MSD Immuno-Dot-Blot Assays

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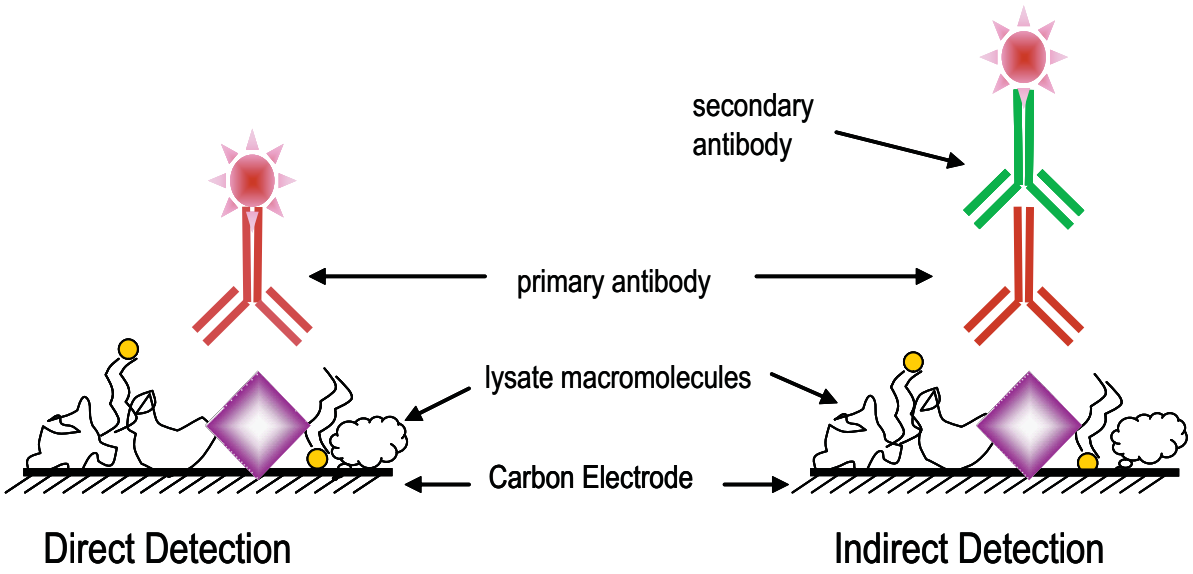
Example: High Throughput Western Blots Replacements

Traditional Western Blots

- High content
- Molecular weight and immunoreactivity
- Labor and protein intensive
- Inherently low throughput– prohibitive for HTS

MSD High Throughput Western Replacements

- Simple workflow, easy to automate
- Reduced protein usage (10-50X)
- Quantitative results
- Monitors abundance and post-translational modifications



Whole cell lysates are non-specifically adsorbed to carbon electrodes at the bottom of MULTI-ARRAY™ plates. After immobilization, a specific primary antibody alone or in combination with a labeled secondary antibody are used to report the species of interest.

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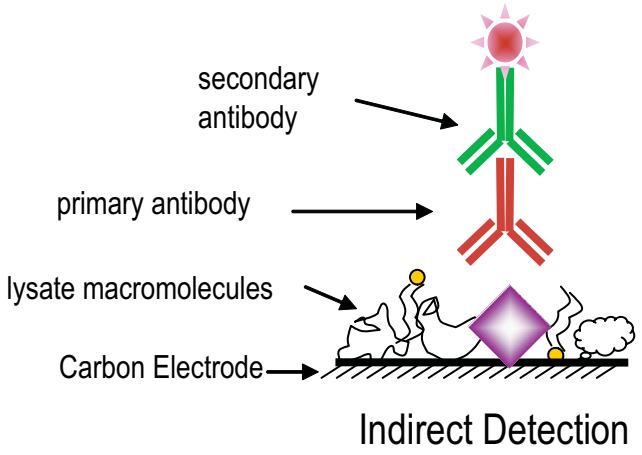
Sample Type: Cell and Tissue Lysates

- We have used RIPA buffers in our system with good success for nuclear and membrane proteins.
- We have noticed that DTT levels greater than 1 mM can reduce signals.
- Denaturing reagents can be used at low concentrations. SDS can be used at levels below 0.1%.
- When studying phosphorylation events, use of Tris-based buffers is essential.
- Perform a lysate titration to find the optimal range of the assay.
- Include appropriate controls of non-phosphorylated lysates or samples diluted with the same matrix.
- MSD Complete Lysis Buffer:

