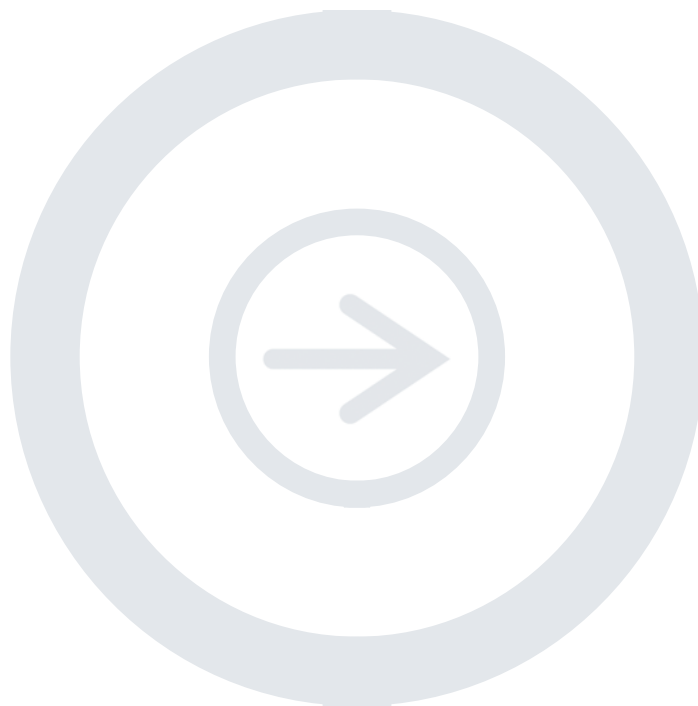


# Good Westerns Gone Bad:

---

## Maximizing sensitivity on chemiluminescent Western blots

Developed for:  
Odyssey® Fc Imager and  
C-DiGit® Blot Scanner



***LI-COR***®

Published January 2015. The most recent version of this document is posted at <http://www.licor.com/bio/support>

## Maximizing sensitivity on chemiluminescent Western blots

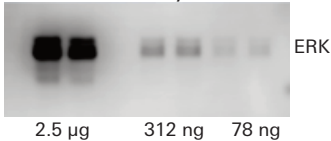
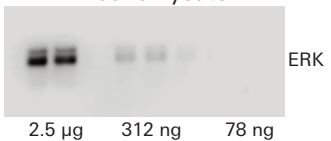
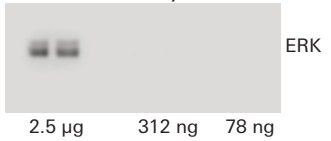
The most important procedural difference between using film and digital imaging is related to the timing of image acquisition. Film is more tolerant in relation to processing time, as you can always expose for a longer period of time if needed, whereas digital imaging requires that you capture the most photons within a finite imaging window. This window begins immediately after the addition of room temperature substrate (*NOTE: Do not compromise the five-minute incubation time!*). **Longer acquisitions in digital imaging do not correlate to results of longer exposures on film, as background noise can begin to contribute signal that impacts your overall limits of detection (LOD).**

If you are seeing weak signal in your data, the following information will help assess possible contributing factors.

### Possible cause # 1: Substrate does not have a fast enough rate of reaction (e.g., SuperSignal® West Pico)

**Solution:** Use WesternSure® PREMIUM or SuperSignal West Femto substrates

**Why this matters:** Different substrates have different rates of reaction. Some are developed to give off a lot of light quickly; others give off small amounts of light over longer periods of time. An alternate substrate may be required for digital imaging when imaging blots with low protein abundance. **See Figure 1.**

Figure 1	Optimal Blot	Satisfactory Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>2.5 µg 312 ng 78 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>2.5 µg 312 ng 78 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>2.5 µg 312 ng 78 ng</p>
Substrate	SuperSignal West Femto	SuperSignal West Dura <sup>1</sup>	SuperSignal West Pico
Substrate Volume	3.0 mL substrate	3.0 mL substrate	3.0 mL substrate
Imaging Method	Substrate placed directly on C-DiGit® Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.
Scan Setting	High	High	High
Performance	LOD – 78 ng	LOD – 312 ng	LOD – 2.5 µg

**Figure 1. Performance differences of three different substrate classifications using C-DiGit Blot Scanner.** All images are normalized to the Lookup Table (LUT) settings of the optimal blot for accurate visual comparison.

