nm solid state laser diodes
- ☑ *Resolution*
  21 – 337 µm

Protein Analysis Protocol



New Publishing Guidelines



Quantitation: Linear relationship between signal intensity and antigen

"Limited linear range" for X-ray film

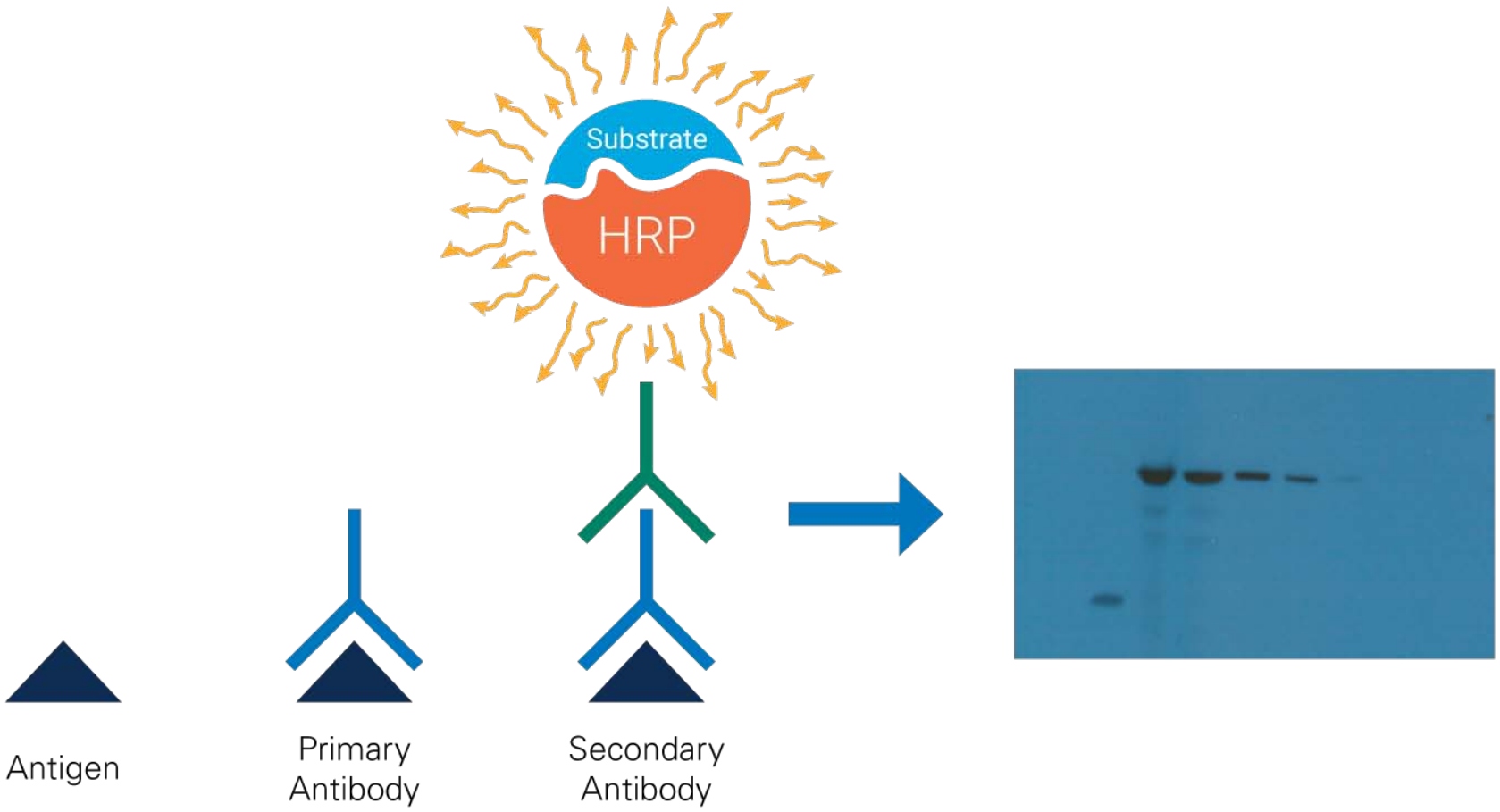
Reproducibility: Include uncertainty and reproducibility of data

Normalization: Total protein loading preferred to housekeeping proteins

Fosang AJ, Colbran RJ. Transparency Is the Key to Quality. (2015) *J Biol Chem* 290: 29692-29694.


Analysis
Imaging
Chemistry
Technique

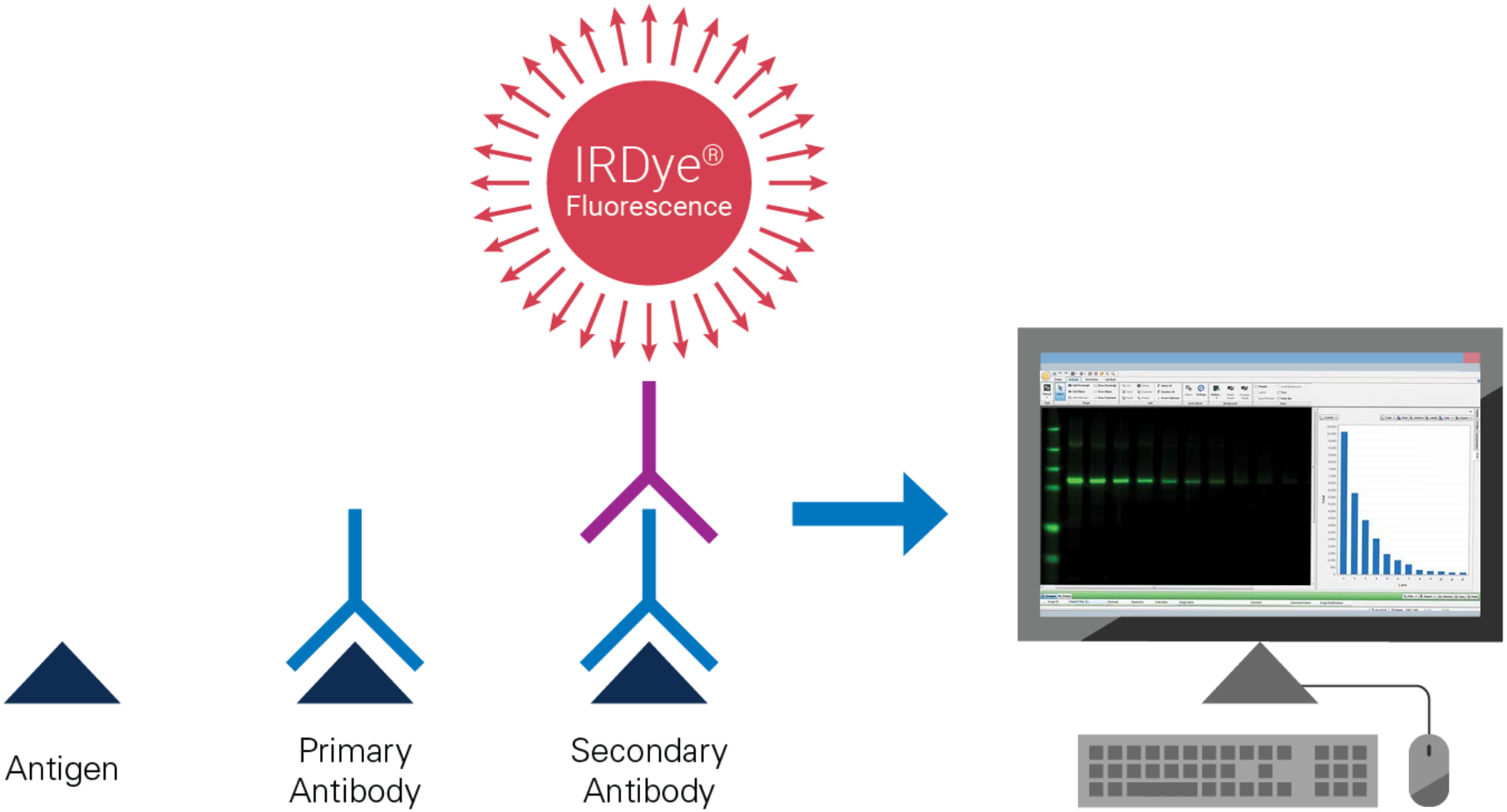
Chemiluminescent Film Detection



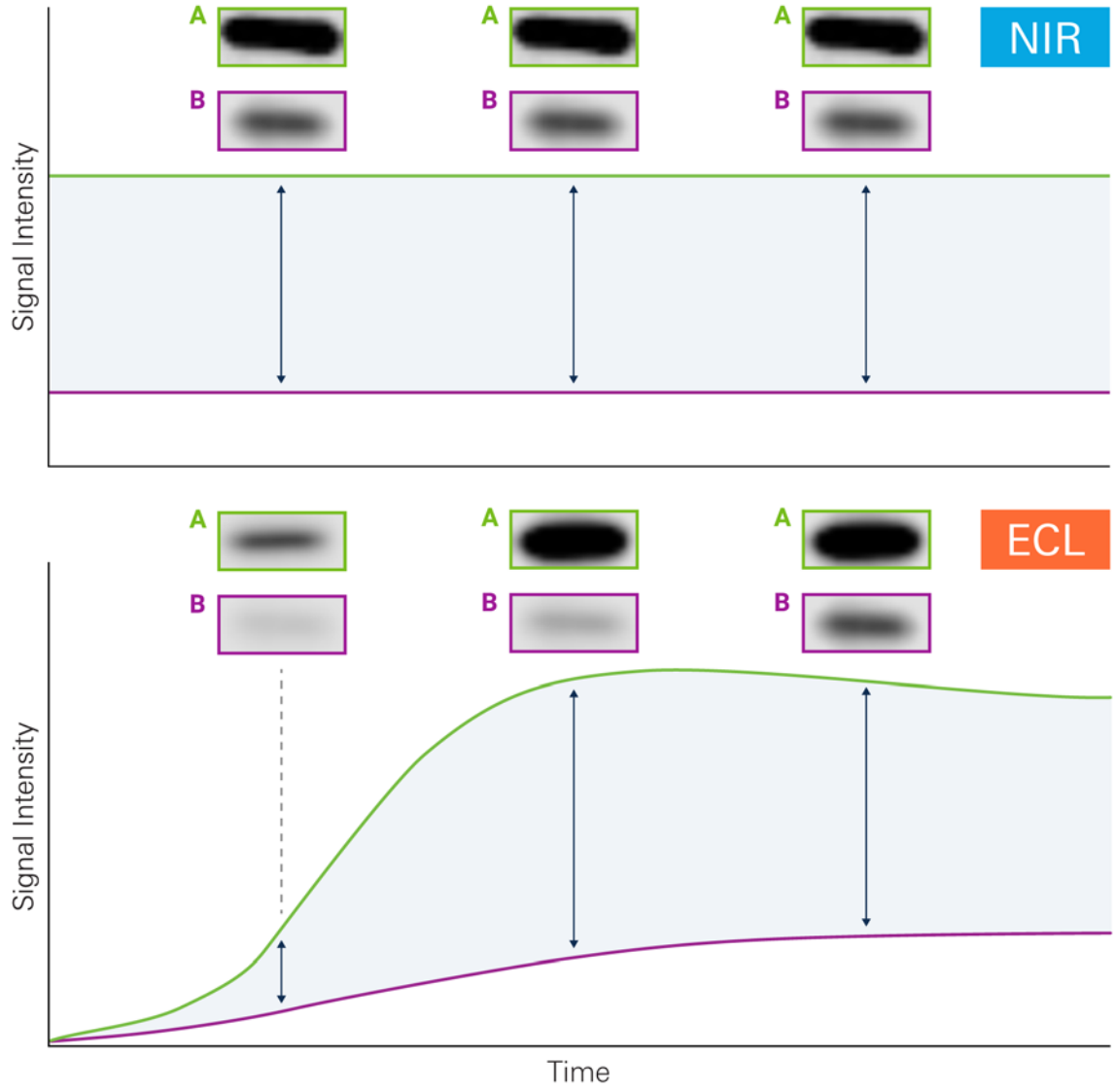
Issues with Chemiluminescence

- ✗ Enzymatic reaction
- ✗ Signal not directly proportional to antigen
- ✗ Multiple targets require stripping and reprobing

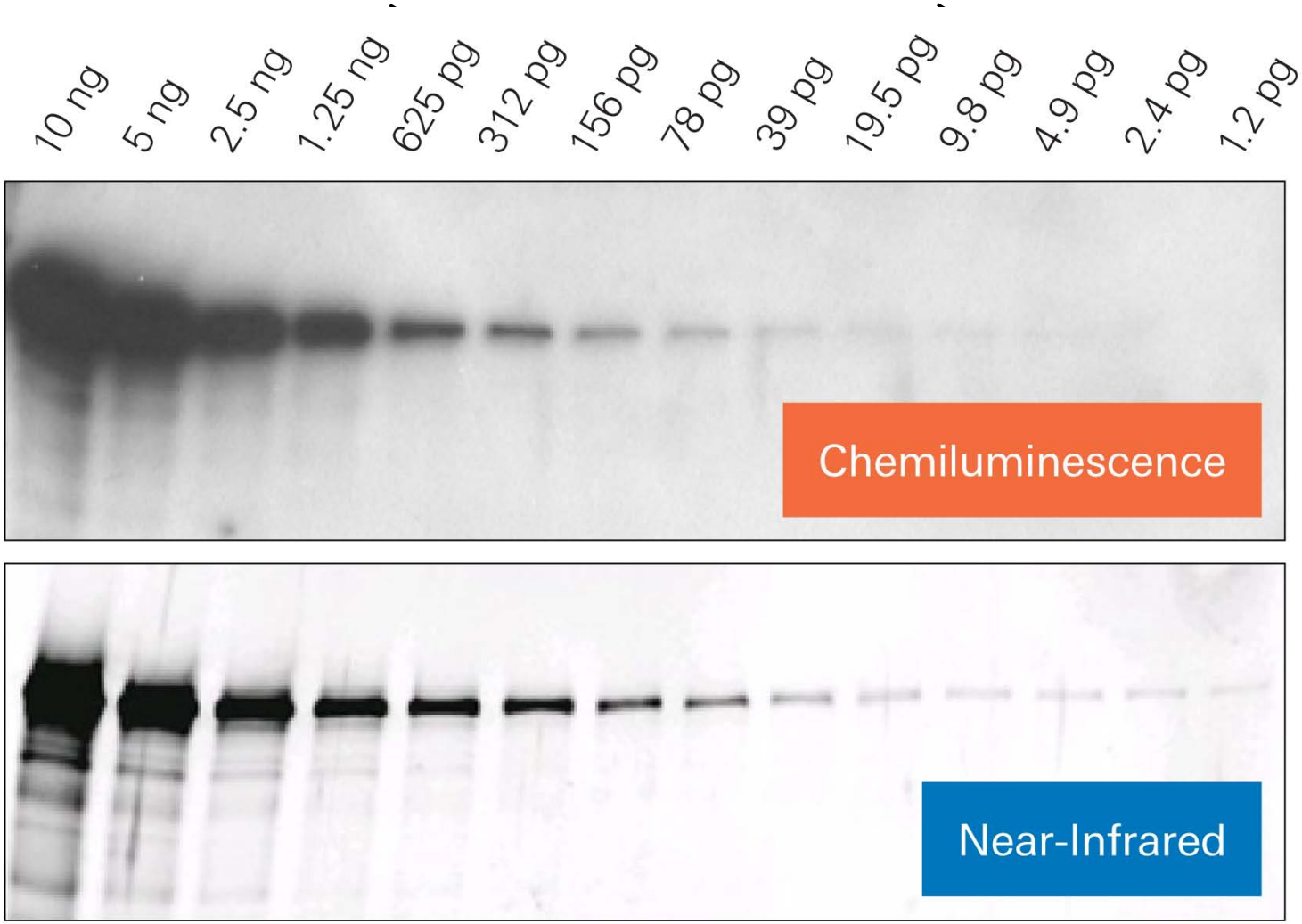
Near-Infrared Fluorescent Detection



Stable Signals



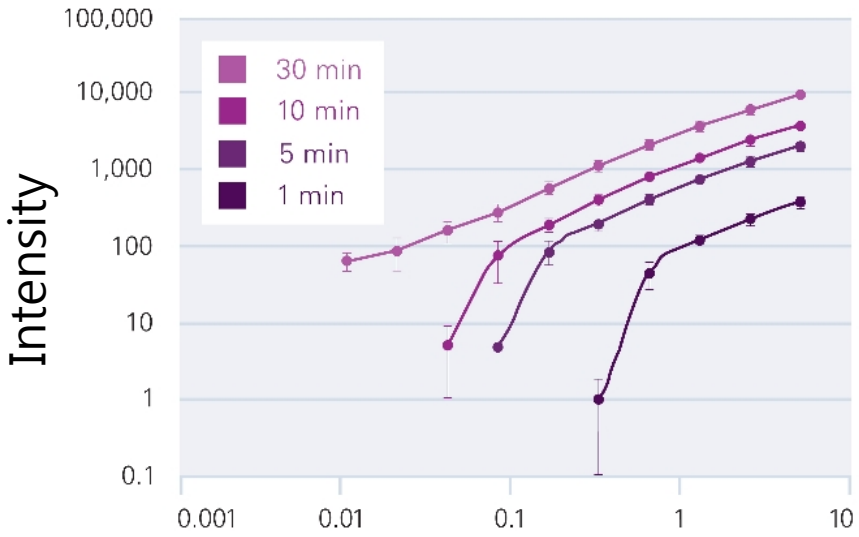
Sensitivity Without Saturation



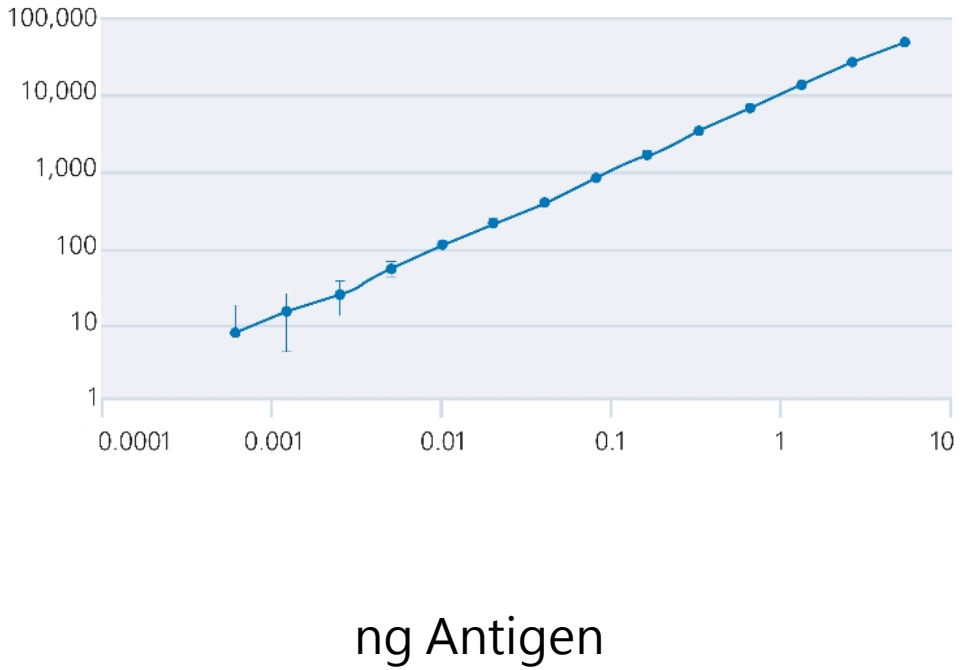
Serial dilutions of purified transferrin were detected on nitrocellulose with rabbit anti-Tf primary and IRDye® Secondary Antibody.