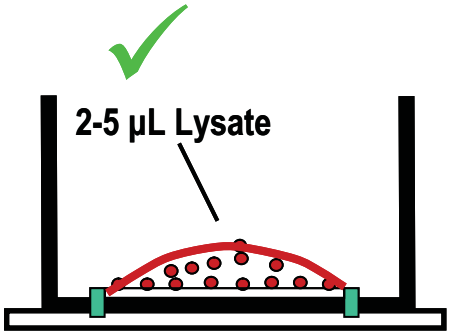
  - 10 ml Tris Saline Lysis Buffer
    - 150 mM NaCl
    - 20 mM Tris, pH 7.5
    - 1 mM EDTA
    - 1 mM EGTA
    - 1% Triton X-100
  - 200 µl Protease inhibitor (50X stock)
  - 100 µl Phosphatase inhibitor 1 (Sigma, Catalog # P-2850, 100X stock)
  - 100 µl Phosphatase inhibitor 2 (Sigma, Catalog # P-5726, 100X stock)

IP Western-Like Assay: Indirect labeled antibody (Spotting)

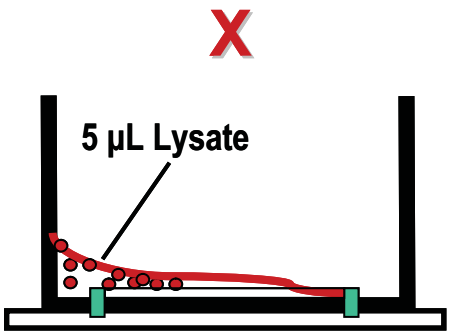
- ***Coating:*** Prepare lysate in RIPA buffer or MSD Complete Lysis Buffer (1% Triton), with a concentration of 1-2 µg/µL total protein. Add 2-5 µL of lysate to a large spot High-Bind plate, pipetting to the center of the wells. Perform a 1:2 lysate titration to determine the linear range of the assay (a plateau in signal is often observed over 2 µg/well). Some lysate preparations contain high surfactant concentrations, and in these cases a 5 µL addition volume will breach the electrode. Use lysis buffer as a test to determine the correct addition volume prior to using lysates. The volume of high surfactant concentration buffers may need to be reduced to 1 or 2 µL per well. Incubate uncovered for 1 hour.
- ***Blocking:*** Add 150 µL of Blocker A (3% Blocker A (w/vol) in Tris Wash Buffer). Seal and incubate on shaker for 1 hour.
- ***Detection antibody addition:*** Wash plates 3 times with 1X Tris Wash Buffer. Dilute detection antibodies to 1-3 µg/mL in a common volume of Tris Wash Buffer containing 1% Blocker A. Dilute secondary antibody to the same concentration as the primary detection antibody (in the same solution). Add 25 µL of antibody solution to wells. Seal and incubate on shaker for 1-2 hours. (This step may proceed overnight).
- ***Read:*** Wash plates 3 times with Tris Wash Buffer. Prepare 1X Read Buffer T by diluting the 4X stock in distilled water. Add 150 µL to each well and read on the SECTOR™ Imager.



Indirect Detection



Nice dome situated over the electrode, but not breaching to the edge



Breaching can be caused by excessive volume, surfactant, or poor spotting technique.

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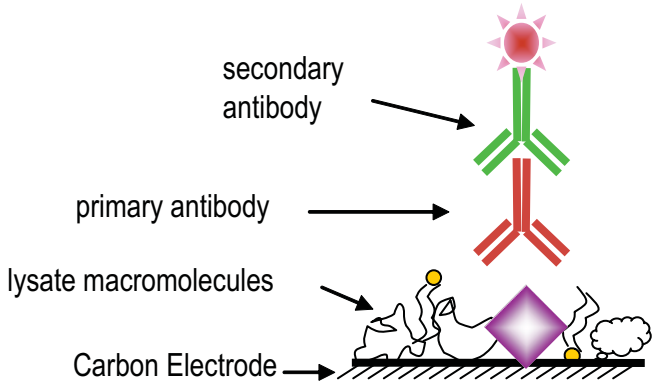
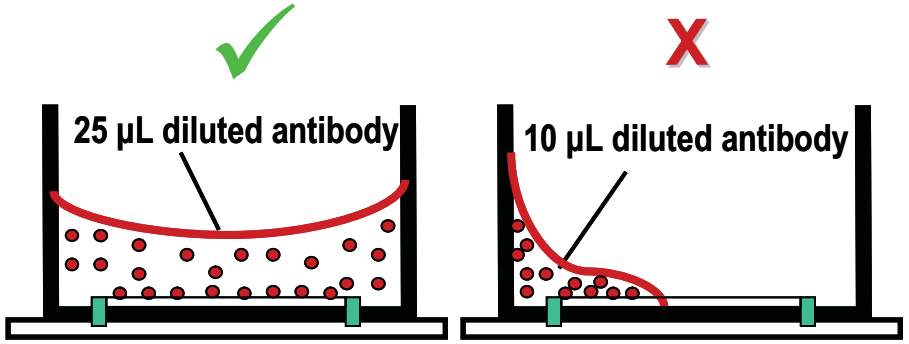
IP Western-Like Assay: Indirect labeled antibody (Solution)