<sup>1</sup> Comparable to WesternSure® PREMIUM

## Possible cause # 2: Not enough substrate was added to the blot

### Precaution/Solution:

#### For C-DiGit® Blot Scanner:

- Add at least 3 mL (7 x 4 cm blot, 0.1 mL/cm<sup>2</sup>) of substrate to glass surface of the scanner, place blot protein side down into the substrate, place 1-ply sheet protector on top, incubate 5 min, then scan on High;

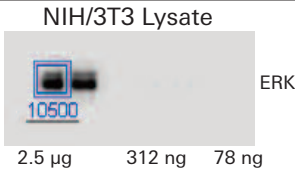
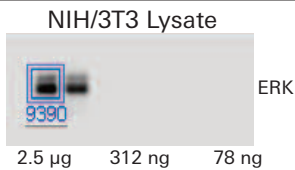
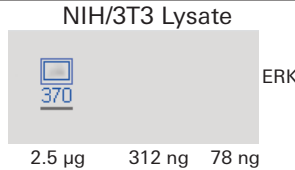
**OR,**

- Add at least 3 mL (7 x 4 cm blot, 0.1 mL/cm<sup>2</sup>) of substrate to blot surface, incubate 5 min, remove excess substrate, place blot protein side down onto the glass surface, cover with 1-ply sheet protector on top, then scan on High.

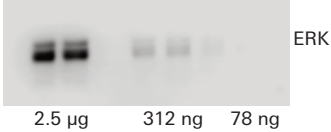
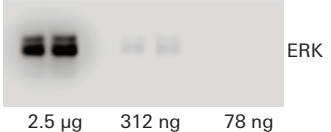
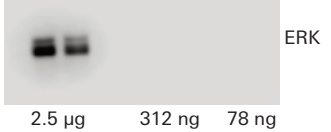
#### For Odyssey® Fc Imager:

- Add at least 3 mL (7 x 4 cm blot, 0.1 mL/cm<sup>2</sup>) of substrate to blot surface, incubate 5 min, remove excess substrate, place blot protein side up onto the imaging tray, then image.

**Why this matters:** If you do not add enough substrate to your blot, the light-generating luminol will be depleted, leading to fewer photons (light) being released. **See Figures 2 and 3.**

Figure 2	Optimal Blot	Satisfactory Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10500</p> <p>2.5 µg 312 ng 78 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>9390</p> <p>2.5 µg 312 ng 78 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>370</p> <p>2.5 µg 312 ng 78 ng</p>
Substrate	SuperSignal® West Pico	SuperSignal West Pico	SuperSignal West Pico
Substrate Vol.	<b>3.0 mL substrate</b>	<b>1.5 mL substrate</b>	<b>0.75 mL substrate</b>
Imaging Method	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.
Scan Setting	High	High	High
Performance	Bright signal	Moderate signal	Low signal

**Figure 2. Performance differences when incubating the blot in different volumes of SuperSignal West Pico.** Three blots have the same LOD (2.5 µg/well); however, signal intensity varies. Blots were all imaged on the C-DiGit Blot Scanner. Images are normalized to the LUT of the optimal blot.