Increased Reproducibility

Chemiluminescence/CCD Detection

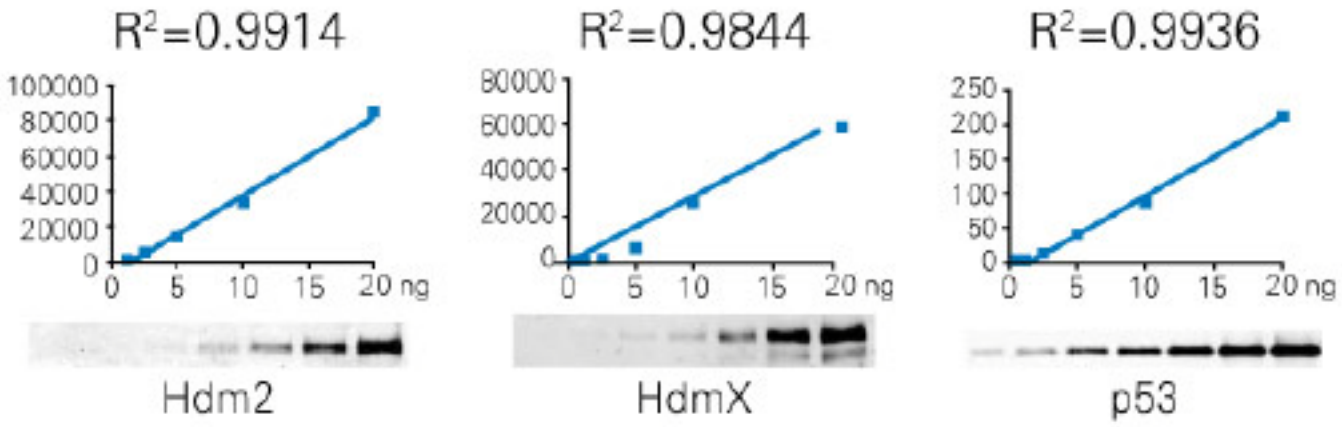


Odyssey® Near-Infrared Detection

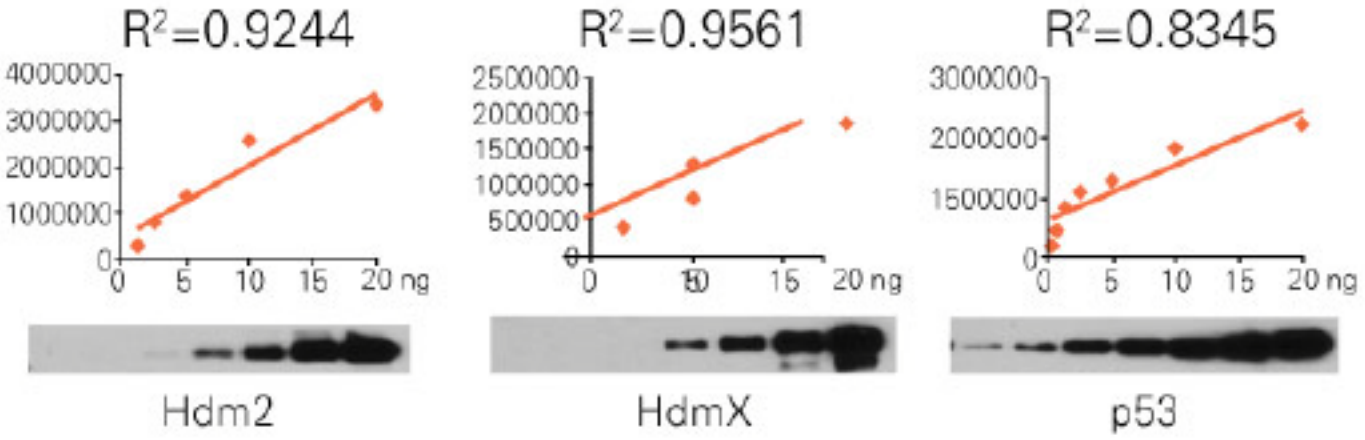


Increased Linearity

LI-COR

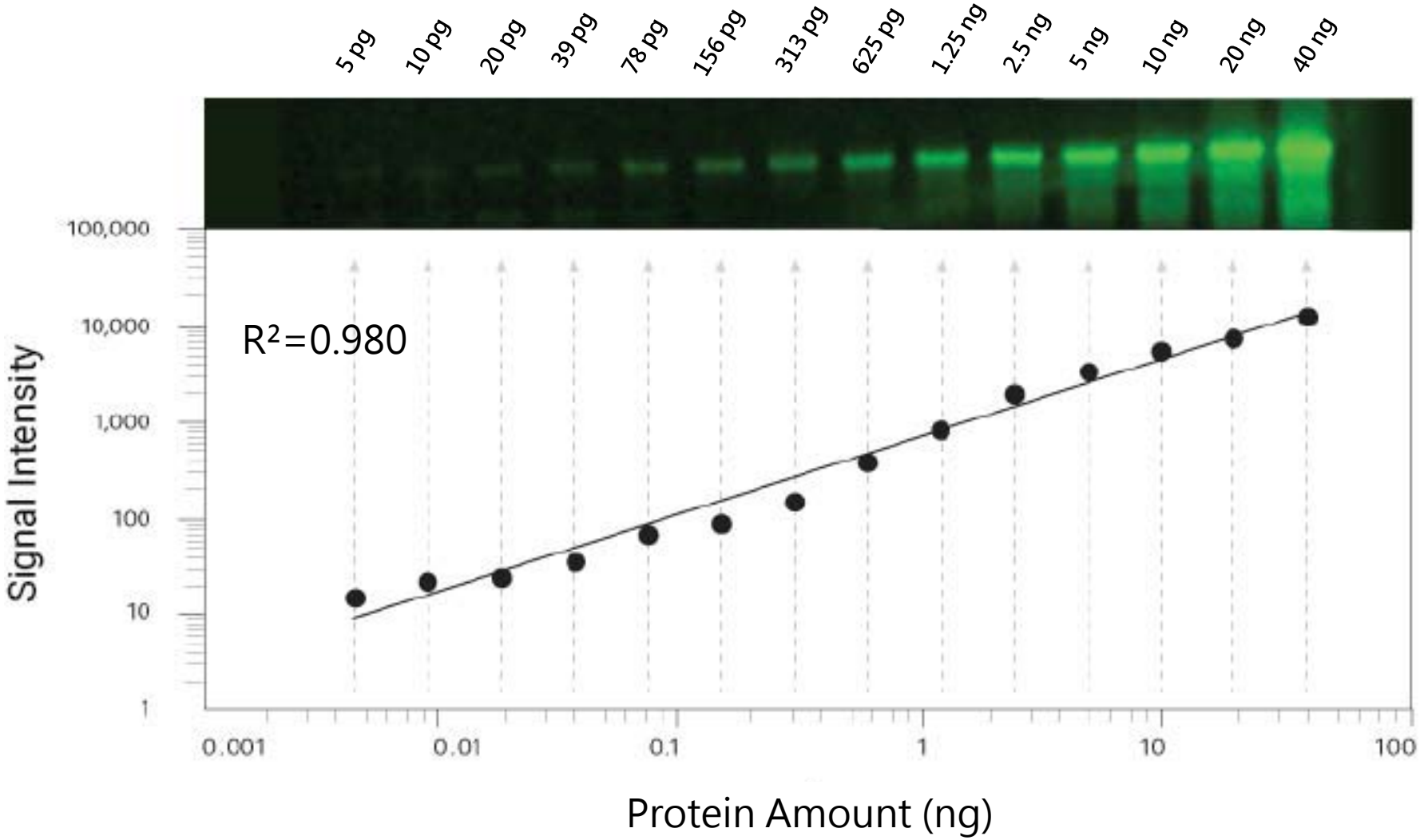


ECL

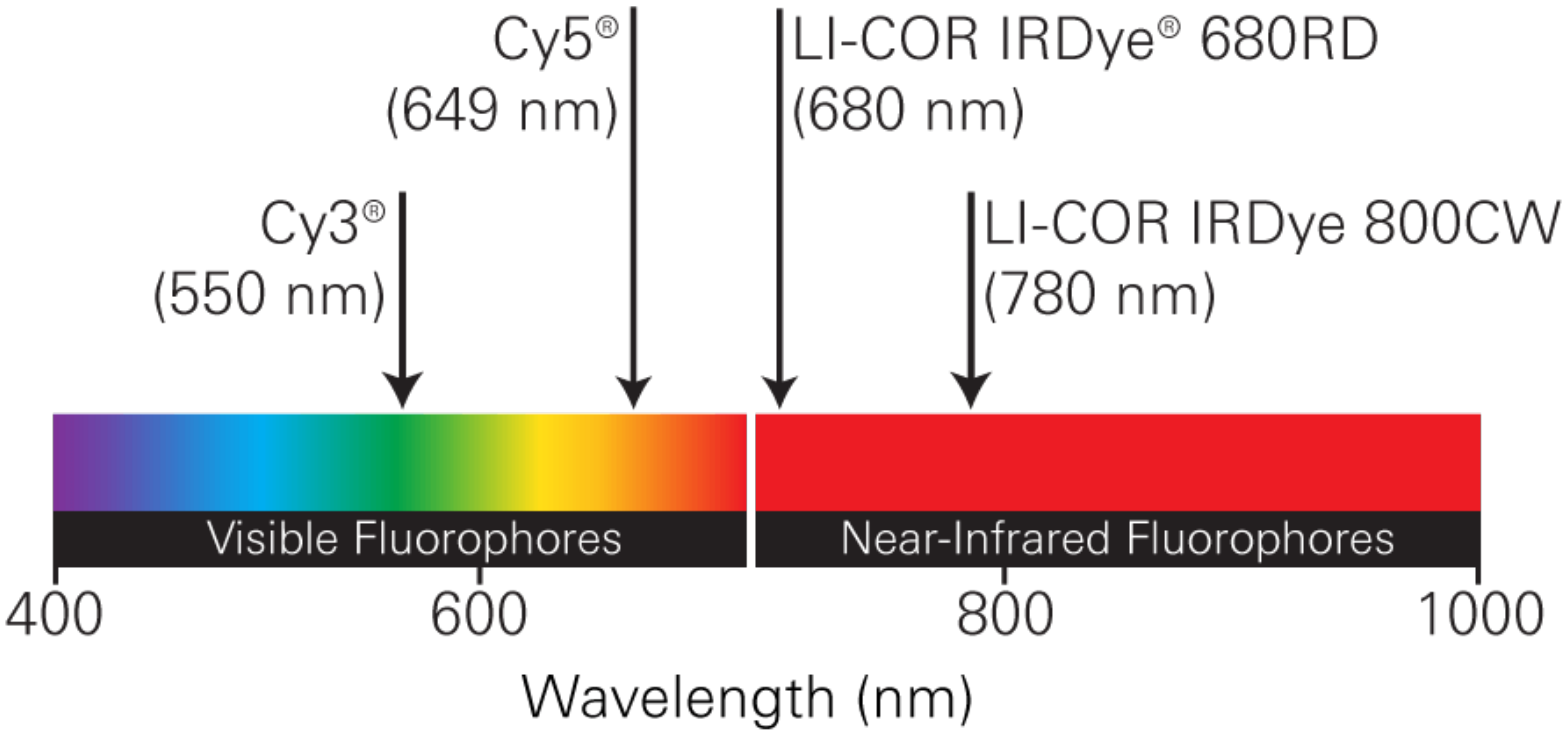


Wang, Y. V., Wade, M., Wong, E., Li, Y.-C., Rodewald, L. W., & Wahl, G. M. (2007). Quantitative analyses reveal the importance of regulated Hdmx degradation for P53 activation. PNAS , 12365-12370.