- ***Coating:*** Prepare lysate in RIPA buffer or MSD Complete Lysis Buffer (1% Triton), with a concentration of 0.2-0.5 μg/μL total protein. Add 25 μL of lysate to a large spot High-Bind plate, pipetting to the center of the wells. . Perform a 1:2 lysate titration to determine the linear range of the assay . Incubate uncovered for 1-2 hour, sealed for 4 hours at room temperature, or overnight, sealed at 4°C.
- ***Blocking:*** Remove lysates. Add 150 μL of Blocker A solution (3% Blocker A in Tris Wash Buffer). Seal and incubate on ashaker for 1 hour.
- ***Detection antibody addition:*** Wash plates 3 times with 1X Tris Wash Buffer. Dilute detection antibodies to 1-3 μg/mL in a common volume of Tris Wash Buffer containing 1% Blocker A. Dilute secondary antibody to the same concentration as the primary detection antibody (in the same solution). Add 25 μL of antibody solution to wells. Seal and incubate on shaker for 1-2 hours. (This step may proceed overnight).
- ***Read:*** Wash plates 3 times with Tris Wash Buffer. Prepare 1X Read Buffer T by diluting the 4X stock in distilled water. Add 150 μL to each well and read on the SECTOR Imager.



Indirect Detection



Good even immobilization across entire electrode

Uneven use of electrode leading to poor sensitivity, increased variability, and background from exposed carbon

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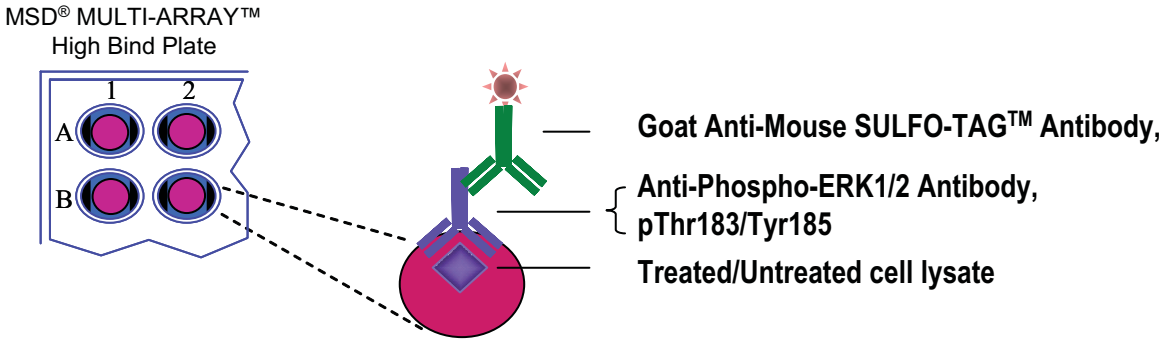
Western Blot Replacement Assays: Notes