<b>Figure 3</b>	<b>Optimal Blot</b>	<b>Satisfactory Blot</b>	<b>Unsatisfactory Blot</b>
<b>Images</b>	<p>NIH/3T3 Lysate</p>  <p>2.5 µg    312 ng    78 ng</p>	<p>NIH/3T3 Lysate</p>  <p>2.5 µg    312 ng    78 ng</p>	<p>NIH/3T3 Lysate</p>  <p>2.5 µg    312 ng    78 ng</p>
<b>Substrate</b>	SuperSignal® West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>
<b>Substrate Vol.</b>	<b>3.0 mL substrate</b>	<b>1.5 mL substrate</b>	<b>0.75 mL substrate</b>
<b>Imaging Method</b>	Substrate placed directly on C-DiGit® Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface.  Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.
<b>Scan Setting</b>	High	High	High
<b>Performance</b>	LOD – 78 ng	LOD – 312 ng	LOD – 2.5 µg

**Figure 3. Performance differences when incubating the blot in different volumes of SuperSignal West Dura<sup>1</sup>.** Blots were all imaged on the C-DiGit Blot Scanner. Images are normalized to the LUT of the optimal blot.

<sup>1</sup> Comparable to WesternSure® PREMIUM

### Possible cause # 3: Membrane was placed on the detection system incorrectly

**Solution:** Ensure that the blot is placed with the proteins facing toward the detection system.

**For the C-DiGit® Blot Scanner:**

- Place blot protein side facing *down*

**For Odyssey® Fc Imager:**

- Place blot protein side facing *up*

**Why this matters:** If the blot is placed incorrectly, you may or may not be able to visualize bands. Even if bands are visualized, they will be substantially reduced in signal. **See Figure 4.**

Figure 4	Correctly Imaged Blot	Incorrectly Imaged Blot
<b>Images</b>		
<b>Substrate</b>	SuperSignal® West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>
<b>Imaging Method</b>	<b>Blot imaged <i>protein side facing down</i></b>	<b>Blot imaged <i>protein side facing up</i></b>
<b>Performance</b>	LOD – 156 ng	LOD – 625 ng

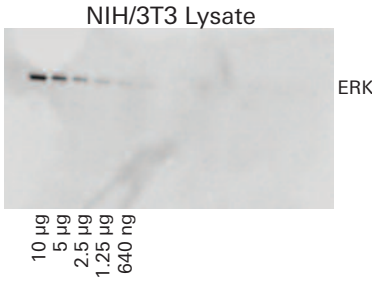
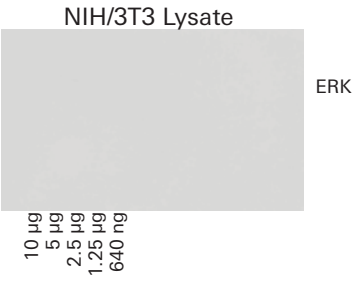
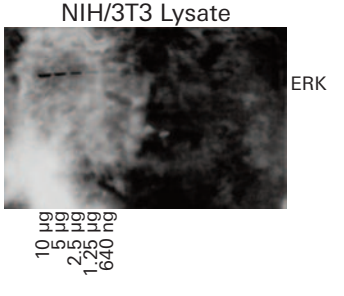
**Figure 4. Performance differences imaging the blot correctly (protein side down), compared to imaging the blot protein side up on the C-DiGit Blot Scanner.** Images are normalized to the LUT of the correctly imaged blot.

<sup>1</sup> Comparable to WesternSure® PREMIUM

### Possible cause #4: Blot was not detected or processed on the same day it was imaged

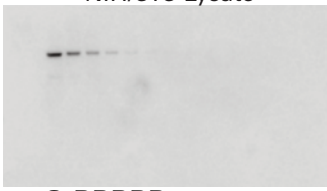

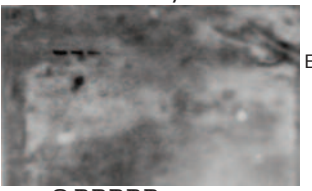
**Precaution/Solution:** Blot should be processed and detected on the same day. The secondary antibody should be incubated the day of imaging and fresh substrate added before imaging.