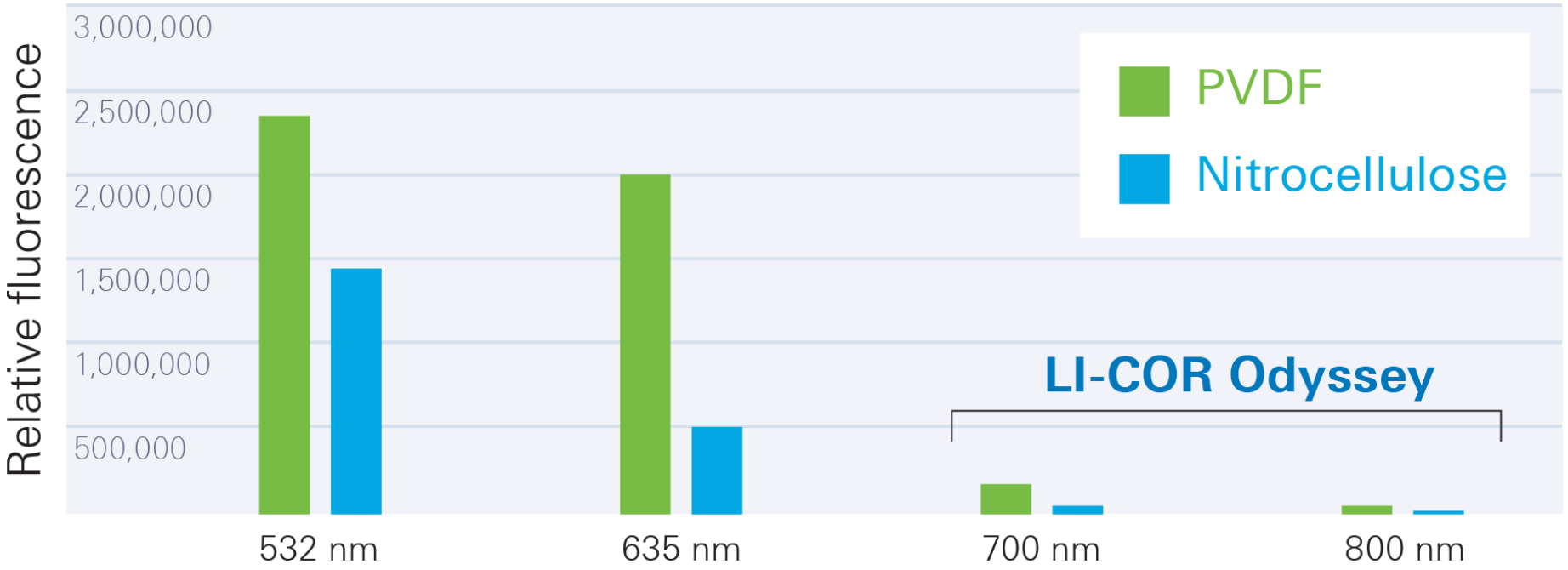
Quantitative Western Blots



Take Your Research Further



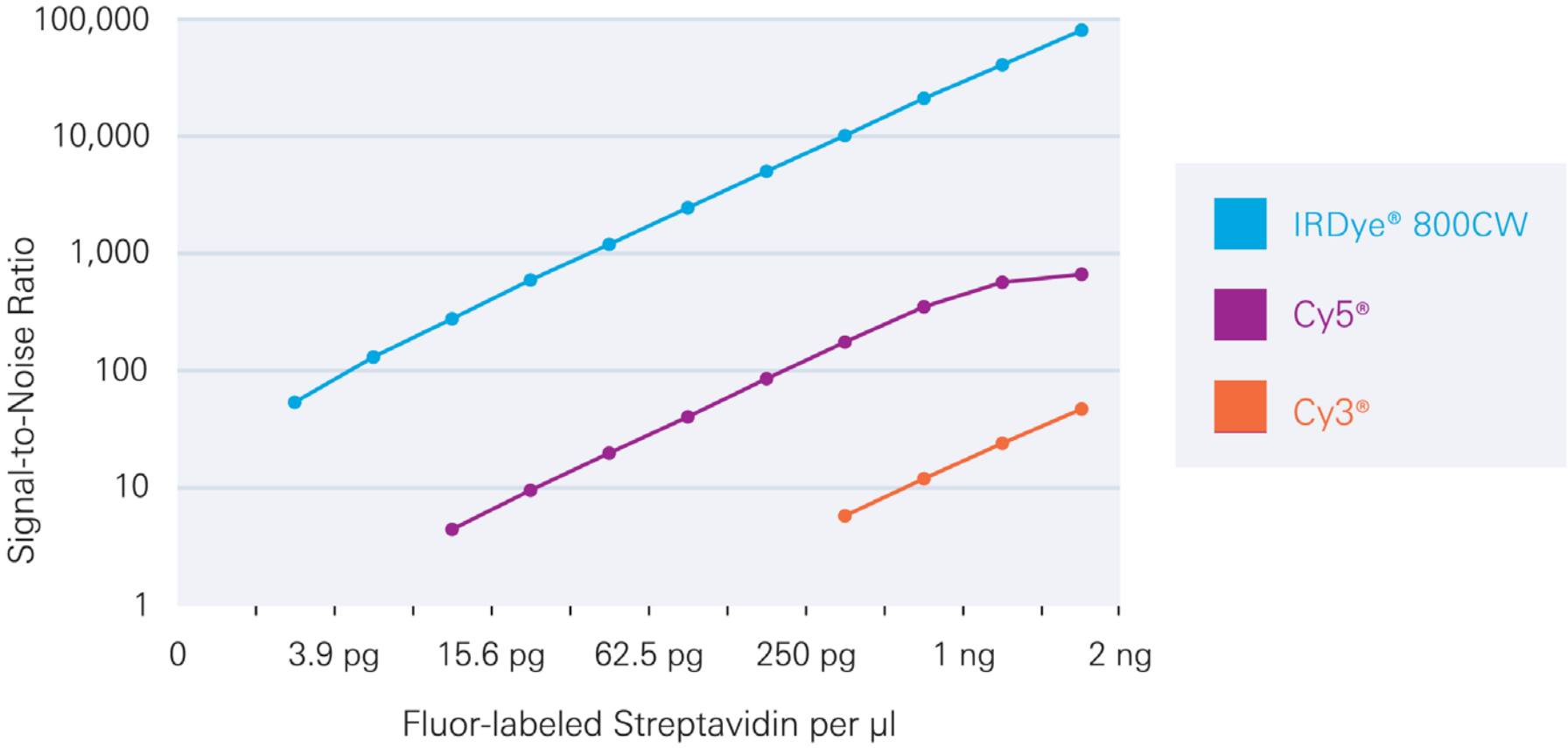
Low Membrane Background in NIR



Visible Fluorescence:
High background

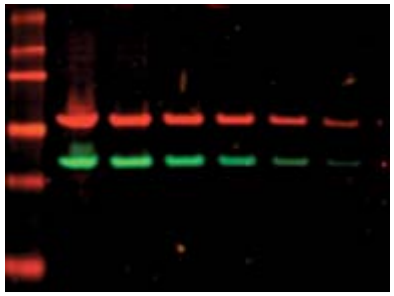
NIR Fluorescence:
Low background

High Signal-to-Noise Ratios

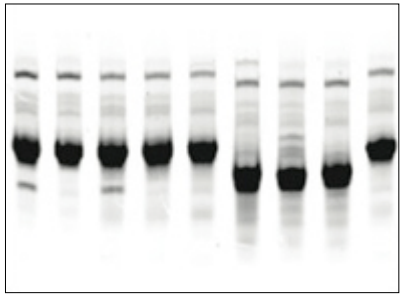


Odyssey® CLx Applications

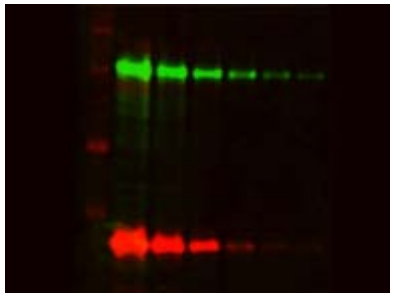
Multiplex Western Blot



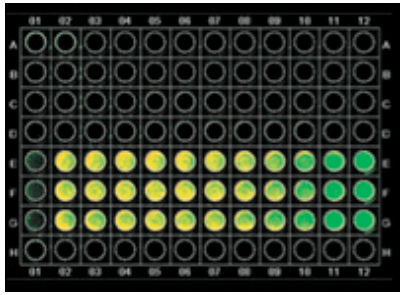
Protein Gel



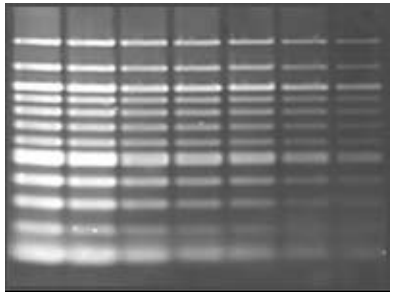
In-Gel Western



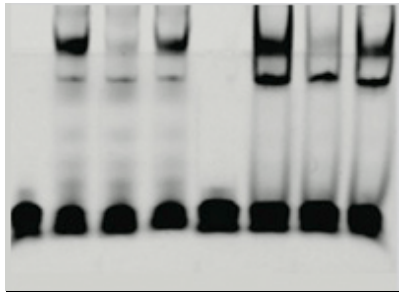
In-Cell Western™ Assay



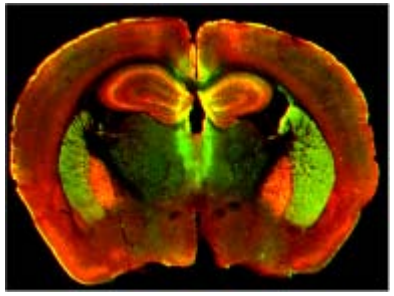
DNA Gel



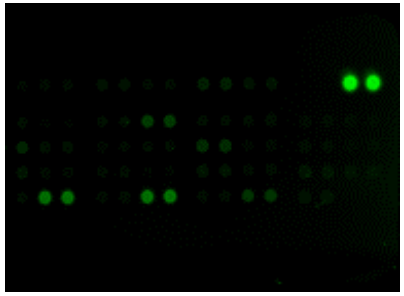
EMSA



Tissue Section



Protein Array

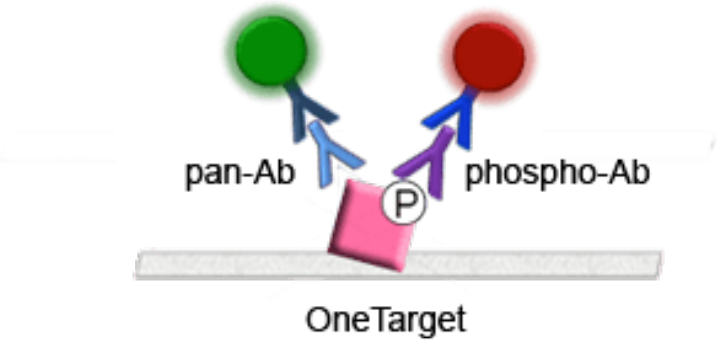


And many others…

Multiplex Western blot

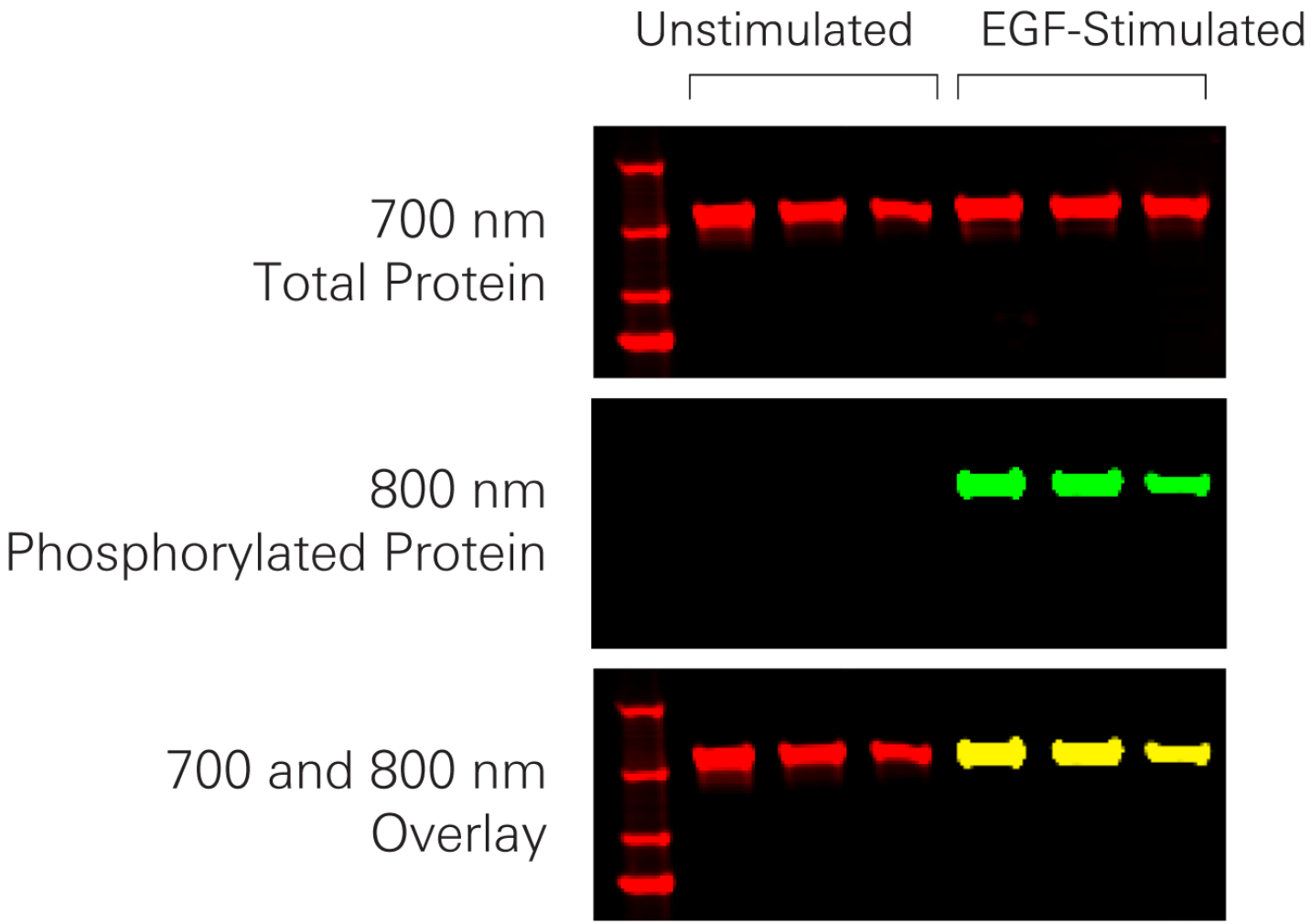
Multiplex Western blot approaches.

Two different targets can be detected



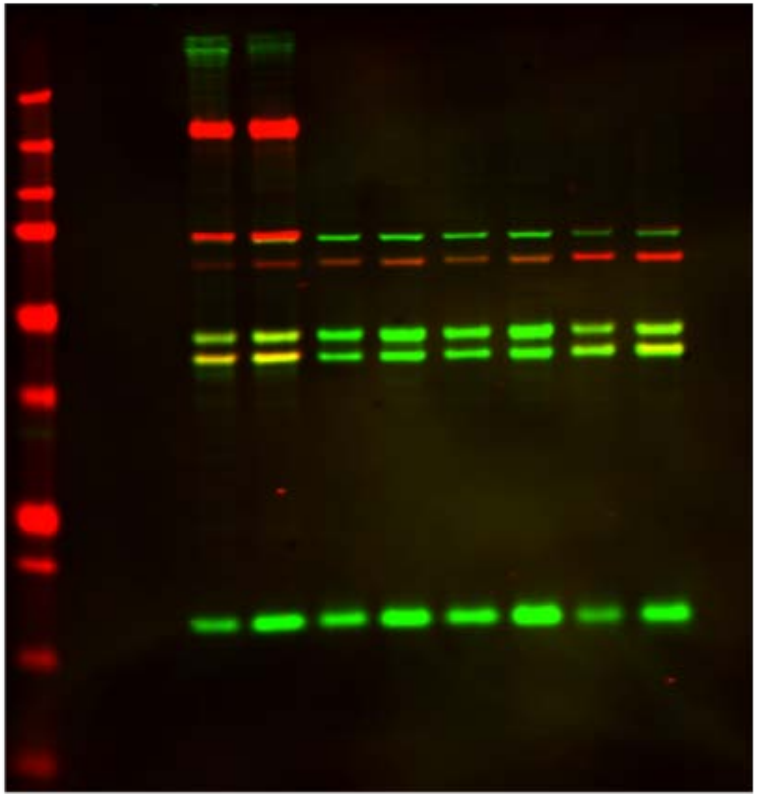
A phospho-antibody can be combined with an antibody against the unmodified target protein (pan-Ab) for multiplex phosphorylation analysis

Detect Multiple Targets



Anti-EGFR and anti-phospho-EGFR antibody specificity in A431 cells

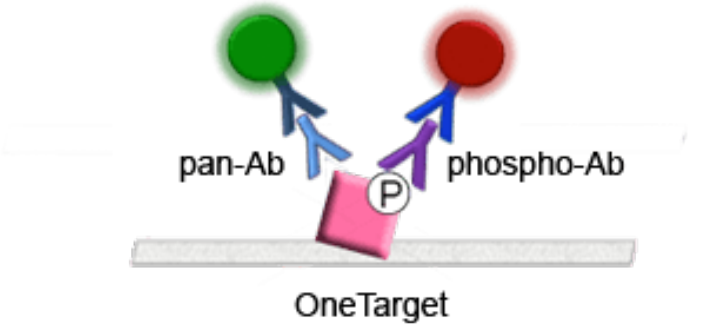
Detect Multiple Targets



pAKT=Green
AKT=Red

ERK=Green
pERK=Red

CoxIV=Green



Normalization Strategy

Quantitative Western Blot

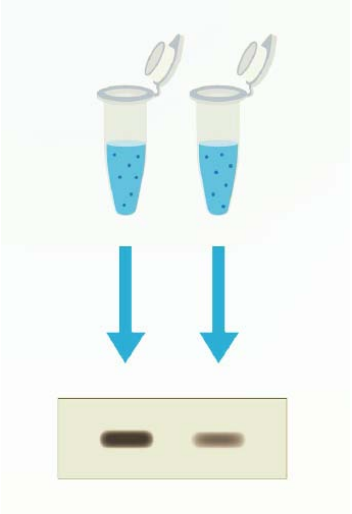
- Quantitative 定量 or Qualitative定性?

- 什麼是 Quantitative Western Blot?
  - 目的在量化蛋白質表現的差異程度

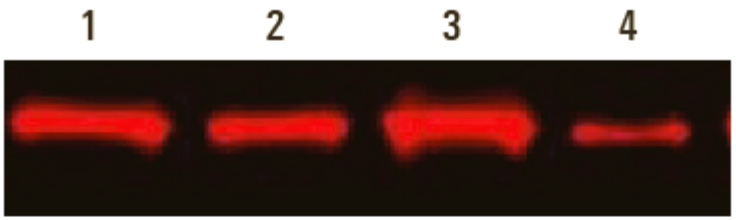
Quantitative Western Blot needs:

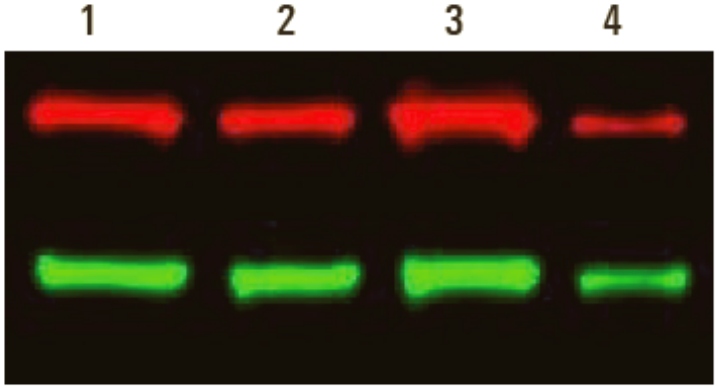
- ✓ **測得的**signal **與樣本的**loading amount 應成比例
- ✓ 應在訊號的**線性範圍內**作標的蛋白的定量
- ✓ 應使用Internal loading controls, 校正來自樣本製備, loading, 轉印等過程中無法避免變異

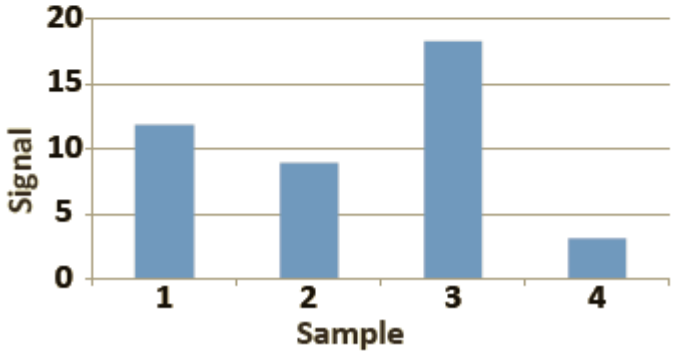
Normalization

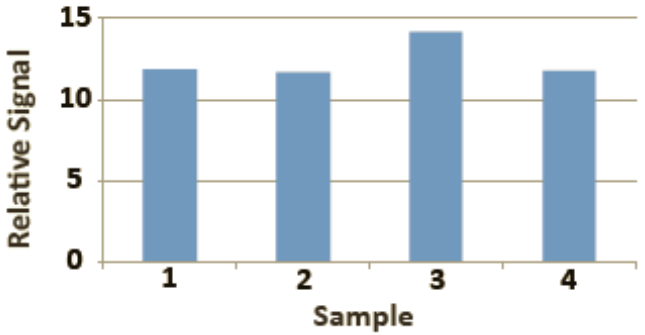


Normalization with Internal Loading Controls









Raw data

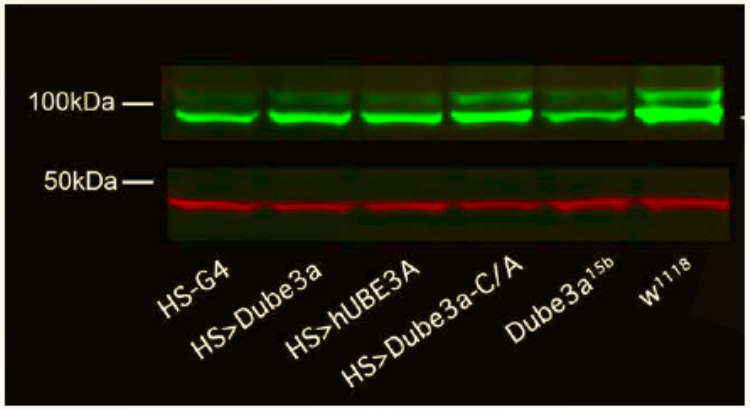
Normalized data

常用的 Internal loading controls

- Internal reference protein
  - *Housekeeping protein (HKP)*
- Pan/phospho analysis (PTM)
- Total protein staining

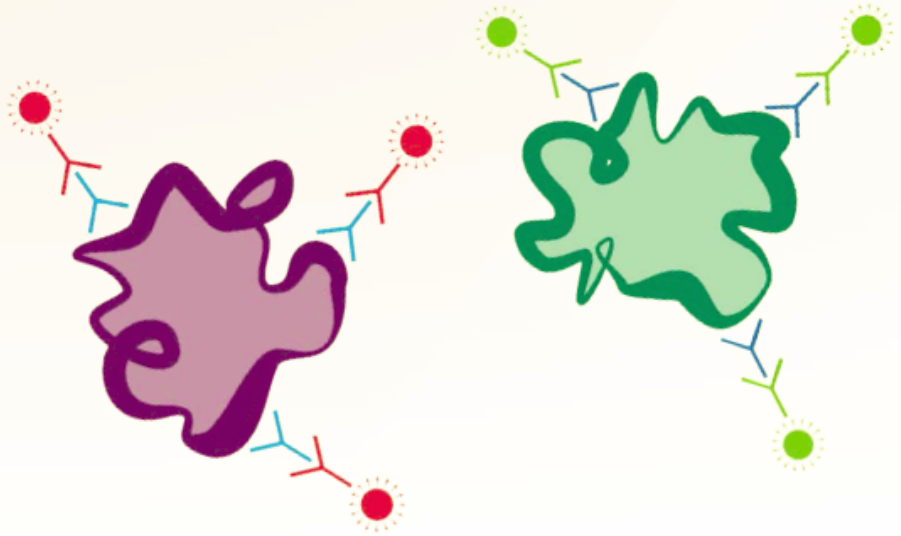
常用的 Internal loading controls

- **Internal reference protein**
  Housekeeping protein (HKP) : a second, unrelated protein found in all samples



Housekeeping protein

Jensen et al. (2013) *PLoS ONE* 8(4): e61952.

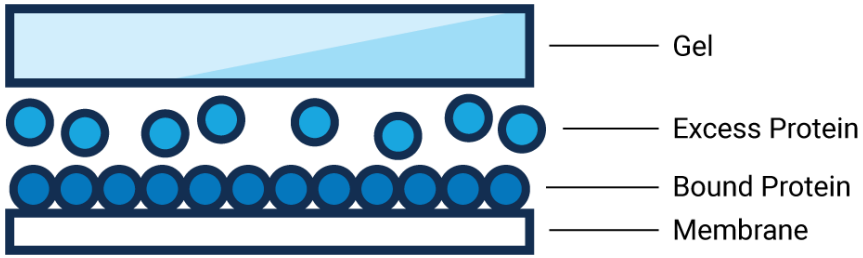


Housekeeping protein Target protein

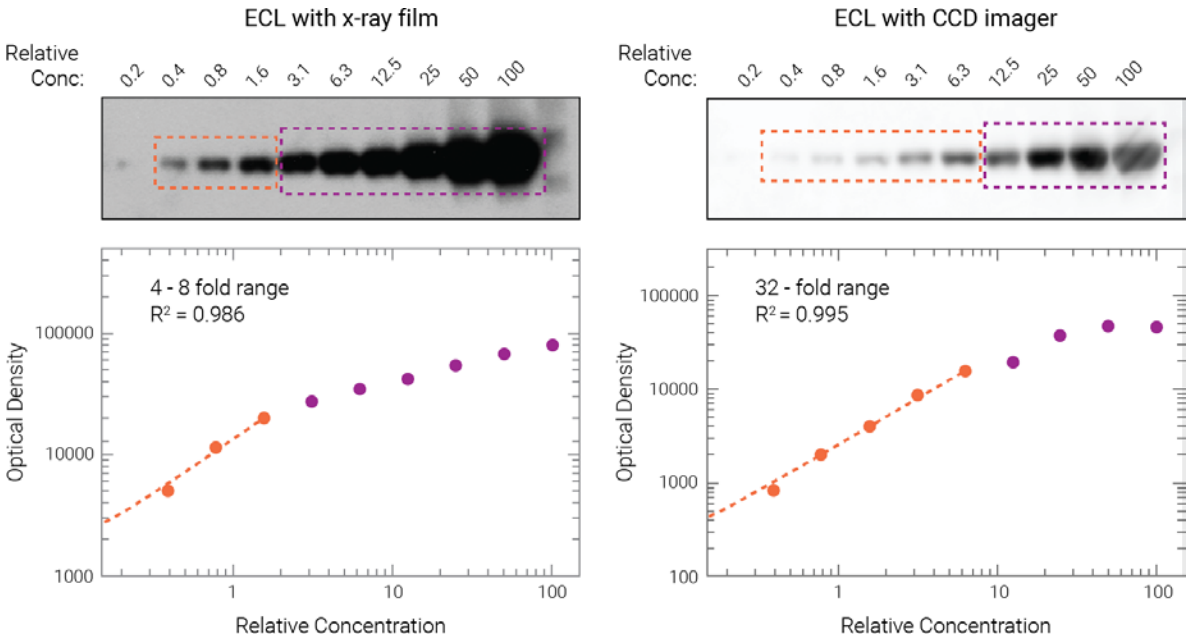
Housekeeping protein (HKP) 的潛在問題

表現量高 -> 訊號容易過飽和

1. Membrane Saturation

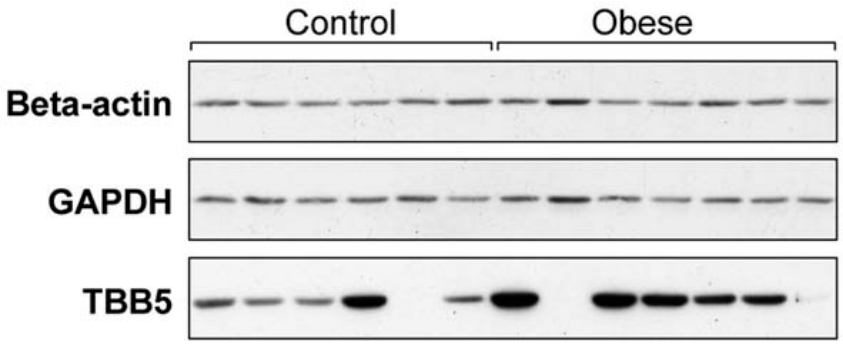


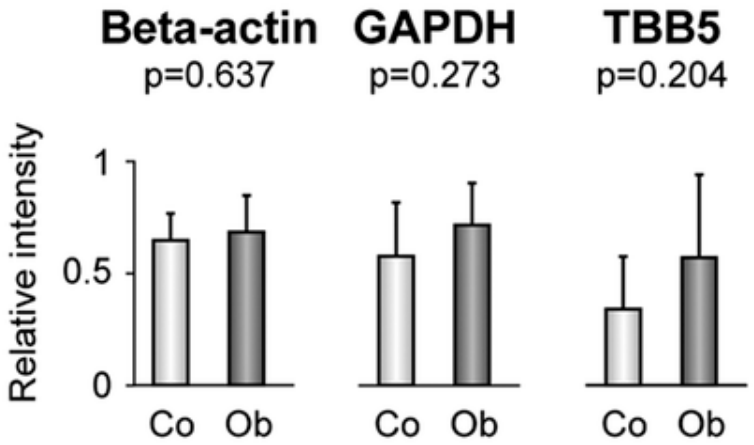
2. Detector Saturation



Housekeeping protein (HKP) 的潛在問題

表現量真的不變嗎?



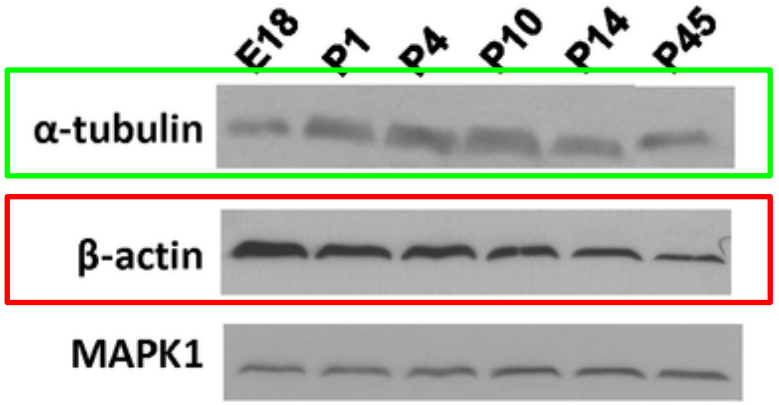


Variable expression of housekeeping proteins is observed in human adipose tissue from non-obese (control) and obese subjects.

Pérez-Pérez R, López JA, García-Santos E, Camafeita E, Gómez-Serrano M, et al. (2012) **PLOS ONE** 7(1): e30326.

Housekeeping protein (HKP) 的潛在問題

表現量真的不變嗎?