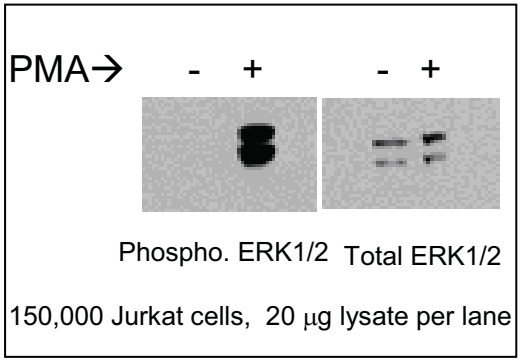
- *Choice of antibody is very important.* If the antibody recognizes multiple bands on the Western Blots, the MSD signal will be an integration of all of the bands. Modulation of the band of interest will be only a fraction of the total signal and can result in high basal signals.
- *The western blot looks good, but I do not get any response on the MSD assay.* Assays recognize native conformation of the proteins and not denatured proteins like western blots. Most antibodies recognize denatured forms of the protein of interest.
- *Assay is not linear with dilutions of the lysate or is flat at the region of interest.* The adsorption of lysate to the electrode is a competition between the protein of interest and the other proteins in the lysate. The signal measured is measuring the amount captured relative to the total protein. This may not be linear with dilution or the total protein may be blocking out the specific protein. There may be an increase in signal with a decrease in lysate amounts.
- *What are the appropriate signal levels*. The signal levels should not matter as long as there is a difference between stimulated and unstimulated lysates. Typically signals between 100 and 1 million are observed. The specificity of the signals is determined by the user
- *There is a high background level that cannot be removed.* If there are multiple bands on the western blot, then those bands will contribute to the overall signal seen in the well. These non-specific bands may not be affected by the stimulus so they contribute to the background.
- *Direct labeling of the detection antibody may improve sensitivity.* If the detection antibody is carrier-free, it can be labeled with MSD's SULFO-TAG NHS-Ester. Often this can improve sensitivity by 3-5 fold.

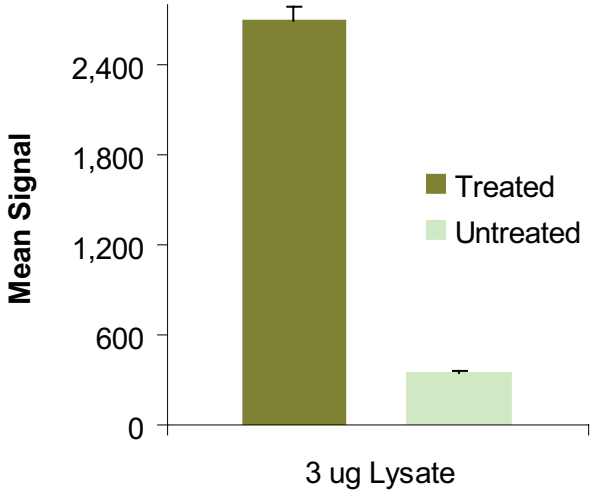
See http://www.meso-scale.com/literature/notes/pdf/WesternBlot.pdf for additional information.

Detection of Phosphorylated Erk1/2 in Whole Cell Lysates – Western ECL



Traditional Western





Logarithmically growing Jurkat cells were treated with 200 nM PMA for 30 minutes. Whole cell lysates were immobilized on MSD MULTI-ARRAY 96-well plates and blocked. Phosphorylated ERK1/2 was detected with 5 nM anti-phospho-ERK1/2 antibody (monoclonal) and SULFO-TAG labeled Goat anti-Mouse antibody.

Lysate	Treated			Untreated			S-B	S/B
(µg)	Ave	Std.Dev.	%CV	Ave	Std.Dev.	%CV		
3	2,687	94	3	347	8	2	2,340	7.7