**Why this matters:** Secondary antibody and/or substrate is not stable enough for acceptable photon emission when digitally imaged after the day it is applied. **See Figures 5 and 6.**

Figure 5	Optimal Blot	Unsatisfactory Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10 µg 5 µg 2.5 µg 1.25 µg 640 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10 µg 5 µg 2.5 µg 1.25 µg 640 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10 µg 5 µg 2.5 µg 1.25 µg 640 ng</p>
Substrate	SuperSignal® West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>
Processing Time	Same day	Next day	Next day
Detection Process	HRP secondary incubated, washed, and substrate added immediately before imaging.	HRP secondary incubated, washed, and substrate added day before imaging.	HRP secondary incubated, washed, and substrate added day before imaging, <b>then re-incubated with HRP secondary and substrate added immediately before imaging.</b>
Storage Conditions		Blot stored overnight dry, at room temperature	Blot stored overnight dry, at room temperature
Performance	LOD – 640 ng	LOD – None detected	LOD – 1.25 µg

**Figure 5. Performance differences when the same blot is imaged immediately after processing vs. stored overnight *dry* and then imaged.** Blots were all imaged on the C-DiGit® Blot Scanner. Images are normalized to the LUT of the optimal blot.

<sup>1</sup> Comparable to WesternSure® PREMIUM

Figure 6	Optimal Blot	Unsatisfactory Blot	Unsatisfactory Blot
Images	<p>NIH/3T3 Lysate</p>  <p>ERK</p> <p>10 1 µg 0.5 µg 0.25 µg 0.125 µg 640 ng</p>	<p>NIH/3T3 Lysate</p>  <p>ERK</p> <p>10 1 µg 0.5 µg 0.25 µg 0.125 µg 640 ng</p>	<p>NIH/3T3 Lysate</p>  <p>ERK</p> <p>10 1 µg 0.5 µg 0.25 µg 0.125 µg 640 ng</p>
Substrate	SuperSignal® West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>
Process Time	Same day	Next day	Next day
Detection Process	HRP secondary incubated, washed, and substrate added immediately before imaging.	HRP secondary incubated, washed, and substrate added day before imaging.	HRP secondary incubated, washed, and substrate added day before imaging, <b>then re-incubated with HRP secondary and substrate added immediately before imaging.</b>
Storage Conditions		<b>Blot stored overnight wet in PBS, at room temperature</b>	<b>Blot stored overnight wet in PBS, at room temperature</b>
Performance	LOD – 640 ng	LOD – None detected	LOD – 1.25 µg

**Figure 6. Performance differences when the same blot is imaged immediately after processing vs. stored overnight wet and then imaged.** Blots were all imaged on the C-DiGit® Blot Scanner. Images are normalized to the LUT of the optimal blot.

<sup>1</sup> Comparable to WesternSure® PREMIUM

## Possible cause # 5: Blot was not kept uniformly wet during the entire image acquisition

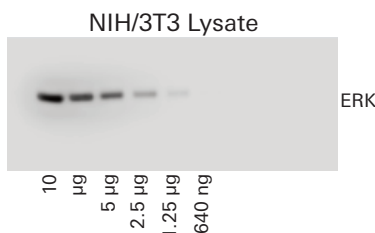
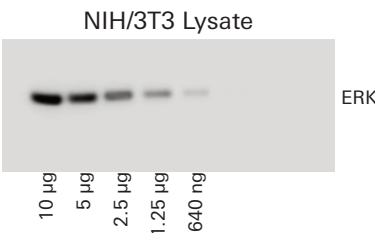
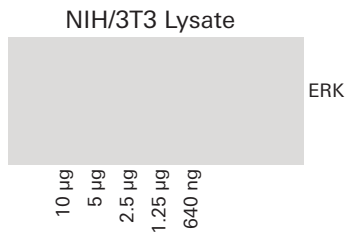
### Precaution/Solution:

- Use more substrate prior to imaging
- Do not completely blot off all of the substrate before imaging

### For C-DiGit® Blot Scanner:

- Wrap the blot in plastic wrap or cover with a plastic sheet protector
- Incubate blot with substrate directly on scanner bed