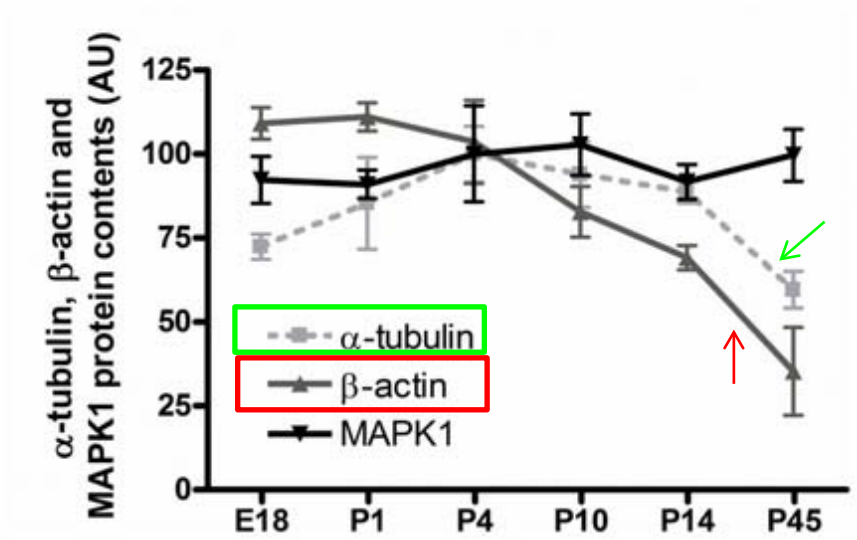
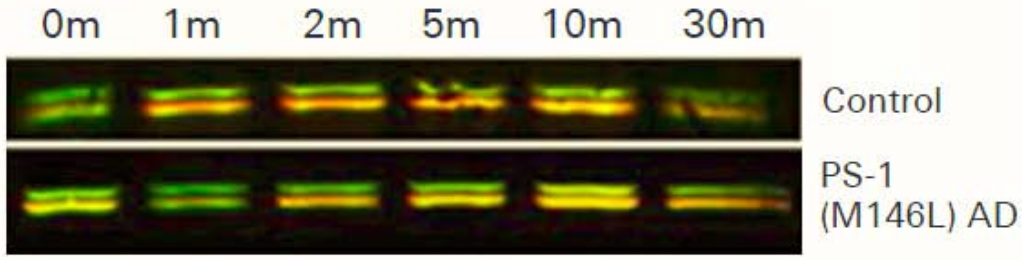


Figure 1. Validation of loading controls for Western blot throughout retinal development.

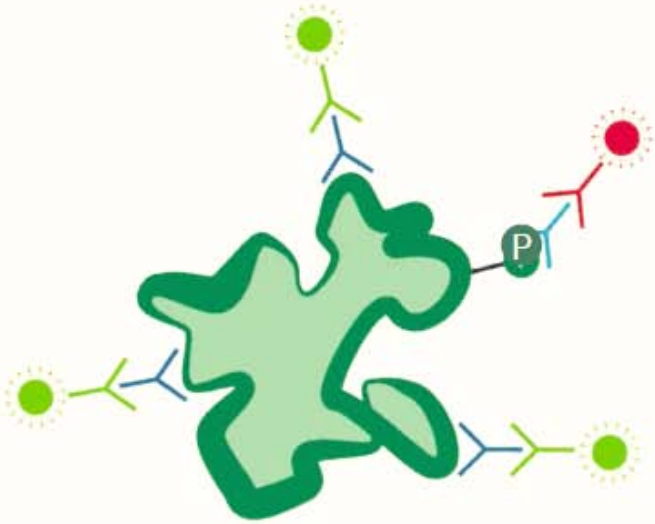
Rocha-Martins M, Njaine B, Silveira MS (2012) Avoiding Pitfalls of Internal Controls: Validation of Reference Genes for Analysis by qRT-PCR and Western Blot throughout Rat Retinal Development. PLOS ONE 7(8): e43028.

常用的 Internal loading controls

- **Pan-phospho analysis (PTM)**
  標的蛋白總量/磷酸化標的蛋白



Multiplex phosphorylation analysis
Mendonsa et al. (2009) *PLoS ONE* 4(2):e4655.



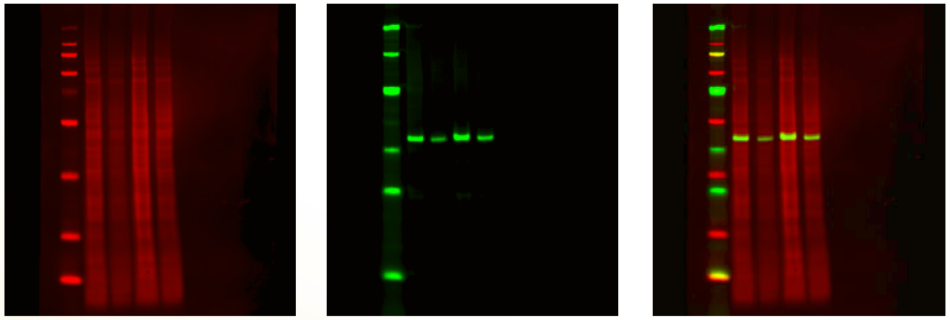
Target protein

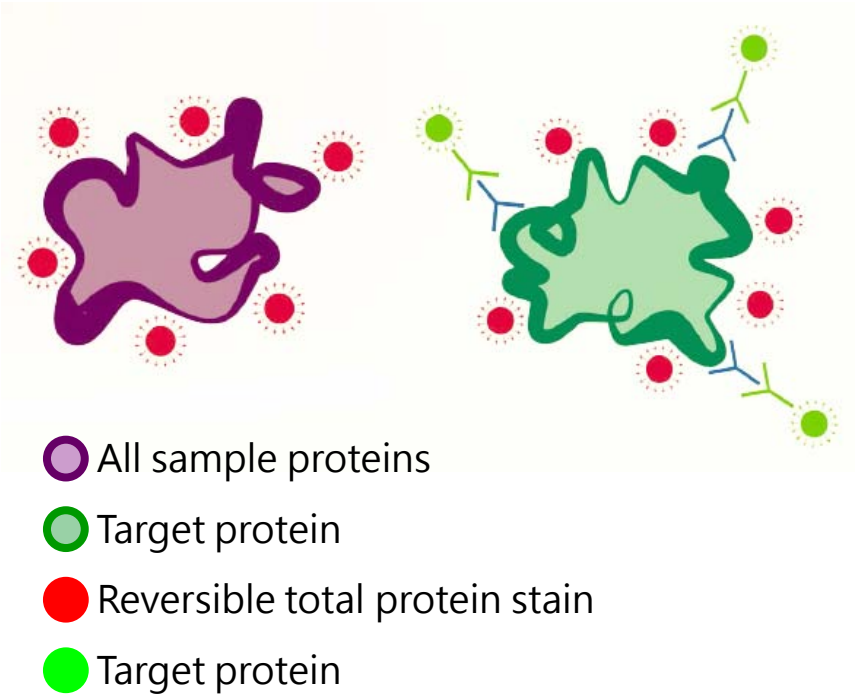
P Phospho-epitope

常用的 Internal loading controls

- **Total Protein Stain**

  轉印後, 染在 membrane上的所有蛋白
  量化每一條lane上的蛋白總量

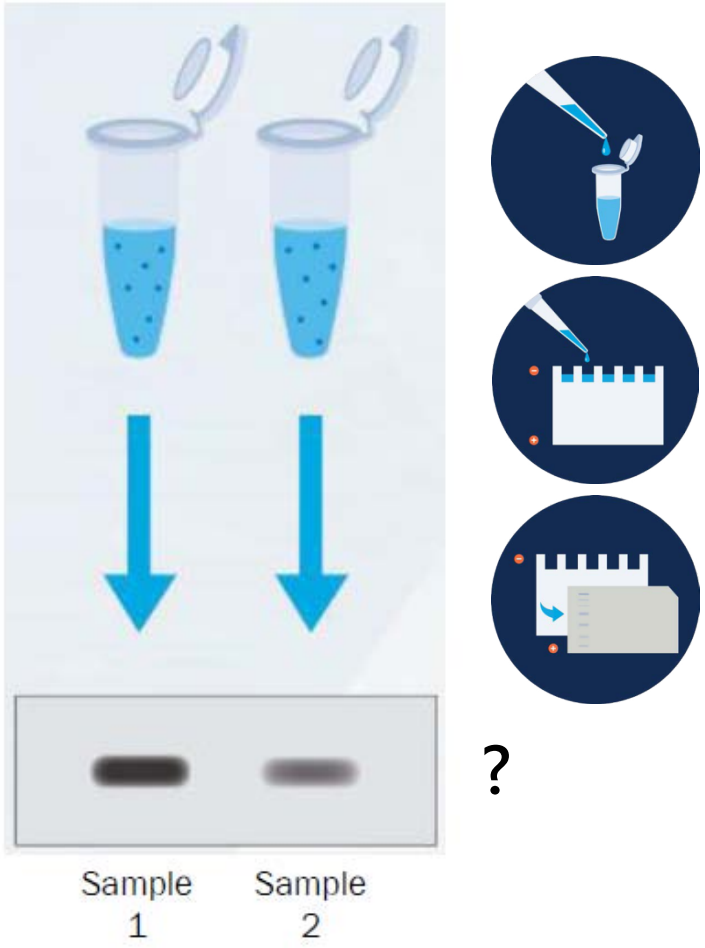


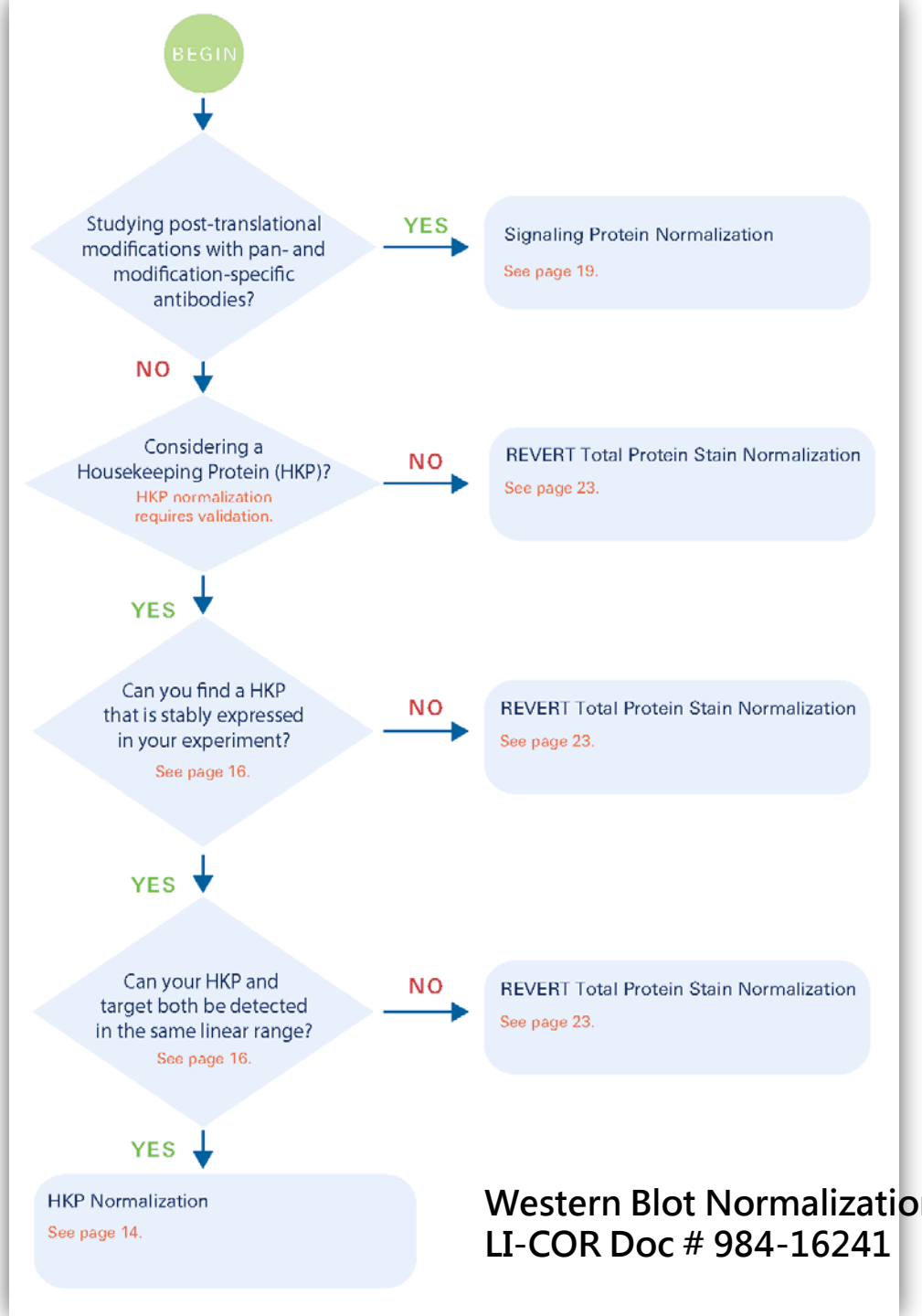


Normalization 是…

Two samples, equal concentration

> Normalization is a Strategy –
> Not Just a Step in the Protocol



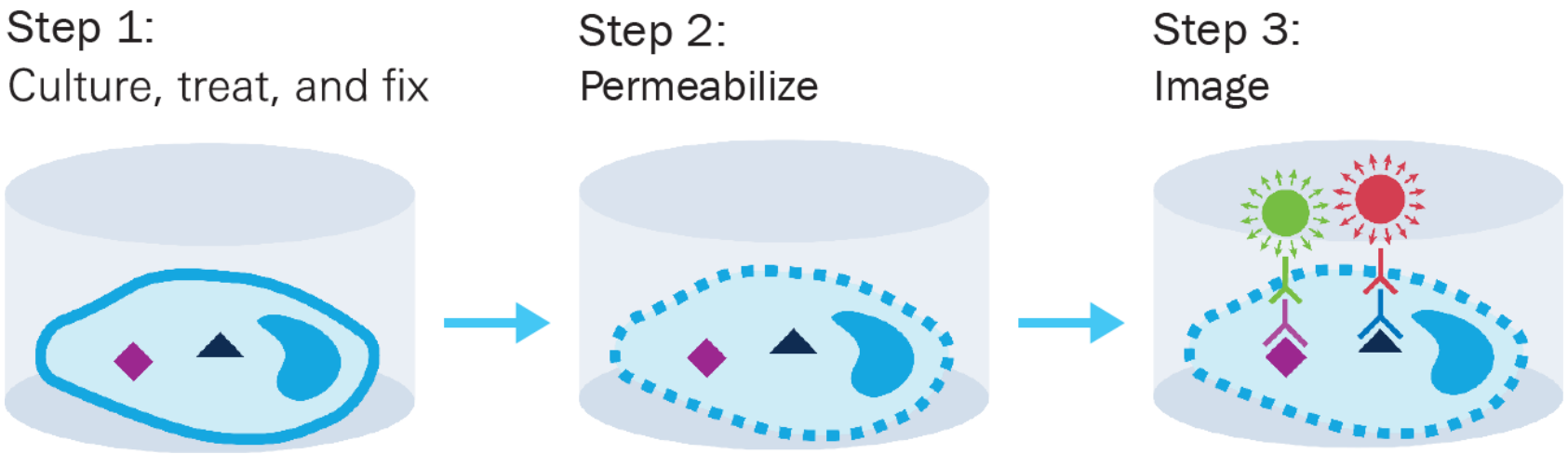


Western Blot Normalization Handbook
LI-COR Doc # 984-16241

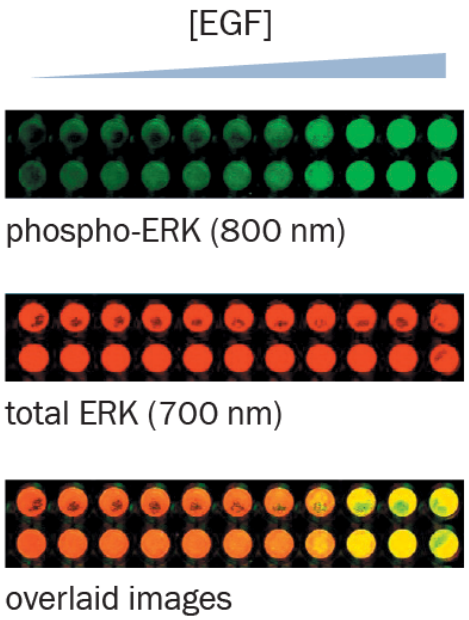
In-Cell Western™ Assay

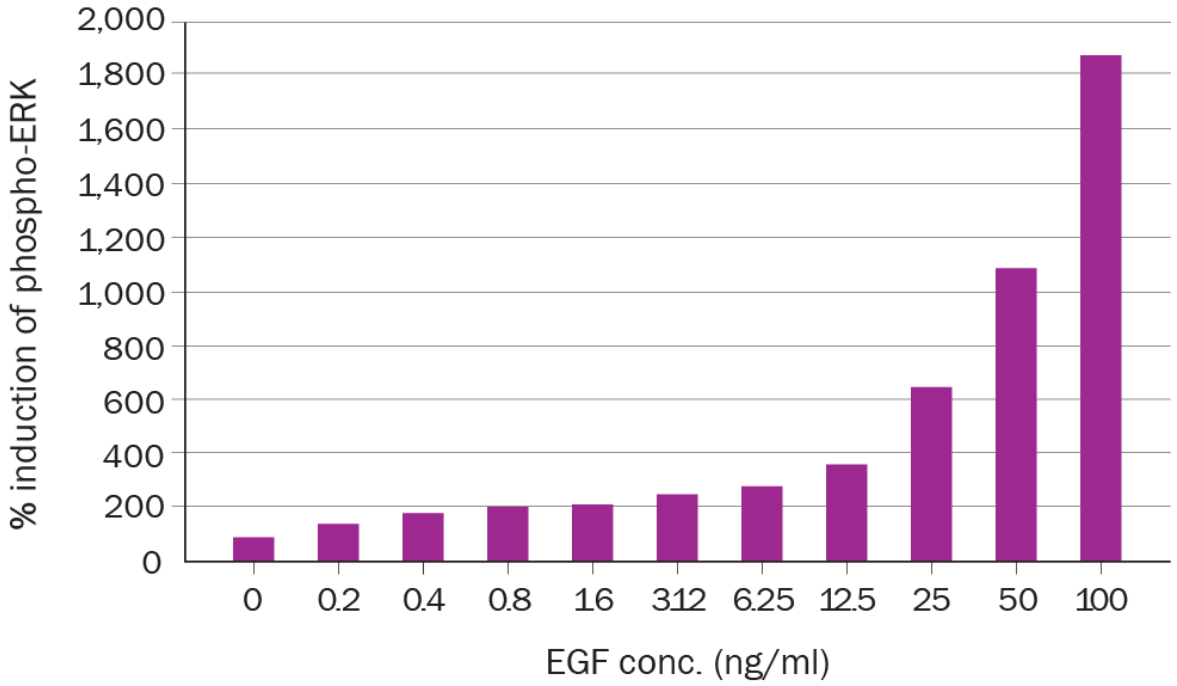
In-Cell Western™ Assay

- A high-throughput, quantitative immunofluorescence assay performed in microplates in any well format



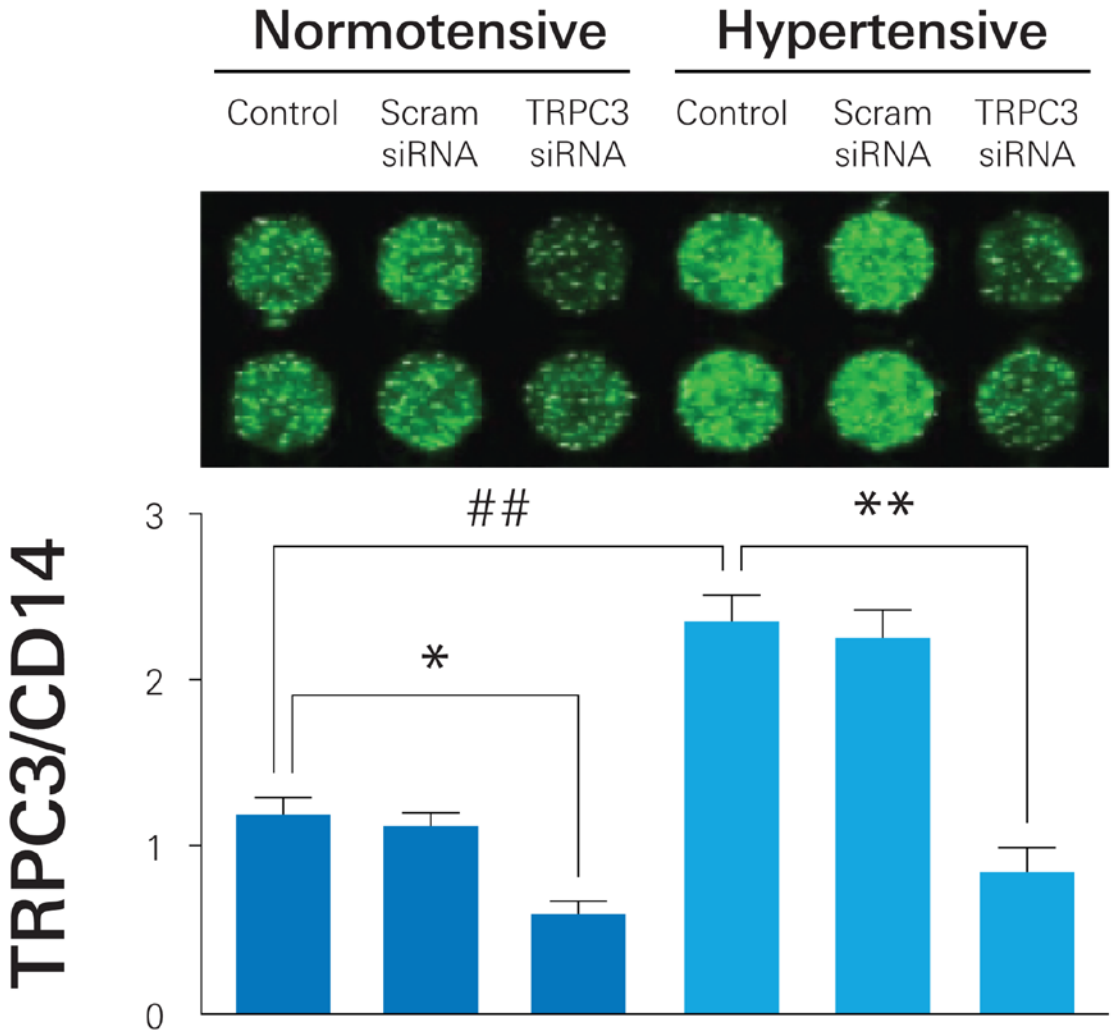
ICW Signaling Pathways





Chen H, Kovar J, Sissons S, Cox K, Matter W, Chadwell F, et al. (2005). A Cell Based Immunocytochemical Assay For Monitoring Kinase Signaling Pathways And Drug Efficacy. *Anal. Biochem.*, 136-142.