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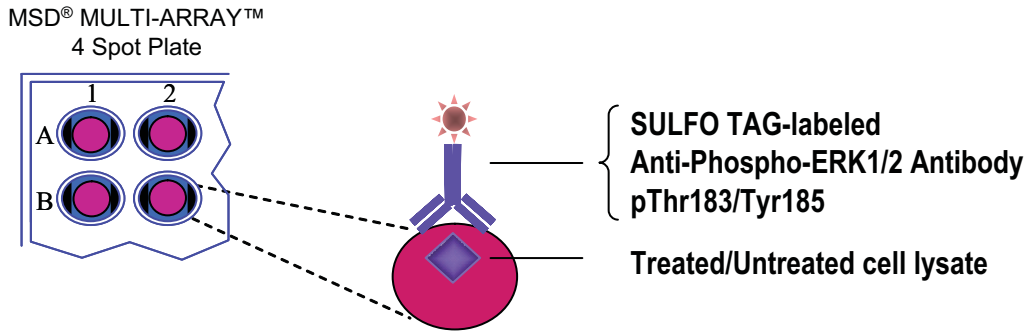
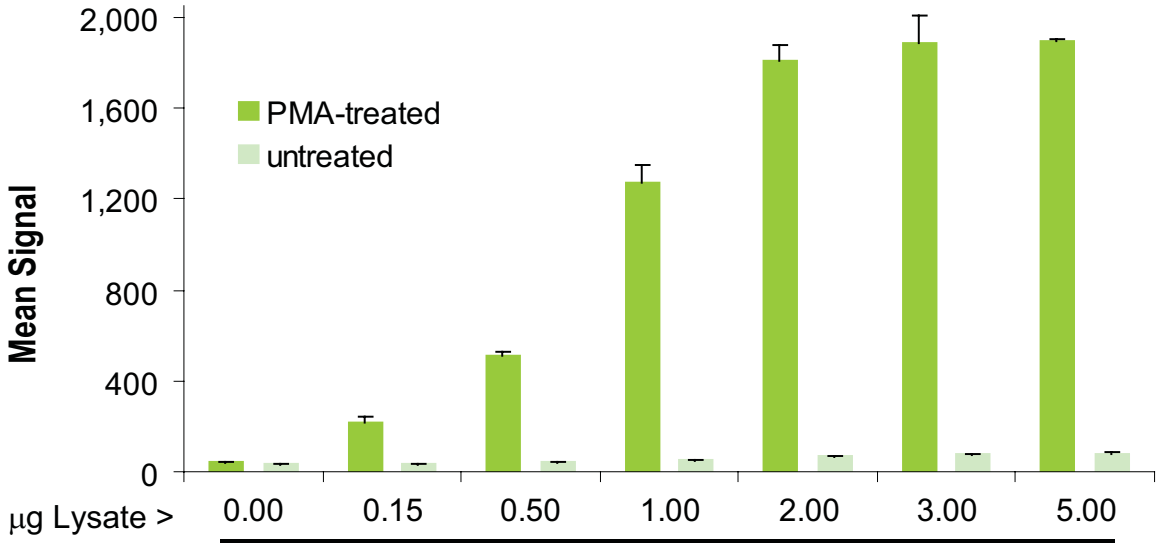
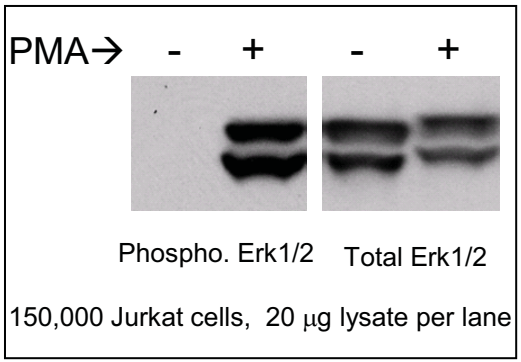
Detection of Phosphorylated Erk1/2 in Whole Cell Lysates – Western ECL





Lysate	PMA-treated			untreated			S-B	S/B
≅g	Ave	S.D.	%CV	Ave	S.D.	%CV		
0.00	40	3	7	36	1	3	4	1.1
0.15	220	23	10	32	3	10	187	6.8
0.50	509	16	3	41	1	1	468	12.3
1.00	1,268	84	7	53	1	2	1,215	23.9
2.00	1,812	64	4	68	2	3	1,744	26.6
3.00	1,889	114	6	74	6	9	1,816	25.6
5.00	1,896	10	1	79	4	5	1,817	24.1

Traditional Western



Logarithmically growing Jurkat cells were treated with 200 nM PMA for 30 minutes. Whole cell lysates were immobilized on MSD MULTI-ARRAY 96-well plates and blocked. Phosphorylated Erk was detected with 10 nM anti-phospho-ERK1/2 antibody (monoclonal) labeled with SULFO-TAG reagent.

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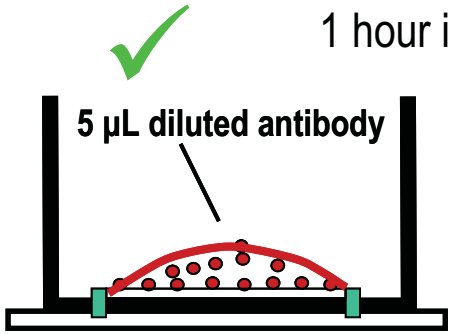
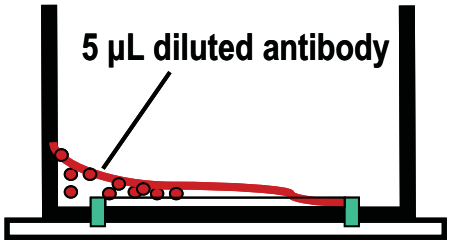
Other Technology Points and Concepts

Plate Preparation: Spotted vs. Solution Coated

- Example: High Bind 96-well Plate (spotting on electrode)
  1. Add 5 µL of 25 µg/mL antibody diluted in PBS or TBS directly to the center of the electrode.
  2. Leave unsealed at room temperature. Allow to dry overnight. Can be used with only 1 hour incubation.