**Why this matters:** If enough substrate is not added, the membrane will not stay wet, and there will be no enzymatic activity. **See Figure 7.**

Figure 7	Optimal Blot	Optimal Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10 µg 5 µg 2.5 µg 1.25 µg 640 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10 µg 5 µg 2.5 µg 1.25 µg 640 ng</p>	 <p>NIH/3T3 Lysate</p> <p>ERK</p> <p>10 µg 5 µg 2.5 µg 1.25 µg 640 ng</p>
Blot Condition	Wet	Damp	Dry
Imaging Method	Imaged in 3.0 mL of SuperSignal® West Dura <sup>1</sup> substrate placed on the scan bed of the C-DiGit Blot Scanner with 1-ply sheet protector on top.	Excess SuperSignal West Dura <sup>1</sup> substrate removed, then imaged on the scan bed of the C-DiGit Blot Scanner with 1-ply sheet protector on top.	Blot dried before imaging.
Performance	LOD – 640 ng	LOD – 640 ng	LOD – None detected

**Figure 7. Performance differences when the same blot is imaged wet, damp, and dry.** Blots were all imaged on the C-DiGit Blot Scanner. Images are normalized to the LUT of the optimal wet blot.

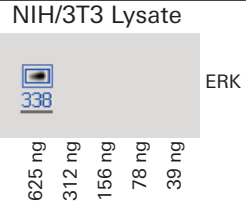
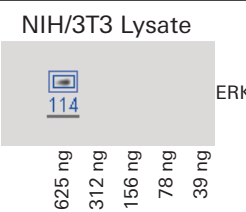
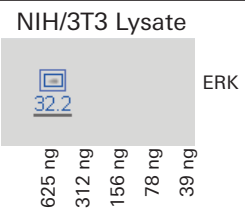
<sup>1</sup> Comparable to WesternSure® PREMIUM

## Possible cause # 6: Blot was exposed to film BEFORE imaging on a digital imager

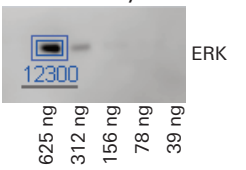
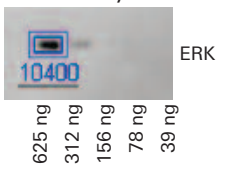
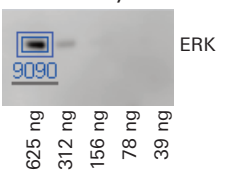
**Precaution/Solution:** Image on digital imager first, then expose blot to film.

**Why this matters:** Digital imaging requires capturing the most photons being generated, which typically is immediately after a 5-minute substrate incubation. Time may be more of an issue with some substrates. **See Figures 8, 9, and 10.**


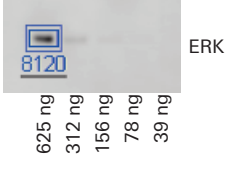
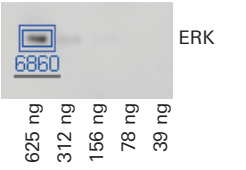


Figure 8	Optimal Blot	Unsatisfactory Blot	Unsatisfactory Blot
Images			
Imaging time	Immediately after incubation with SuperSignal® West Pico	26 min after incubation	51 min after incubation
Scan Setting	High	High	High
Performance	LOD – 625 ng, Signal – 338	LOD – 625 ng, Signal – 114	LOD – 625 ng, Signal – 32.2

**Figure 8. Performance differences of a Western blot detected with SuperSignal® West Pico when the same blot is imaged over time.** Blot was incubated 5 min in substrate before imaging on the C-DiGit® Blot Scanner. Images are normalized to the LUT of the optimal blot.