ICW siRNA Knockdown



Zhao Z et al. (2012) Increased Migration of Monocytes in Essential Hypertension Is Associated with Increased Transient Receptor Potential Channel Canonical Type 3 Channels. *PLoS ONE.* 7(3): e32628.

Applications for LI-COR Products

AFLP	Microsatellites	Reverse Phase (Lysate) Array
Biodistribution*	Microscopy	Small Animal Imaging*
Chemiluminescent Western Blot	Microwestern Array	Structural Imaging*
Coomassie Protein Gel Staining	Nucleic Acid Gel Documentation	TILLING
DNA Sequencing	On-Cell Western Assay	Tissue Section Imaging
Ecotilling	Protease Assay	Transcription Factor Assay
ELISA	Protein Array	Transporter Targeting*
EMSA/Gel Shift Assay	Protein Gel Documentation	Vascular/Lymphatic Imaging*
Glycoprotein Detection	Quantitative Western Blot	Western Blot
In-Cell Western™ Assay	Receptor Targeting*	
In-Gel Western	Reporter Gene Assay	
In Vivo Imaging	RNAi Analysis	

Odyssey CLx | Odyssey Fc | Odyssey Sa

C-DiGit Scanner | Pearl Impulse | 4300 | IRDye® Reagents

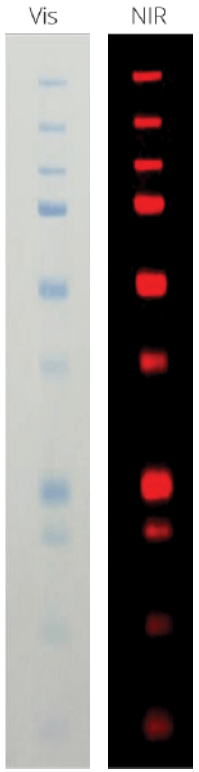
Western Blotting Hints/Tips

Good Westerns Gone Bad: Tips to Make Your NIR Western Blot Great

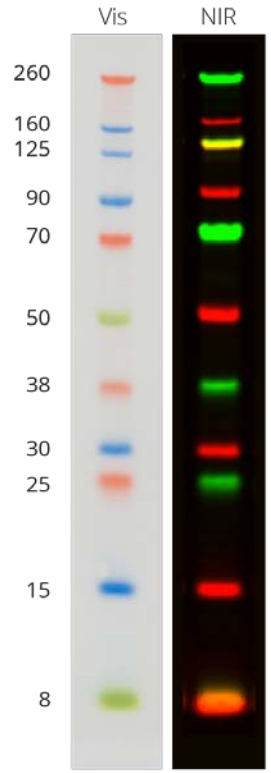
SDS-PAGE

SDS-PAGE

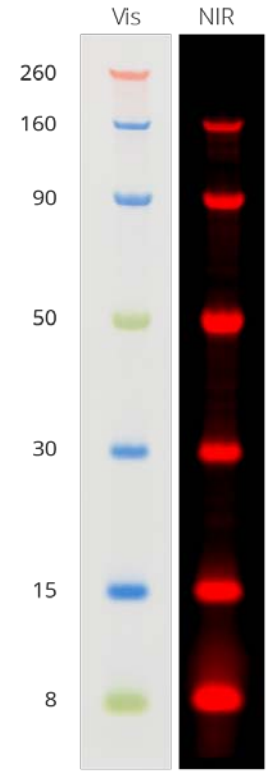
- Protein MW Standards
  - 1/3 to 1/4 amount for current MW standard



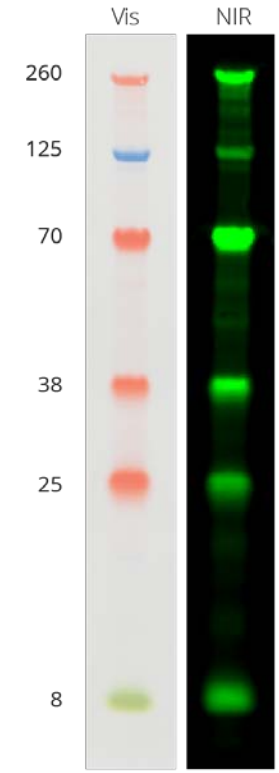
Odyssey Protein MW Marker
LI-COR P/N 928-40000



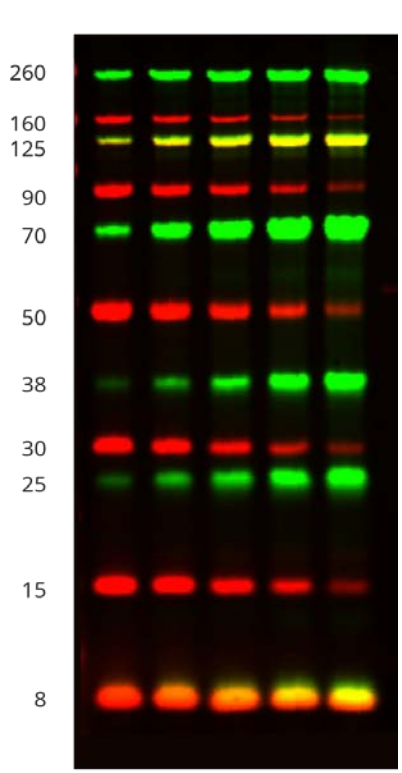
Chameleon™ Duo
LI-COR P/N 928-60000



Chameleon 700
LI-COR P/N 928-70000



Chameleon 800
LI-COR P/N 928-80000



Chameleon Kit
LI-COR P/N 928-90000

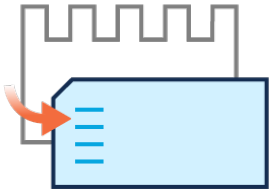
SDS-PAGE

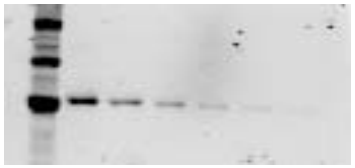
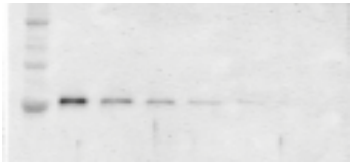
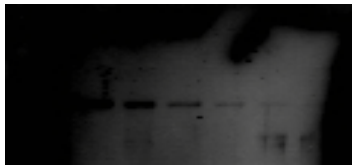
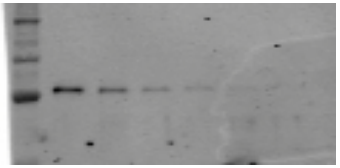
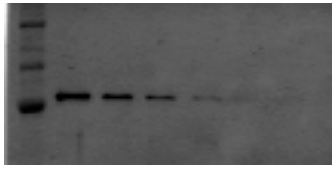
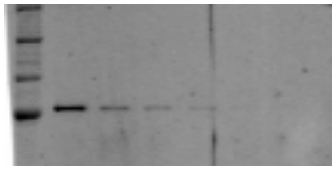
- Sample loading buffer
  - Run dye front off or cut off
  - LI-COR 4X Protein Sample Loading Buffer

P/N 928-40004

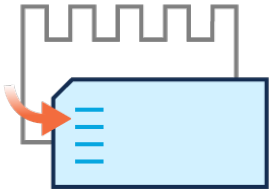
Transfer

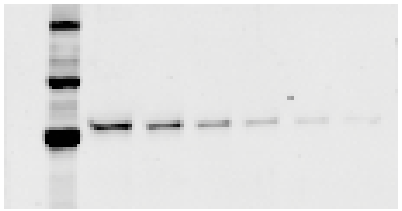
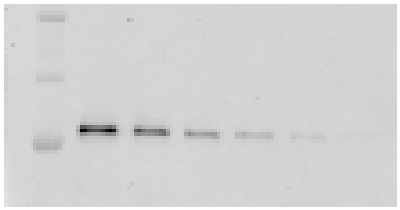
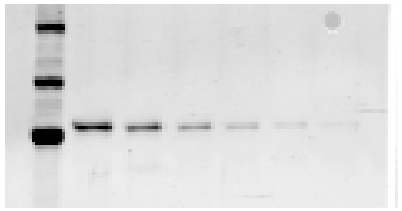
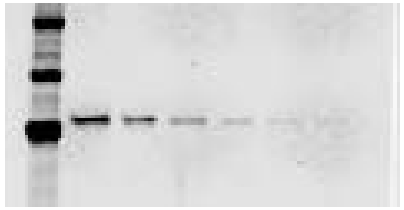
PVDF



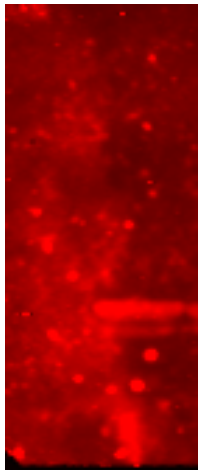
	700 nm Channel	800 nm Channel
Millipore Immobilon™ FL		
Millipore Immobilon™ P		
Bio-Rad Immun-Blot®		
Pall BioTrace® PVDF		
Perkin Elmer PolyScreen®		
Amersham Hybond™-P		

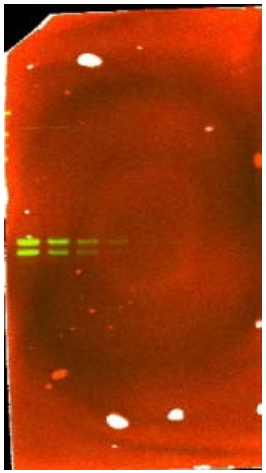
Nitrocellulose

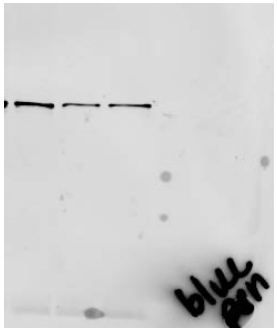


	700 nm Channel	800 nm Channel
Osmonics® NitroBind		
Bio-Rad unsupported		
Odyssey Nitrocellulose		

Transfer

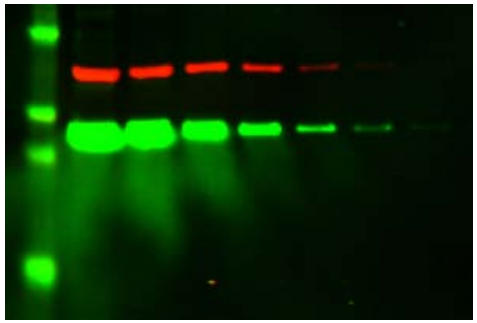






Pro-Tip:

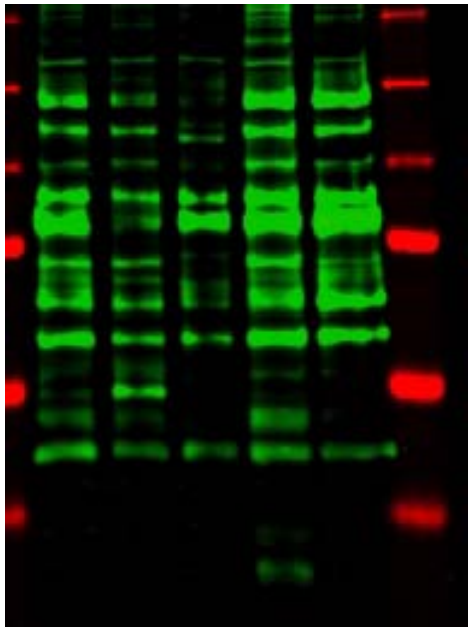
- Remove gel fragments
- Dry membrane (great for pausing!)
- Avoid writing on membranes
- Clean incubation boxes with methanol
- Normalize with REVERT™ Total Protein Stain



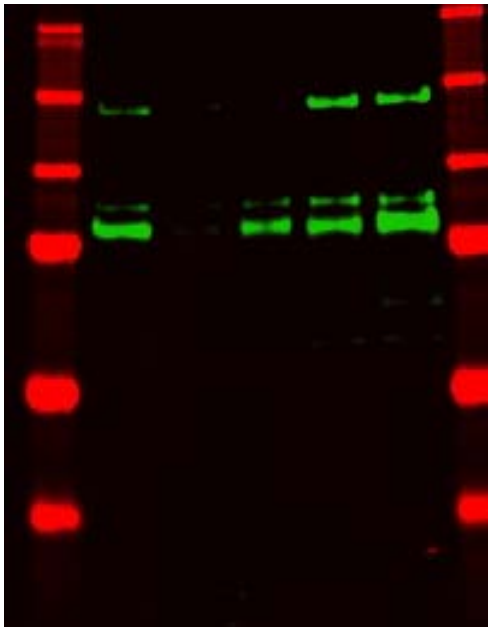
Blocking

Blocker Selection

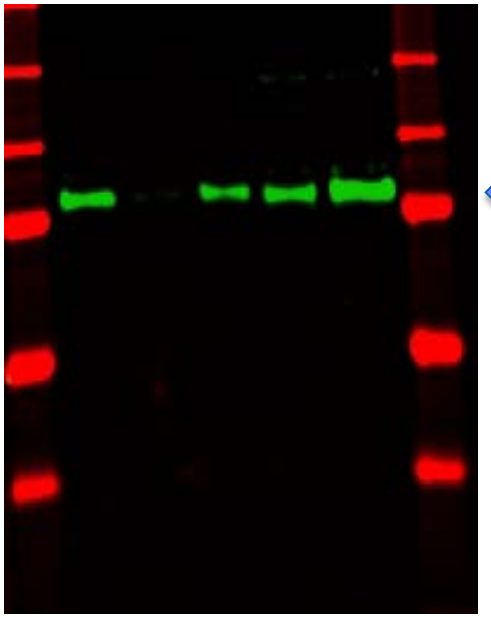
5% BSA Blocker



5% Milk Blocker

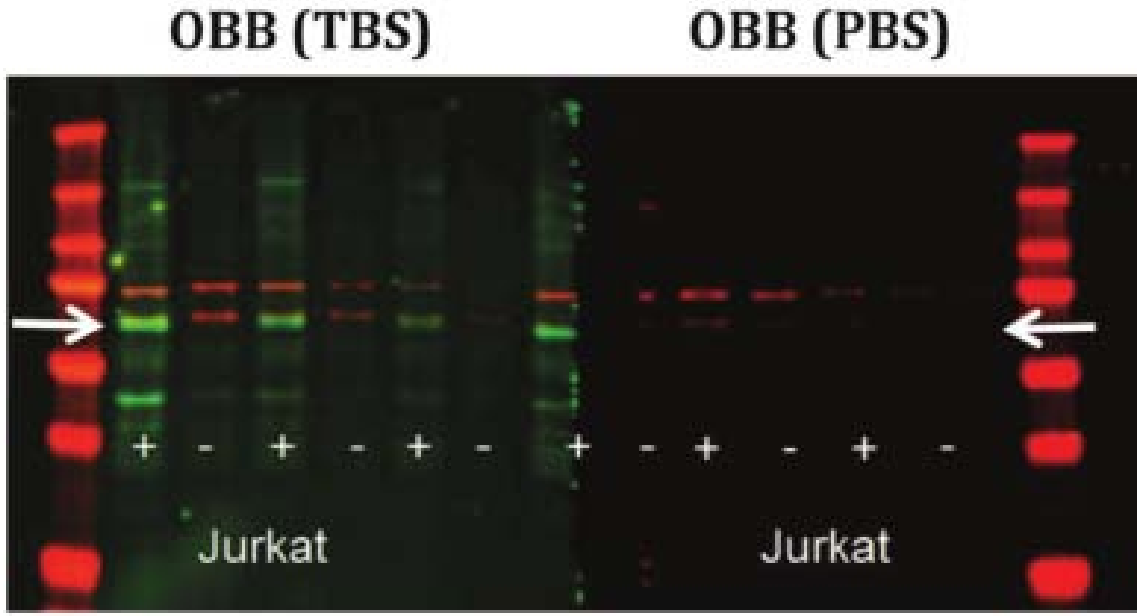


Odyssey Blocking Buffer



PKC-α

Blocker Selection

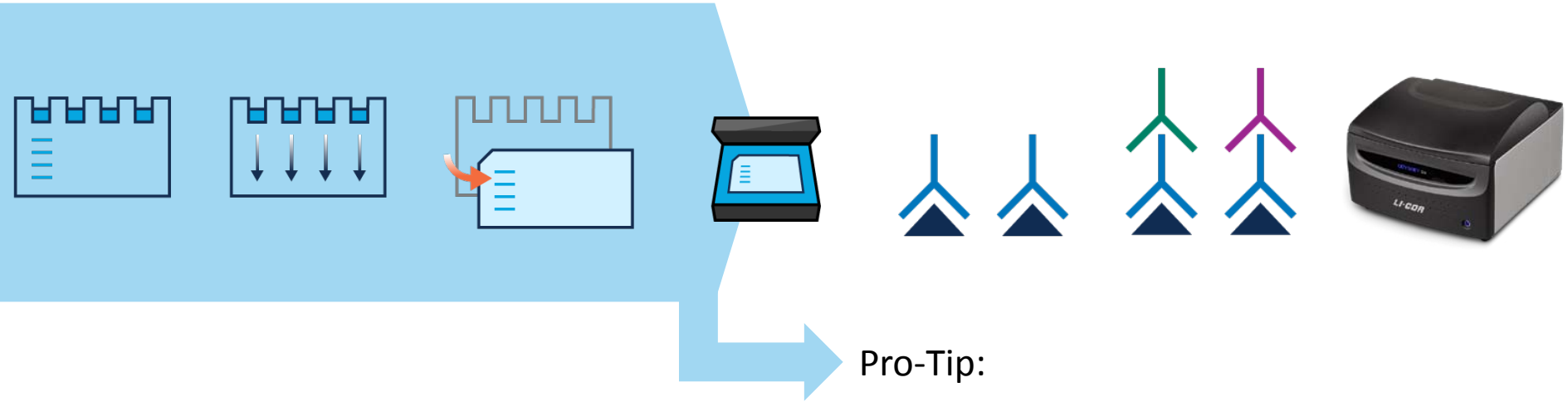


Blocking Buffer Optimization Kit
LI-COR P/N 927-40040

Detergents

		Tween-20 0.1–0.2%	SDS 0.01–0.02%
	Blocking	✕	✕
	Primary		
Secondary	PVDF		
	Nitrocellulose		

Block

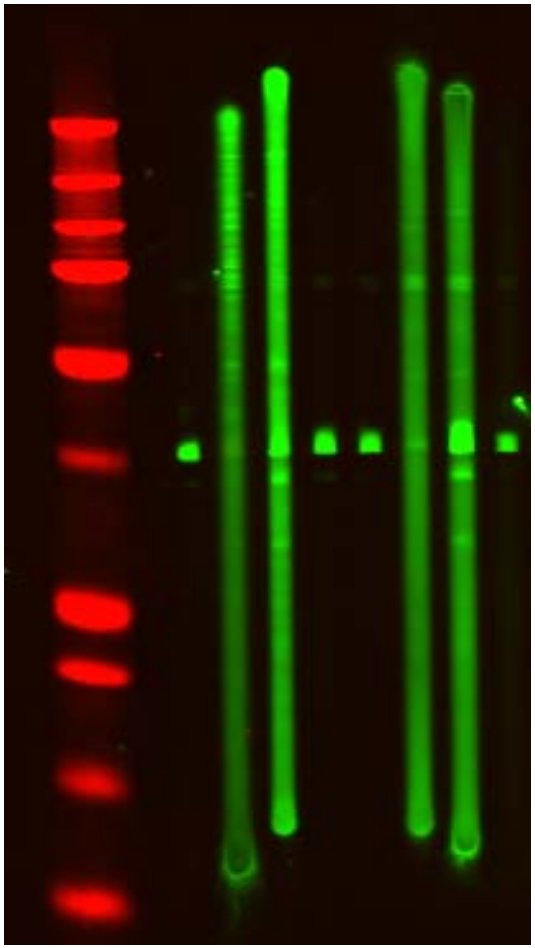


Pro-Tip:

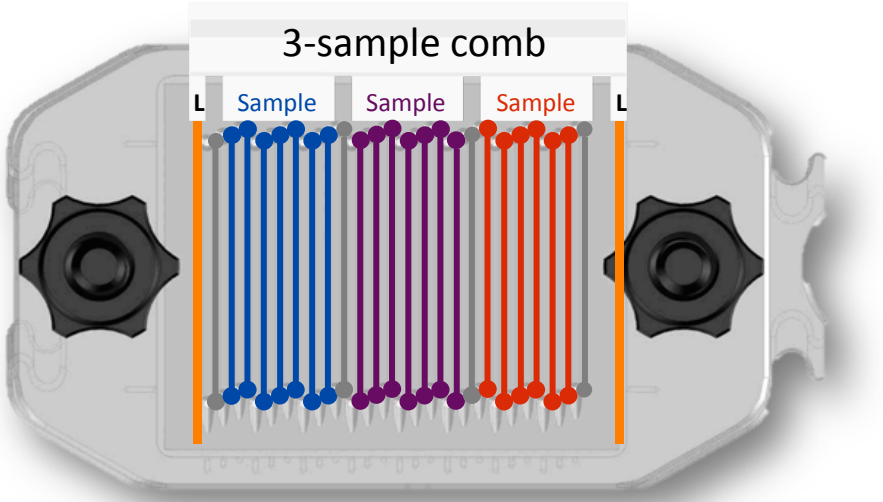
- Use fresh blocking buffers
- Keep time/temperature consistent

Primary

Primary Antibody Selection

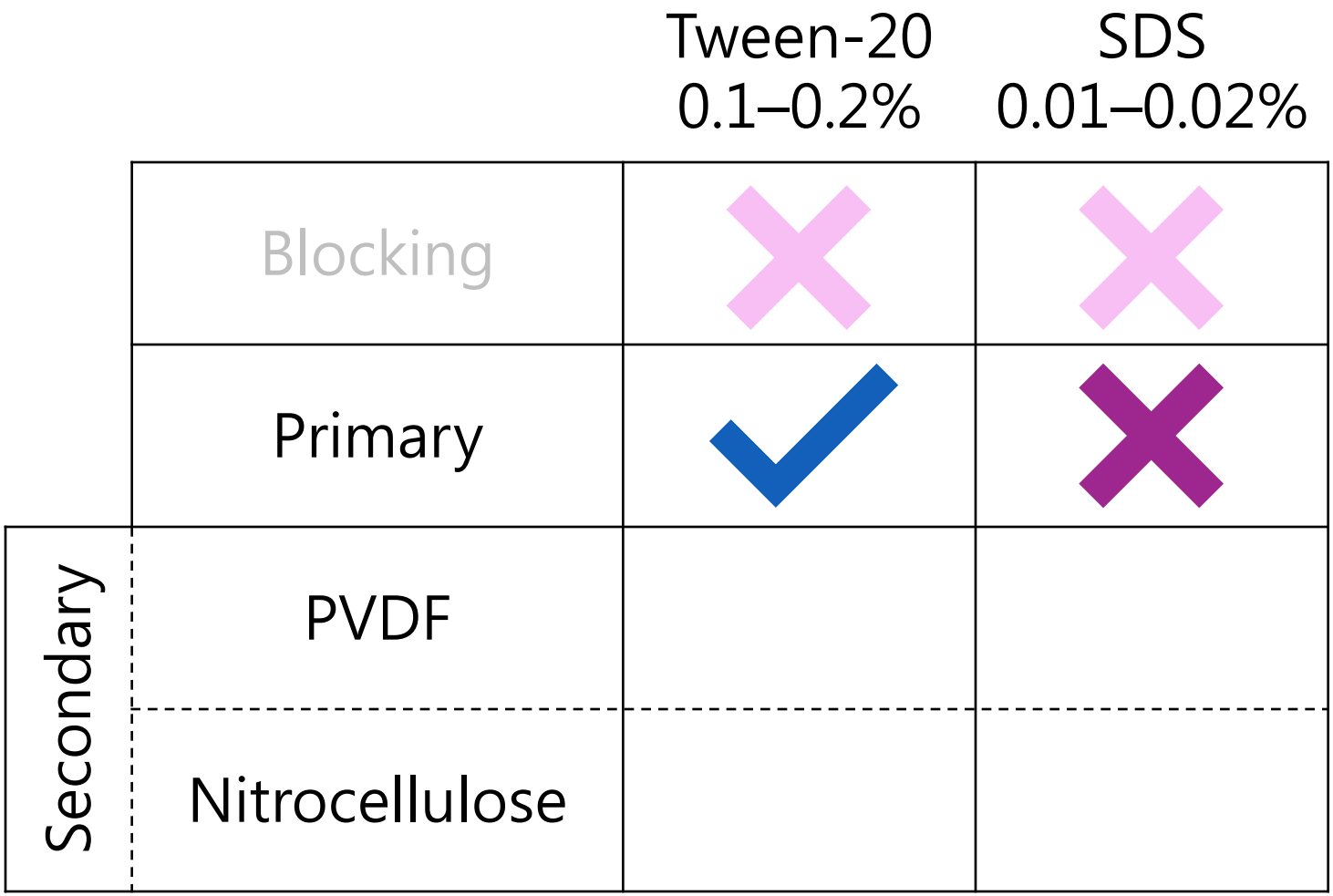


	Antibody	Host	Manufacturer	Part #
1	α-GAPDH	Mouse	Ambion	4300
2	GAPDH	Sheep	AbCam	ab35348
3	GAPDH	Rabbit	Rockland	600-401-A33
4	GAPDH	Mouse	AbCam	ab8245
5	GAPDH	Chicken	ProSci Inc.	XW-7214
6	GAPDH (N-14)	Goat	Santa Cruz Bio	sc-20356
7	GAPDH (V-18)	Goat	Santa Cruz Bio	sc-20357
8	α-GAPDH	Mouse	Sigma	G8795

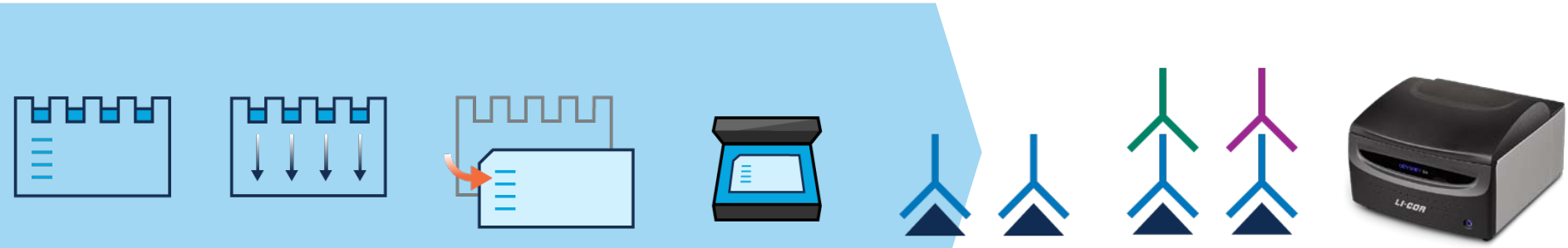


P/N 921-00000

Detergents



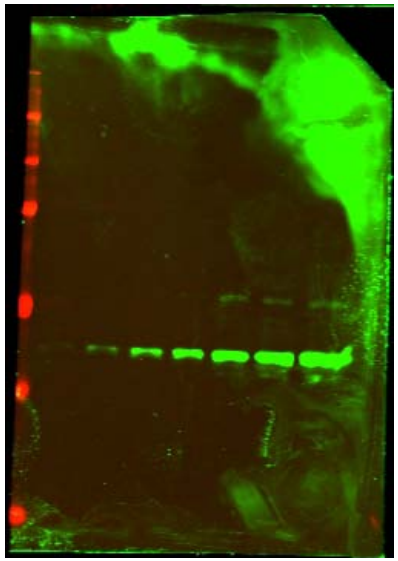
Primary



Pro-Tip:

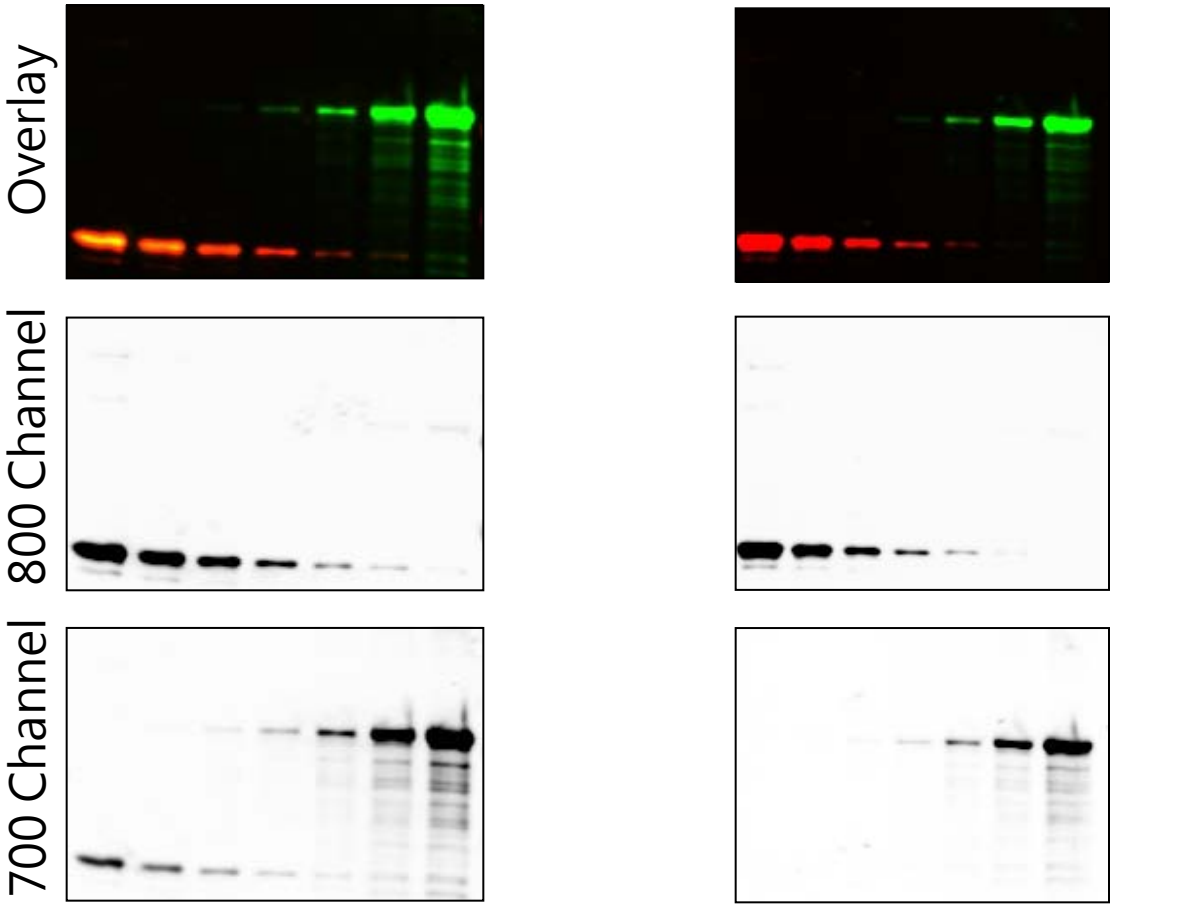
- Keep incubation time/temperature consistent
- Use fresh primary dilutions

- Wash with TBS or PBS + 0.1% Tween-20
- Wash 4x 5 minutes…don't over-wash!!



Secondary

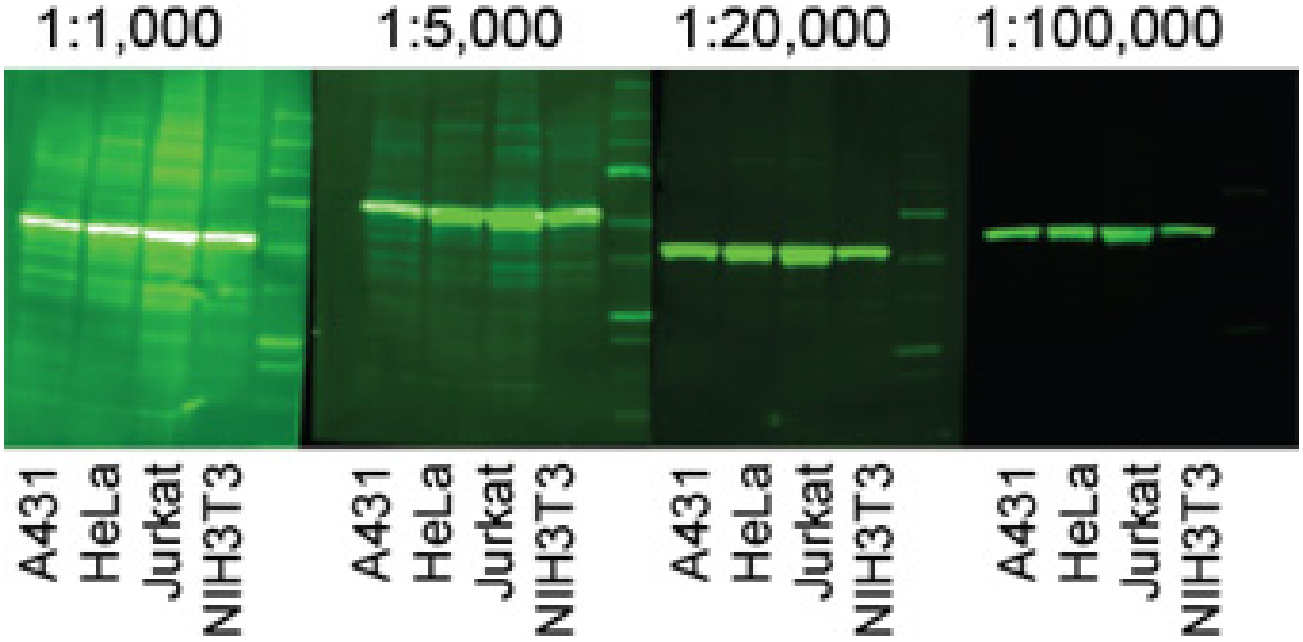
Secondary Antibody



NO CROSS ADSORPTION

HIGHLY CROSS ADSORBED

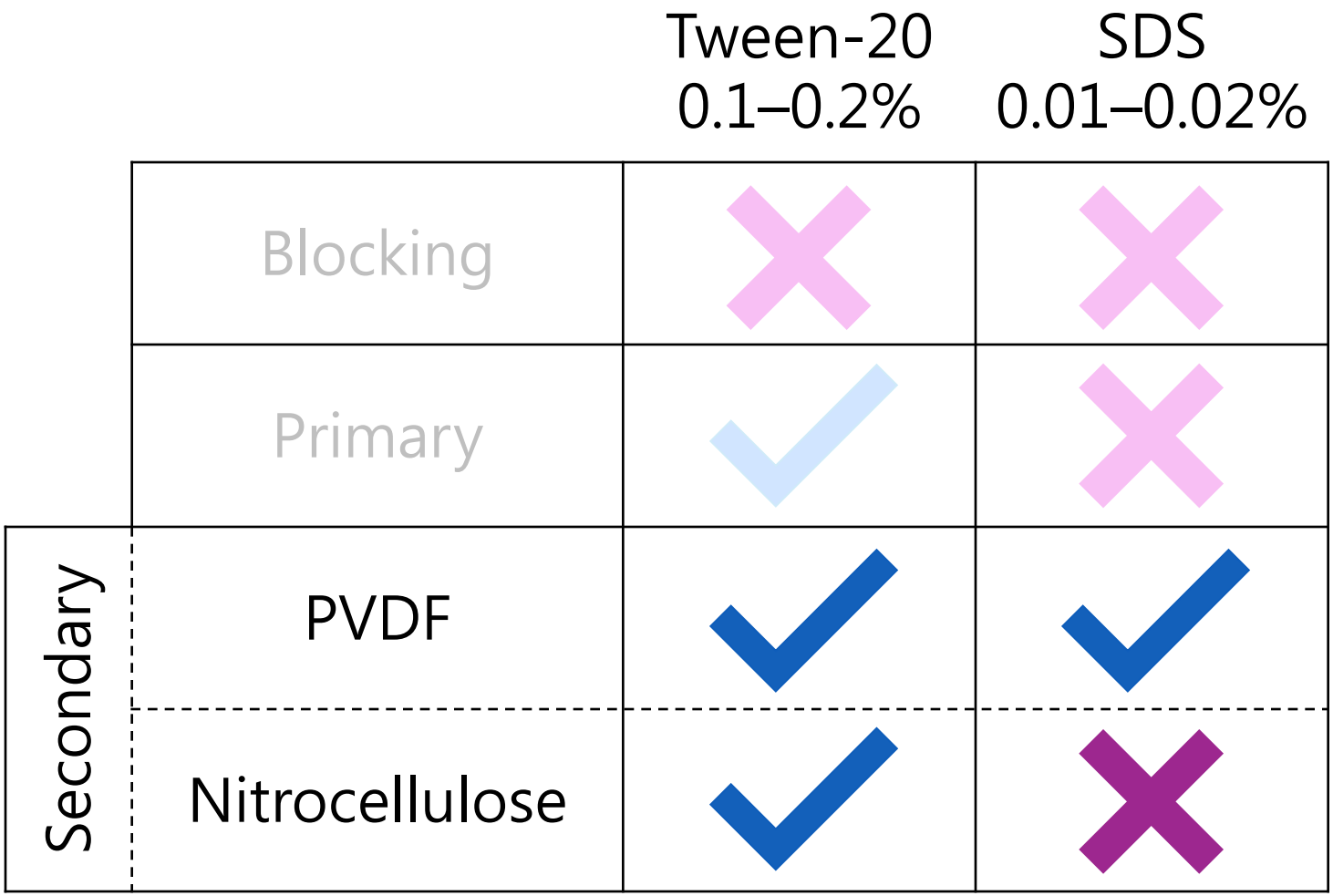
Secondary Antibody Dilution



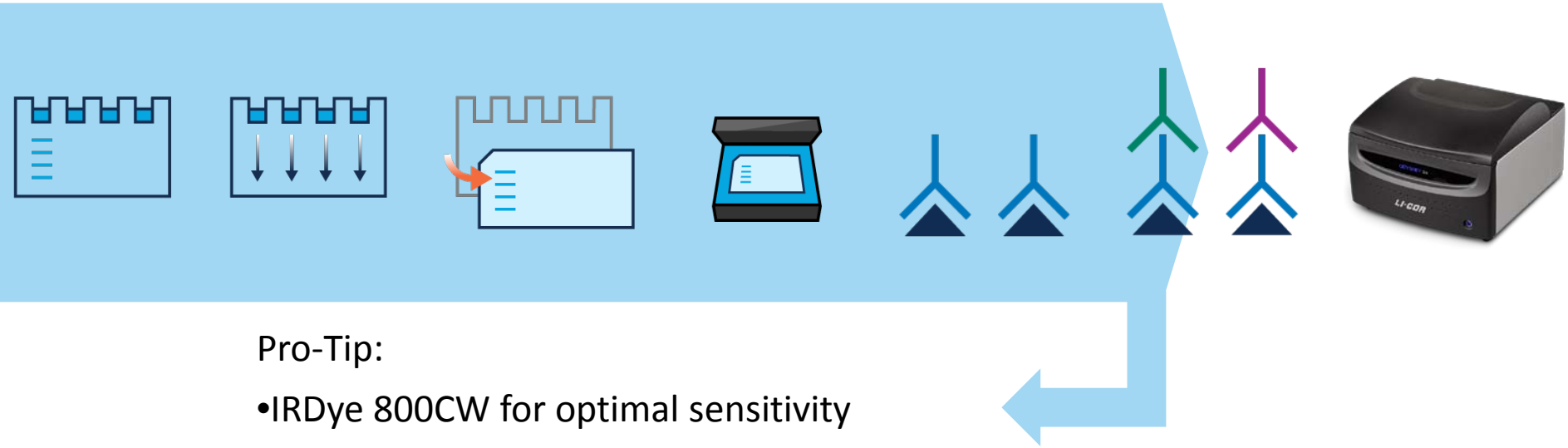
Start at 1:20,000

Working Range 1:10,000 – 1:40,000

Detergents



Secondary

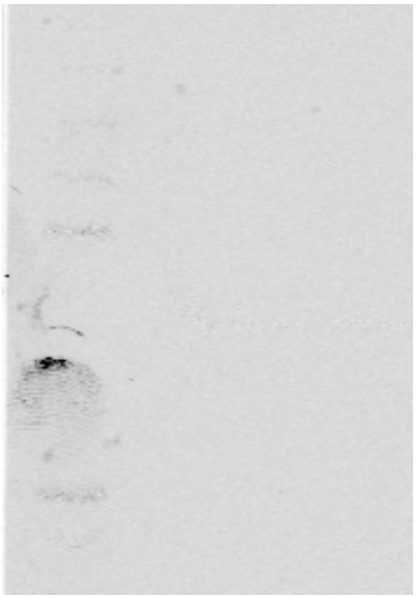


Pro-Tip:

- IRDye 800CW for optimal sensitivity
- Incubate 1 hour at RT for best results

- Wash with TBS or PBS + 0.1% Tween-20
- Wash 4x 5 minutes…don't over-wash!!