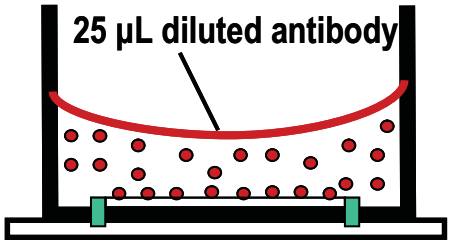


Nice dome situated over the electrode, but not breaching to the edge

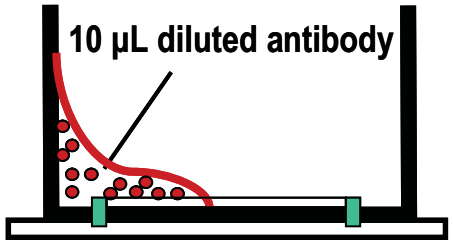


Breaching can be caused by excessive volume, surfactant, or poor spotting technique.

- Example: Solution Coating a High Bind Plate.
  1. Add 25 µL of antibody diluted to 1 µg/mL.
  2. Place on shaker for 60 minutes to make sure it covers the bottom. Seal and incubate overnight at room temperature.



Good even immobilization across entire electrode



Uneven use of electrode leading to poor sensitivity, increased variability, and background from exposed carbon

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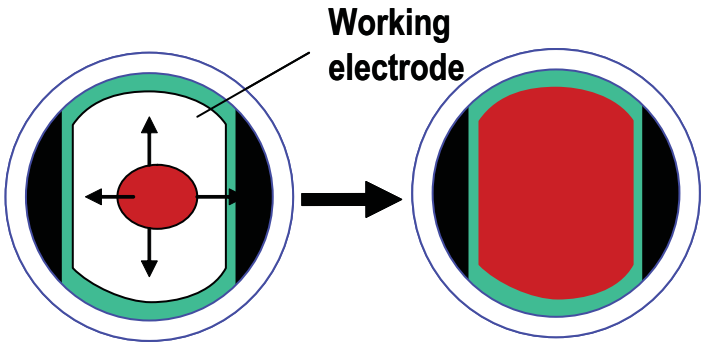
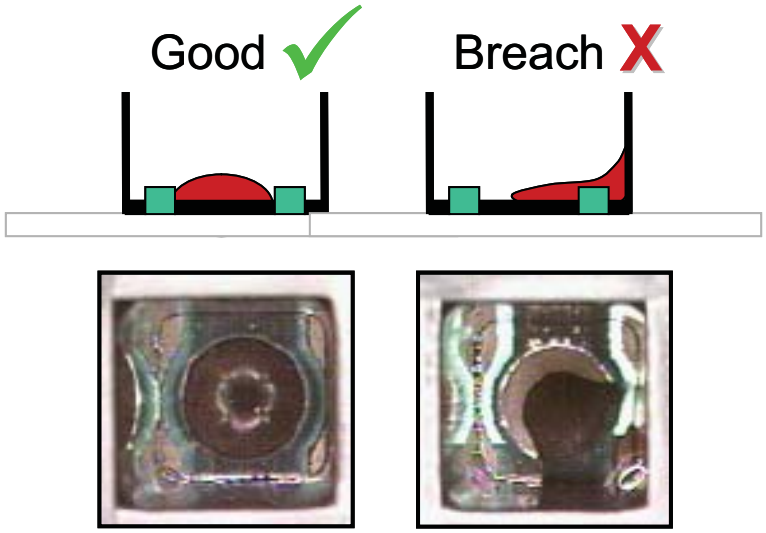
Plate Preparation: Spotting Uncoated Plates

- Necessary when there is no linker, or same antibody used for capture and detection without labeled antibodies.
- Dispense the fluid by touching the plastic tip to the electrode and then dispensing the fluid.
- Volumes are critical so that the fluid does not breach the working electrode: 2-5 μL for a 96 well plate; 0.25-1.5 μL for a 384 well plate, 0.5-1.0 μL for a 96 well small spot plate.
- Surfactant is necessary for coating Standard plates since the surface is hydrophobic. Try a concentration of 0.015% Triton in the coating buffer of choice (PBS, TBS, Hepes).
- Coat Standard plates and allow them to air dry overnight.



Spread of solution to completely cover the working electrode.