Figure 9	Optimal Blot	Satisfactory Blot	Satisfactory Blot
Images			
Imaging time	Immediately after incubation with SuperSignal West Dura <sup>1</sup>	24 min after incubation	48 min after incubation
Scan Setting	High	High	High
Performance	LOD – 156 ng, Signal – 12,300	LOD – 156 ng, Signal – 10,400	LOD – 156 ng, Signal – 9,090

**Figure 9. Performance differences of a Western blot detected with SuperSignal West Dura<sup>1</sup> when the same blot is imaged over time.** Blot was incubated 5 min in substrate before imaging on the C-DiGit Blot Scanner. Images are normalized to the LUT of the optimal blot.

Figure 10	Optimal Blot	Satisfactory Blot	Satisfactory Blot
Images			
Imaging time	Immediately after incubation with SuperSignal West Femto	24 min after incubation	48 min after incubation
Scan Setting	High	High	High
Performance	LOD – 156 ng, Signal – 11,500	LOD – 156 ng, Signal – 8,120	LOD – 156 ng, Signal – 6,860

**Figure 10. Performance differences of a Western blot detected with SuperSignal West Femto when the same blot is imaged over time.** Blot was incubated 5 min in substrate before imaging on the C-DiGit Blot Scanner. Images are linked to the LUT of the optimal blot.

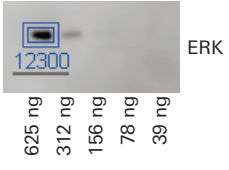

<sup>1</sup> Comparable to WesternSure® PREMIUM

## Possible cause # 7: Blot was imaged using incorrect sensitivity setting

### Precaution/Solution:

- On the C-DiGit<sup>®</sup> Blot Scanner, use High Sensitivity setting (12-min scan) for more sensitive detection
- On the Odyssey<sup>®</sup> Fc Imager, use a longer integration time (up to 10 min)

**Why this matters:** Digital imaging with the C-DiGit Blot Scanner or Odyssey Fc will not generally reach a saturation point. Begin with a longer acquisition time to ensure best sensitivity, then optimize to shorter scan times. **See Figure 11.**

Figure 11	Optimal Blot	Satisfactory Blot
Images	<p>NIH/3T3 Lysate</p>  <p>ERK</p> <p>12300</p> <p>625 ng 312 ng 156 ng 78 ng 39 ng</p>	<p>NIH/3T3 Lysate</p>  <p>ERK</p> <p>5030</p> <p>625 ng 312 ng 156 ng 78 ng 39 ng</p>
Substrate	SuperSignal <sup>®</sup> West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>
Sensitivity Setting	High	Standard
Performance	Signal – 12,300	Signal – 5,030

**Figure 11. Performance differences of a Western blot detected with SuperSignal West Dura on C-DiGit Blot Scanner when the same blot is imaged on High Sensitivity (12 min) versus Standard Sensitivity (6 min). Images are linked to the LUT of the optimal blot.**


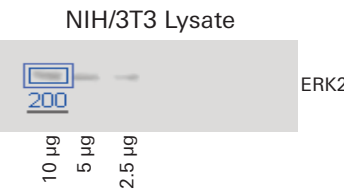
<sup>1</sup> Comparable to WesternSure<sup>®</sup> PREMIUM

## Possible cause # 8: Substrate was too cold

### Precaution/Solution:

- Equilibrate substrate to room temperature before imaging on a digital imager.

**Why this matters:** Enzyme activity is greatly reduced when it is cold. Substrate needs to be equilibrated to room temperature for digital imaging. This is true with film as well, but there may be a period of time after adding substrate and exposing to film during which the substrate has had a chance to equilibrate to room temperature. **See Figure 12.**

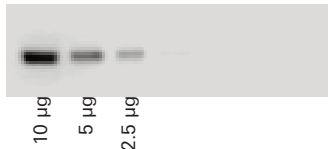
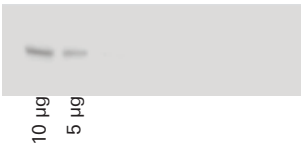
Figure 12	Optimal Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK2</p> <p>1740</p> <p>10 µg 5 µg 2.5 µg</p>	 <p>NIH/3T3 Lysate</p> <p>ERK2</p> <p>200</p> <p>10 µg 5 µg 2.5 µg</p>
Substrate	SuperSignal® West Pico	SuperSignal West Pico
Sensitivity Setting	Standard	Standard
Substrate temperature	Room temperature	Cold
Performance	Signal – 1,740	Signal – 200

**Figure 12. Performance differences of a Western blot detected with SuperSignal West Pico when the substrate has been equilibrated to room temperature versus being imaged with cold (4 °C) substrate.** Image was acquired using Standard (6-min) sensitivity.