Image



Pro-Tip:

- Rinse membrane in TBS or PBS without Tween-20
- Handle membranes with clean forceps

- Image wet or dry, test and be consistent
- Dry membranes for long term storage

LI-COR Applications

REAGENTS & CONSUMABLES

LI-COR Reagents and Consumables

Use LI-COR reagents for a better Western blotting workflow

Step 1

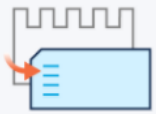
Add Molecular Weight Markers

- WesternSure® Pre-stained Chemiluminescent Protein Ladder
- Chameleon® Pre-stained Protein Ladders
- Odyssey® One-Color Protein Molecular Weight Marker
- All Molecular Weight Markers

Step 2

Perform Electrophoresis

- Sample Loading Buffer



Step 3

Transfer to Membrane

- Membranes and Membrane Kits

Step 4

Add Blocking Buffer

- NIR Blocking Buffers
- Incubation Boxes

Step 5

Incubate with Primary Antibodies

- Actin Primary Antibodies
- Tubulin Primary Antibodies
- COX IV Primary Antibodies

Step 6

Incubate with Secondary Antibodies

- WesternSure Secondary Antibodies
- IRDye® and VRDye™ Secondary Antibodies
- IRDye Streptavidins

Step 7

Add Detection Substrates

- WesternSure Chemiluminescent Substrates
- Odyssey Chemifluorescent Substrate

Step 8

Strip Antibodies

- WesternSure ECL Stripping Buffer
- NewBlot™ Stripping Buffers

Normalization Reagents

- Primary Antibodies for Housekeeping Proteins
- REVERT™ Total Protein Stain
- Odyssey® Loading Indicators

IRDye® and VRDye™ Reagents

- IRDye Infrared Dyes
- IRDye Protein Labeling Kits
- Click Chemistry Reagents
- VRDye Protein Labeling Kit

In-Cell Western™ Assay Reagents

- Sapphire700™ Stain
- CellTag™ 700 Stain

Western Blot Kits

- Odyssey Western Blotting Kit
- Quick Western Kit – IRDye 680RD
- Chemi-IR™ Detection Kit

in vivo Imaging

- BrightSite™ Small Animal Imaging Agents
- CellVue® Cell Labeling Kits
- PSVue® 794 Reagent Kit
- Dye Decontamination Kit

WesternSure® Chemiluminescent Reagents

- WesternSure Pen

Reagent Flexibility

- Compatible with many dyes and stains

Fluorescent Reagent Compatibility

Check **www.licor.com** for the latest list of compatible dyes.

Dye/Stain	Performance	Odyssey Channel
IRDye® 680	+++	700
IRDye® 800CW	+++	800
IRDye® 700DX	++	700
IRDye® 800	+++	800
Alexa Fluor® 647	+/++	700
Alexa Fluor® 660	++	700
Alexa Fluor® 680	+++	700
Alexa Fluor® 700	++	700
Alexa Fluor® **790**	+++	**800**
Atto 680	++	700
Coomassie® (traditional or colloidal)	+++	700
Cy® 5	+/++	700
Cy® 5.5	++	700
DDAO Phosphate (alkaline phosphatase substrate)	++	700**
DRAQ5™	+++	700
NBT/BCIP	++	700**
DyLight™ 700	+++	700
DyLight™ 800	+++	**800**
SYTO®-60	++	700/800*
TO-PRO®-3	++	700
TOTO®-3	++	700
Trypan Blue	++	700

Yellow = Labeled antibody products available directly from LI-COR (www.licor.com)

* Signal appears on both channels
** May not be quantitative

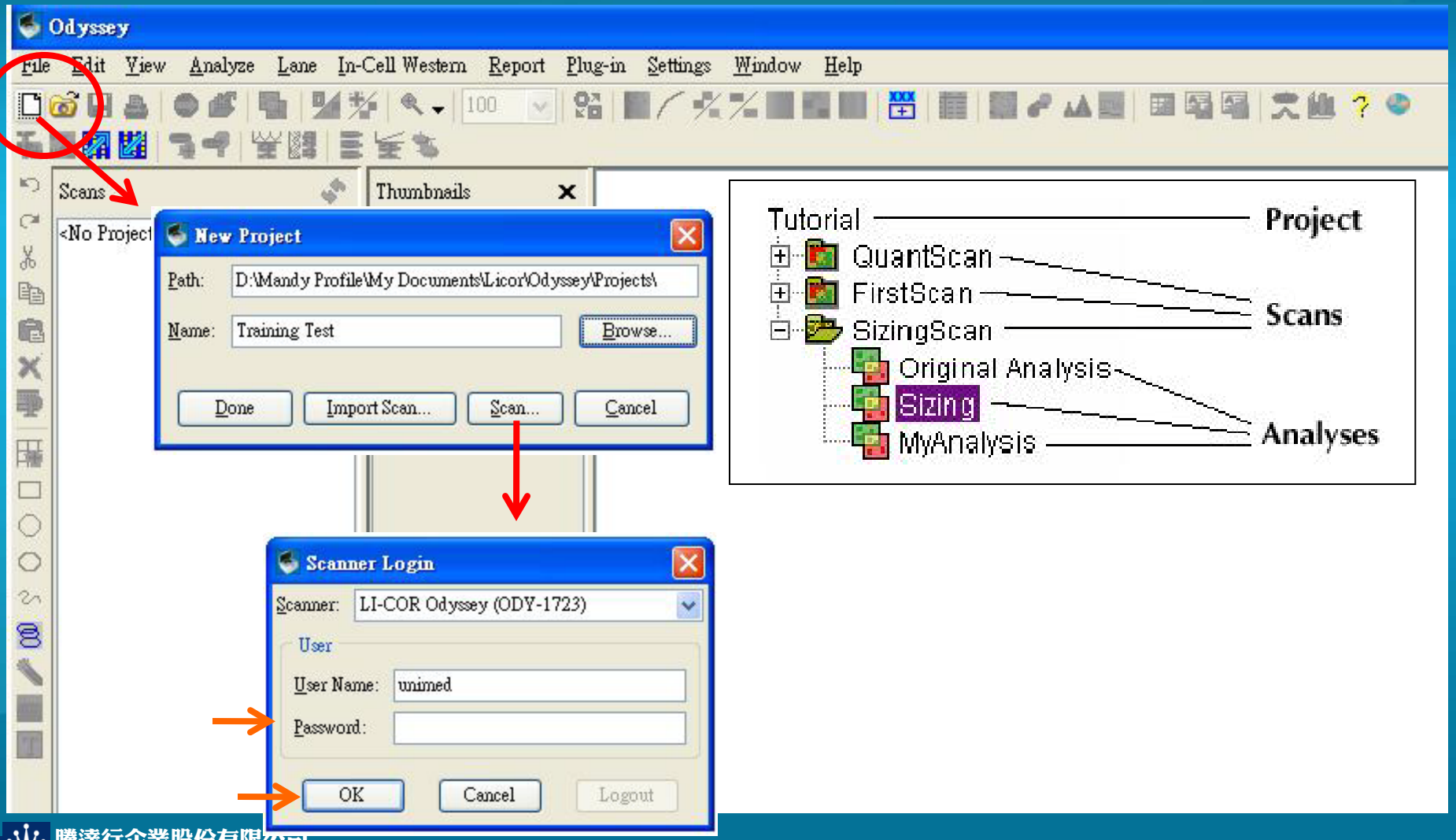
Performance: +++ Excellent
++ Good
+ OK

Scanning Procedures

LI-COR
Experience Excellence

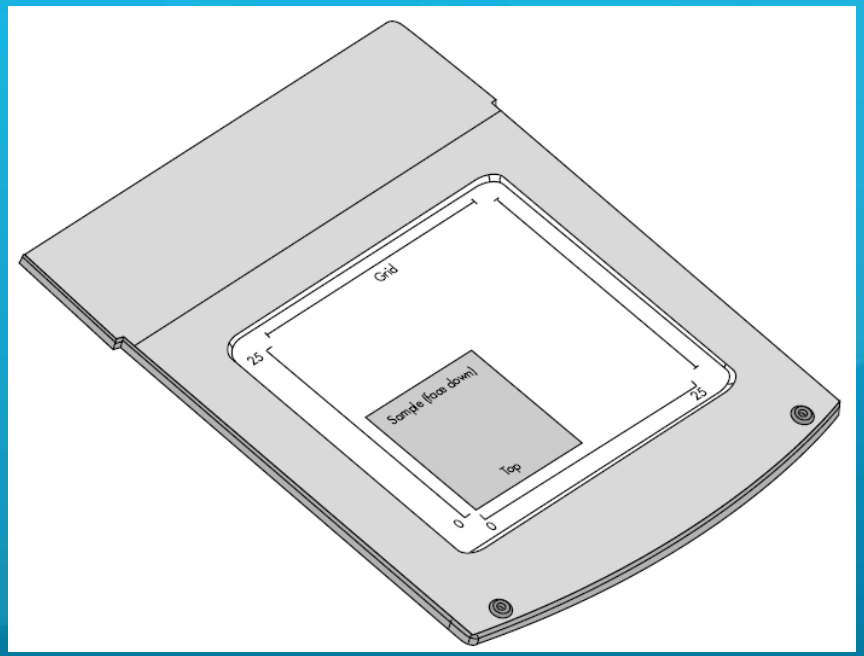
UNIMED
UNIMED HEALTHCARE INC.
UNIMED HEALTHCARE INC.

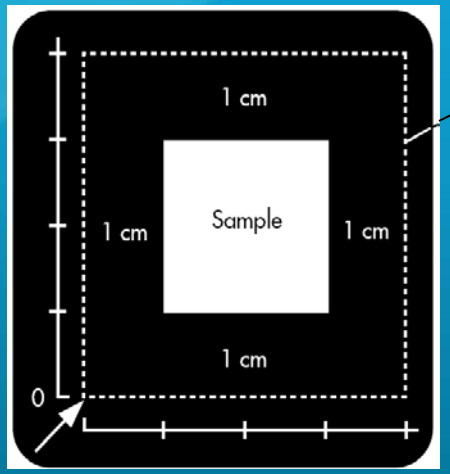
File/New... 開啟新檔案...



Western blot/gel 擺放…

Membrane / gel 正面朝下, 上方靠近操作者, 長的一邊橫放

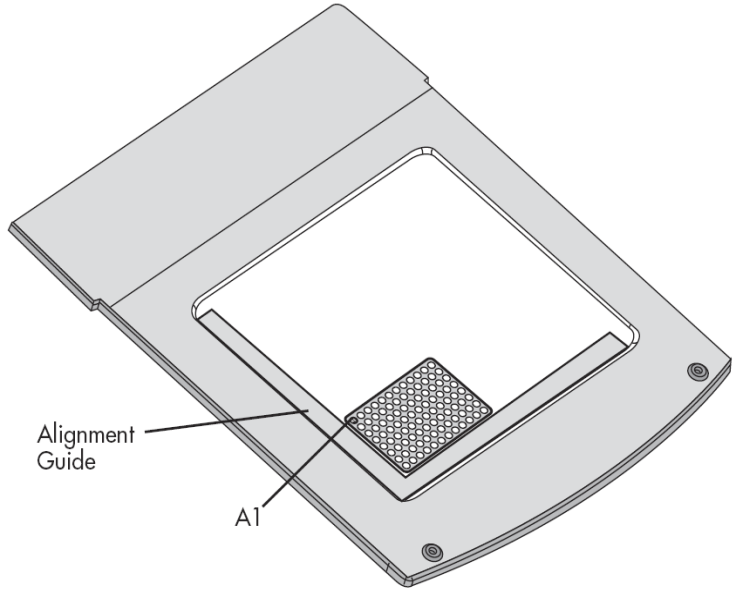


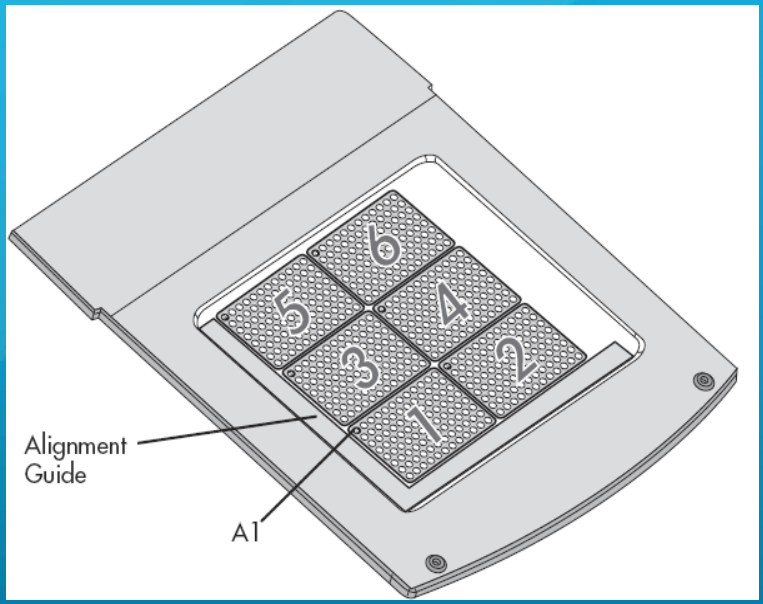


Scan Boundary

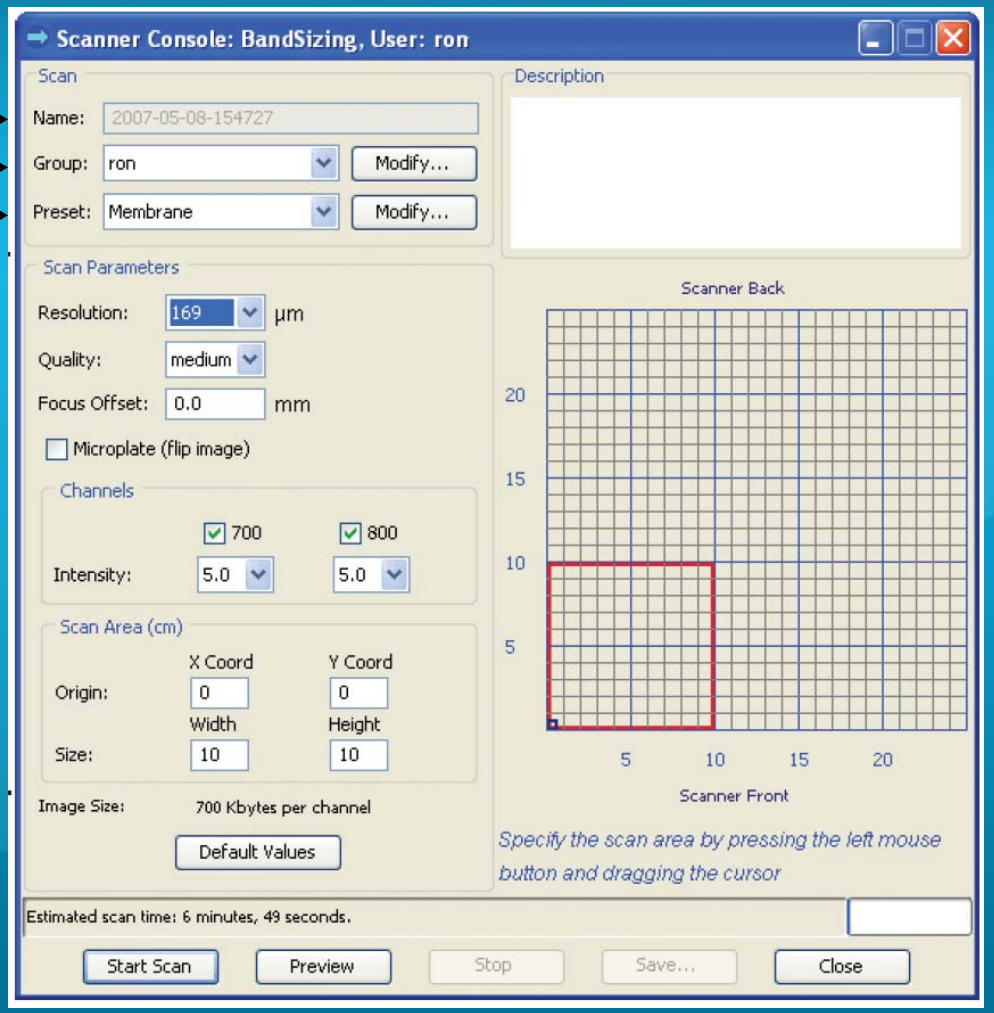
Microtiter plate 擺放…

Plate 正面朝上，緊靠 Alignment Guide 放置





掃描條件設定...

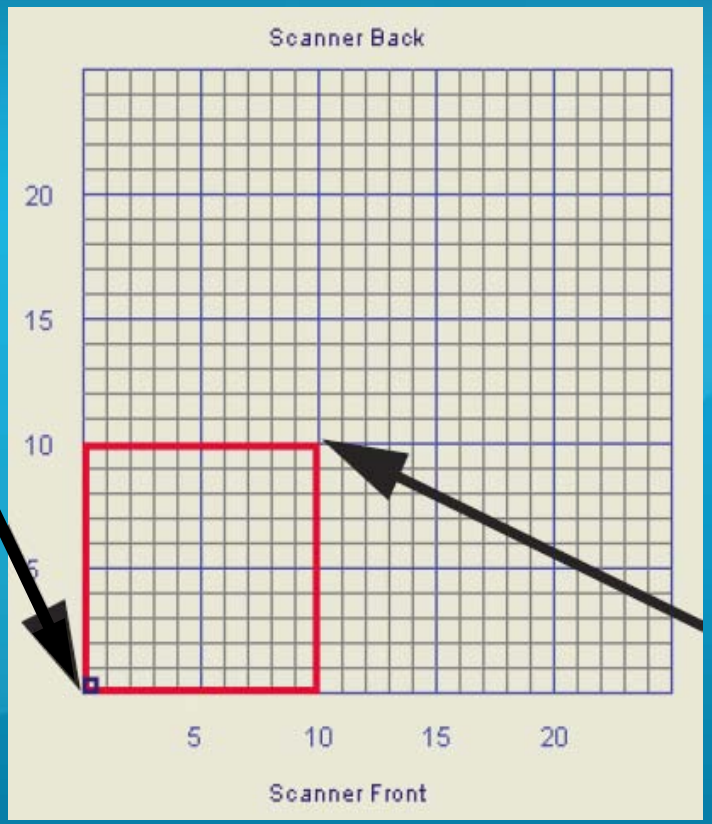


LI-COR Presets:

LI-COR Presets	Membrane	DNA Gel	Protein Gel	Microplate2
Resolution	169	169	169	169
Quality	medium	medium	medium	medium
Focus Offset	0.0	2.0	0.5	3.0 mm
Channels	700, 800	700, 800	700, 800	700, 800
Intensity	5.0	8.0	5.0	5.0
Scan Origin	0,0	0,0	0,0	0,0
Scan Size	10,10	10,10	10,10	13,9

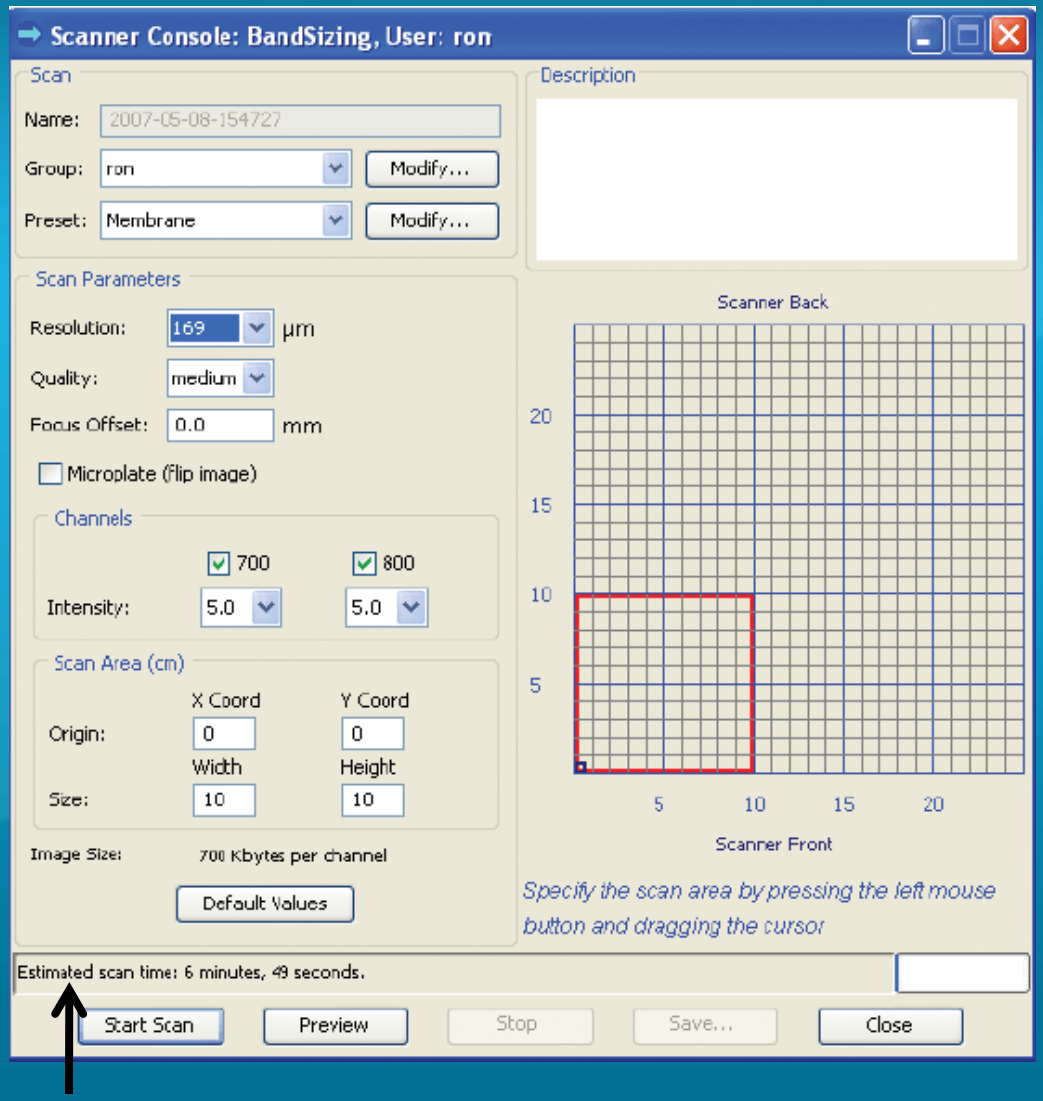
設定掃描範圍...