Photograph of 1 μL of antibody spotted to a 384-well MULTI-ARRAY plate

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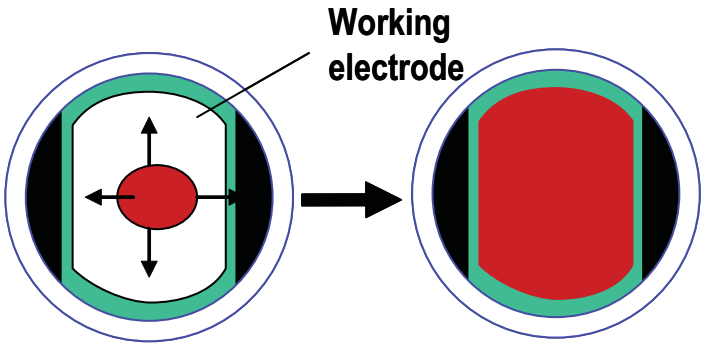
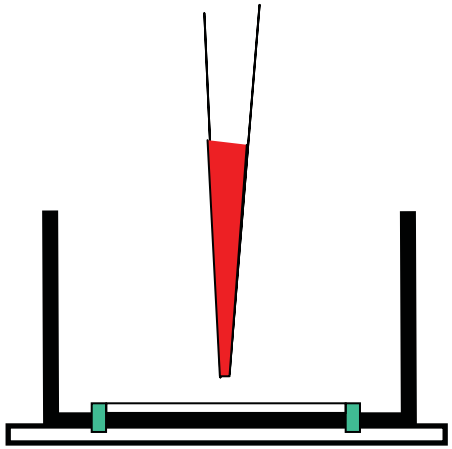
Plate Preparation: Spotting Techniques Procedure

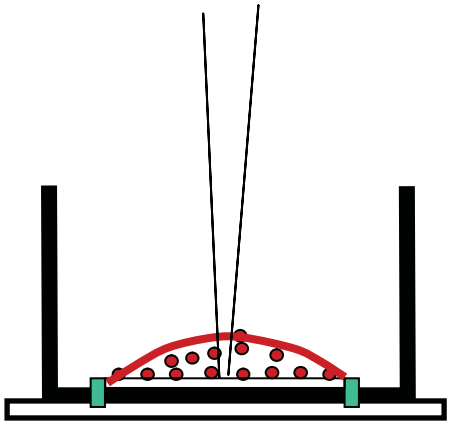
- Practice dispensing onto electrode with blank buffers before spotting lysates, proteins, or antibodies.
- Make sure the buffer is same buffer used for dilution of the lysate, protein, or antibody (lysis buffer, PBS, or TBS).



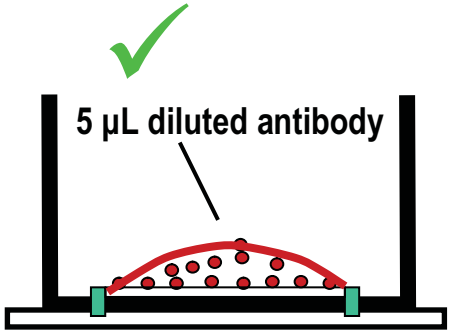
Spread of solution to completely cover the working electrode.



Start dispensing fluid just above the electrode surface.