## Possible cause # 9: Substrate was not incubated for 5 minutes

**Precaution/Solution:** Incubate substrate for five minutes prior to imaging on a digital imager.

**Why this matters:** Five minutes is the typical manufacturer’s recommendation for optimal photon emission, for both film and digital imaging. **See Figure 13.**

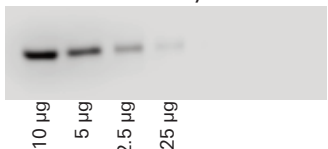
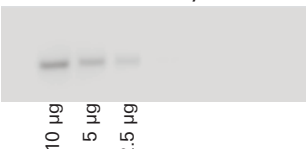
Figure 13	Optimal Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK2</p> <p>10 µg 5 µg 2.5 µg</p>	 <p>NIH/3T3 Lysate</p> <p>ERK2</p> <p>10 µg 5 µg</p>
Substrate	SuperSignal® West Pico	SuperSignal West Pico
Incubation time	5 min	No incubation
Performance	LOD – 2.5 µg	LOD – 5 µg

**Figure 13. Performance differences of a Western blot detected with SuperSignal West Pico when doing a 5-min substrate incubation as opposed to not doing a substrate incubation.**

## Possible cause # 10: Substrate was diluted

**Precaution/Solution:** Do NOT dilute your substrate.

**Why this matters:** Rate of reaction is determined by the ratio of enzyme to substrate. Diluting the substrate will dramatically impact the overall generation of light. **See Figure 14.**

Figure 14	Optimal Blot	Unsatisfactory Blot
Images	 <p>NIH/3T3 Lysate</p> <p>ERK2</p> <p>10 µg 5 µg 2.5 µg 1.25 µg</p>	 <p>NIH/3T3 Lysate</p> <p>ERK2</p> <p>10 µg 5 µg 2.5 µg</p>
Substrate	SuperSignal West Dura <sup>1</sup>	SuperSignal West Dura <sup>1</sup>
Dilution	Not diluted	Diluted 1:1 (in water)
Performance	LOD – 1.25 µg	LOD – 2.5 µg

**Figure 14. Performance differences of a Western blot detected with SuperSignal West Dura that has not been diluted compared to an identical blot in which the substrate has been diluted 1:1 in water.**

<sup>1</sup> Comparable to WesternSure® PREMIUM



Global Headquarters, Serving the United States

4647 Superior Street • Lincoln, NE 68504 • Phone: +1-402-467-0700 • Toll free: 800-645-4267 • Fax: +1-402-467-0819  
biosales@licor.com

### Regional Offices

LI-COR GmbH, Germany, Serving Europe, Africa, and the Middle East.

LI-COR Biosciences GmbH • Siemensstraße 25A • 61352 Bad Homburg • Germany • Phone: +49 (0) 6172 17 17 771  
Fax: +49 (0) 6172 17 17 799 • bio-eu@licor.com

LI-COR Ltd., United Kingdom, Serving Denmark, Finland, Iceland, Ireland, Norway, Sweden, and UK.

LI-COR Biosciences UK Ltd. • St. John's Innovation Centre • Cowley Road • Cambridge • CB4 0WS • United Kingdom  
Phone: +44 (0) 1223 422104 • Fax: +44 (0) 1223 422105 • bio-eu@licor.com

LI-COR Distributor Network: [www.licor.com/bio/distributors](http://www.licor.com/bio/distributors)