Click and hold down the mouse button in the lower left corner of the area to be scanned.

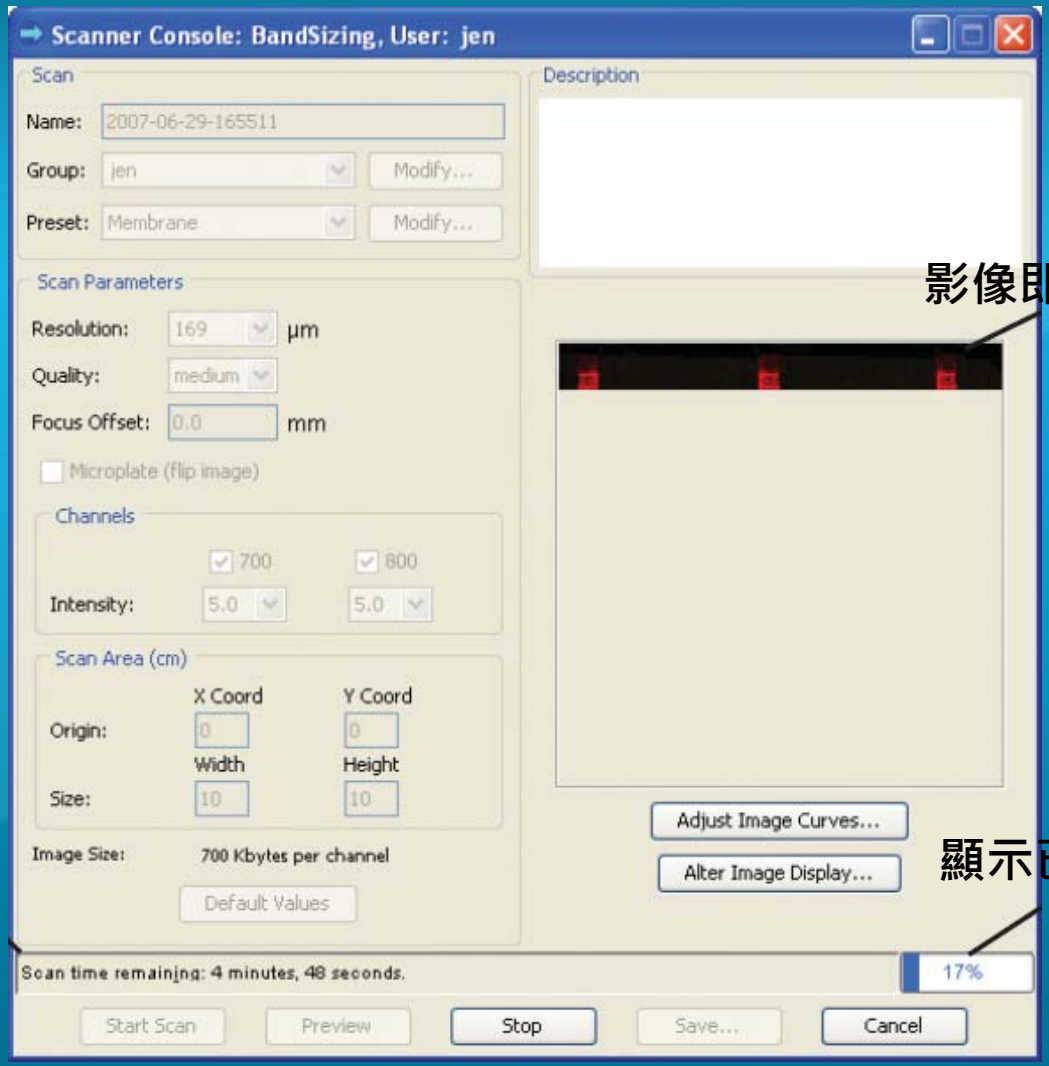


Drag the cursor to the upper right corner of the area to be scanned and release the mouse button.

開始掃描...



掃描期間...

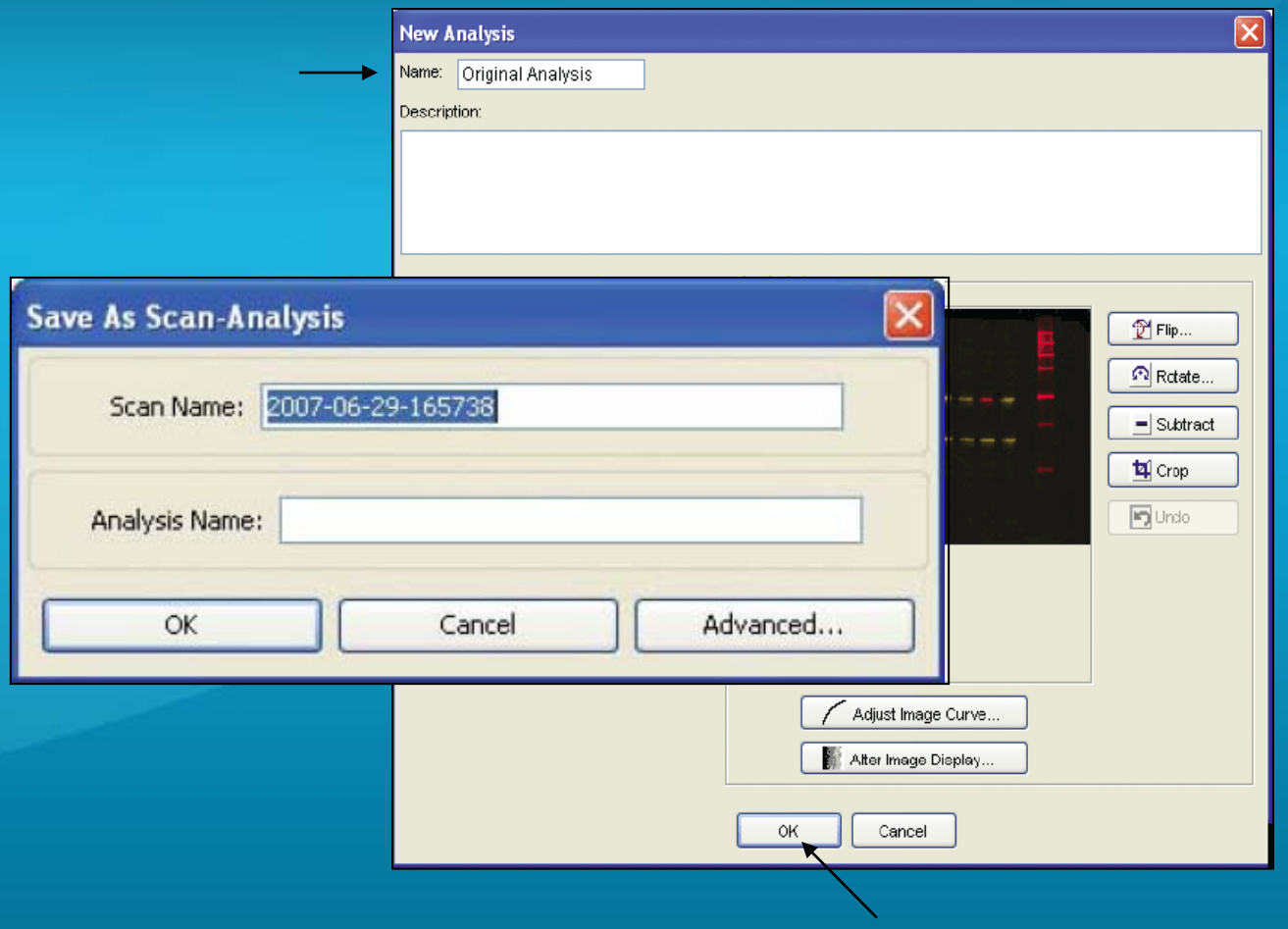


影像即時顯示

顯示剩餘之時間

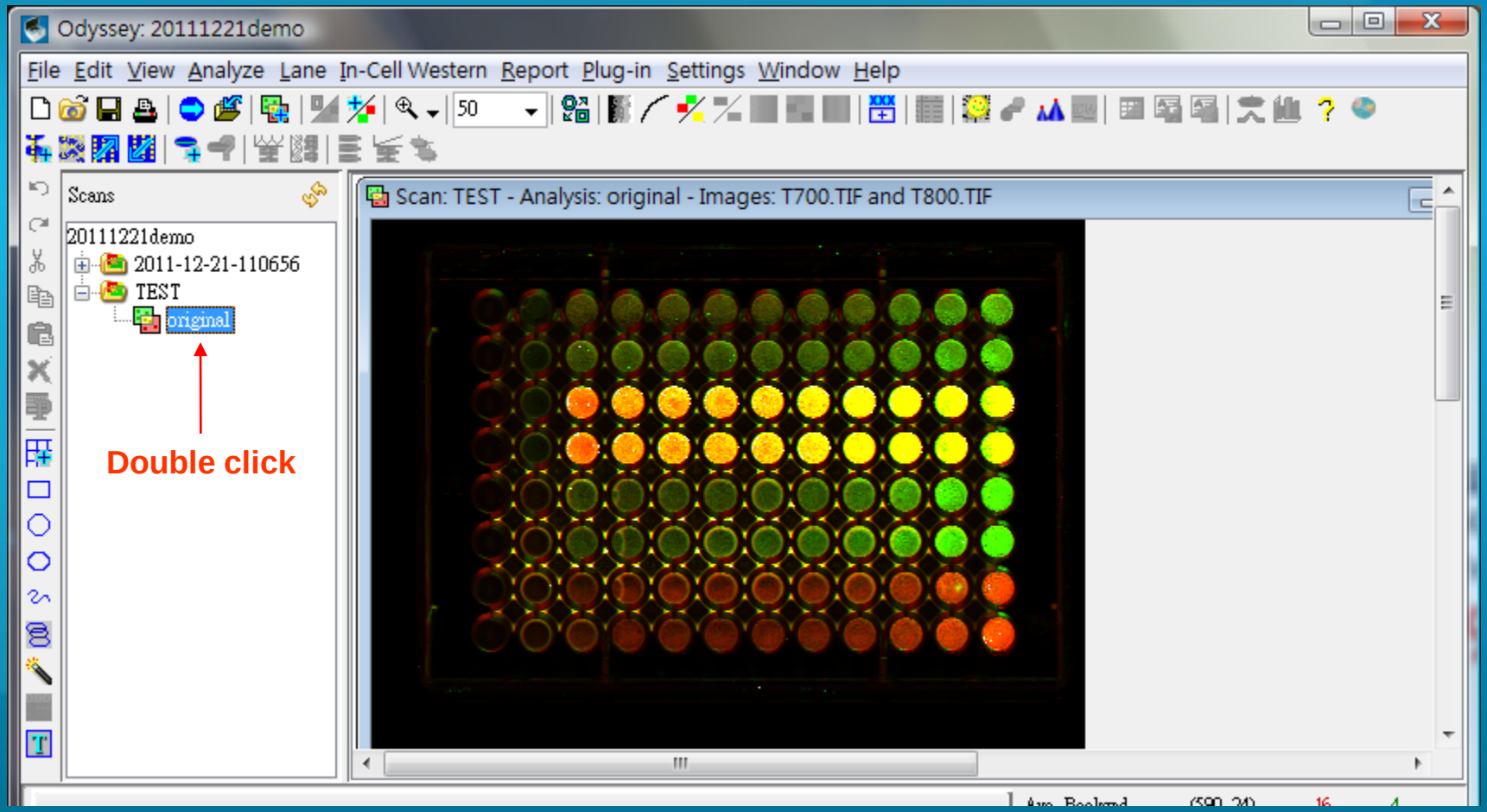
顯示已完成之百分比

掃描完成...


New Analysis
Name:
Original Analysis
Description:
Save As Scan-Analysis
Scan Name:
2007-06-29-165738
Analysis Name:
OK
Cancel
Advanced...
Flip...
Rotate...
Subtract
Crop
Undo
Adjust Image Curve...
Alter Image Display...
OK
Cancel

Post-Scanning

掃描影像檢視...



Post-Scanning

掃描影像檢視...

主工具列

影像對比度調整

影像顯示大小調整

700/800 單色畫面轉換

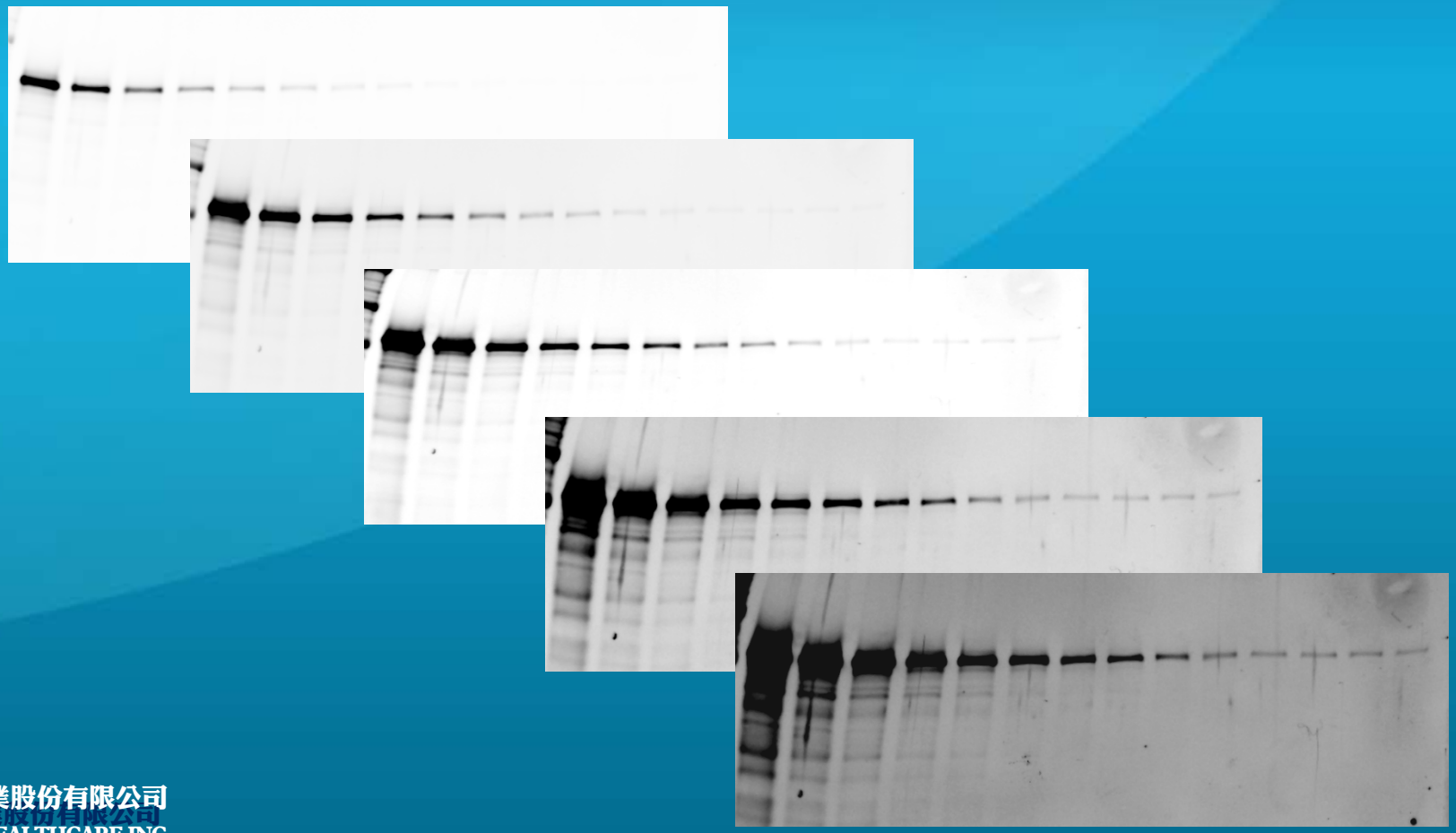
雙色/單色畫面轉換

開啟新掃描

彩色/灰階畫面轉換

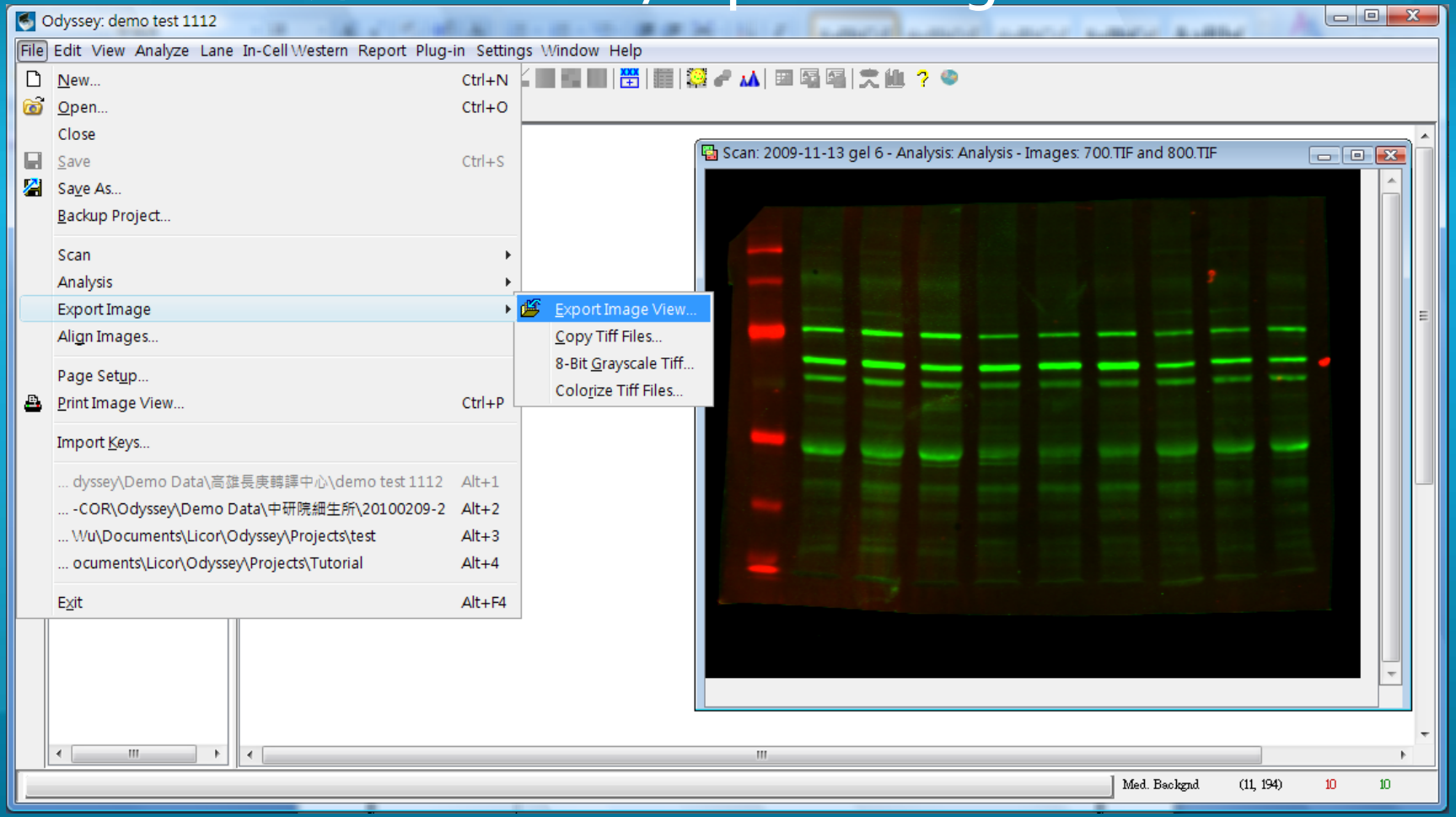
Post-Scanning

影像對比度調整



Post-Scanning

掃描影像輸出... File/Export Image->



We can help you get high-quality data!