Dispense remaining fluid and touch the electrode



Nice dome situated over the electrode, but not breaching to the edge

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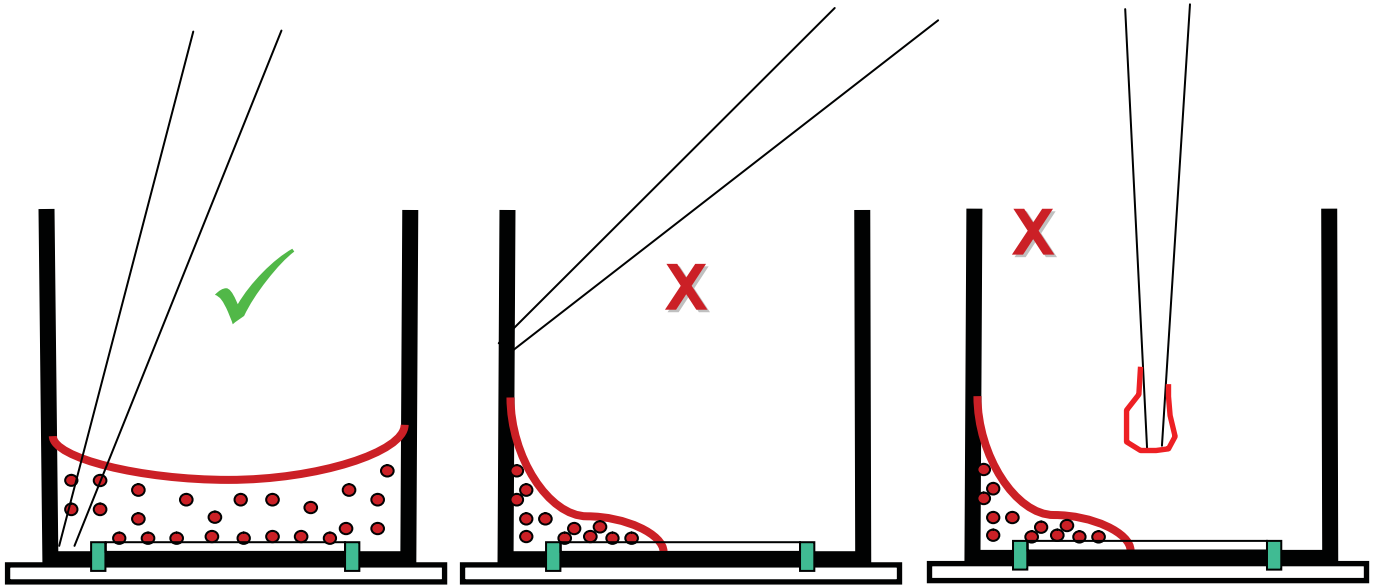
Small Volume Pipetting: Pipetting into Corners for Solution Coating

- You can solution coat by putting 25 µL onto the electrode surface, but be careful of losing fluid that may wick up the side of the pipette tips.
- The plates are robust enough to withstand touching plastic pipettes to the bottom of the plate. When pipetting low volumes into a dry plate, pipette into the bottom corner of the plate, then tap the plate to make sure the solution covers the entire bottom.



Dispense into the bottom corner of the plate.

Pipetting to the side, but on the top causes higher variability because of differences in "dropping" to the surface.

Without touching the fluid or the side, there is risk of leaving fluid on the pipette tip and causing higher variability.

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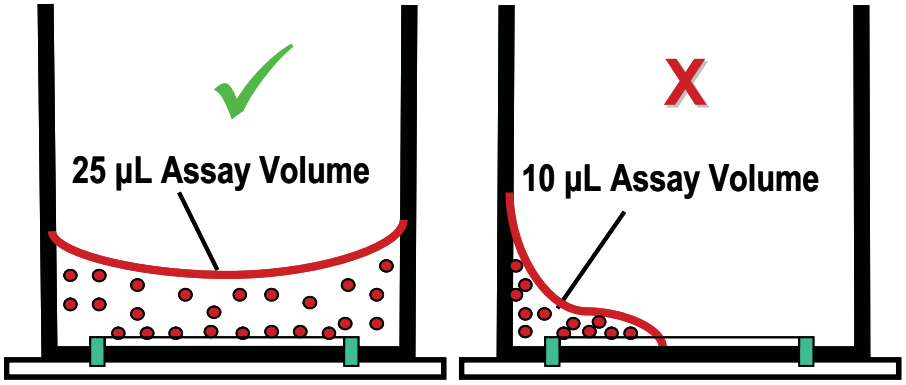
Small Volume Pipetting: Appropriate Volumes

- Lower volumes can be used because binding to the plate occurs only at the bottom of the well. The greater the well volume, the longer it takes for molecules to diffuse to the surface for binding. The reaction time can be accelerated by shaking (explained later).
- Recommended assay volumes range from 20-50 µL for 96 well plates and 10-25 µL for 384 well plates.



Good even coverage over the working electrode

Uneven use of electrode leading to poor sensitivity, increased variability, and background from exposed carbon

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Plate Preparation: Plate Capacity of Uncoated Plates

Approximate Optimal Immobilization Amounts (pmol)			
Plate Type	**96 Well**	**384 Well**	**96 Well Small Spot (4 spot)**
Standard Plate	**1**	**0.2**	**0.2**
High Bind Plate	**5**	**1**	**1**

- It is important to convert from concentration to absolute amounts of protein. The coating solution concentration must be at least 1.5 μg/mL for spotting uncoated plates.
- When coating from crude samples (lysates, serum, antibodies with BSA), there is competition between the protein of interest and other proteins. Proteins in serum cannot be directly spotted on the uncoated plate surface. When spotting lysates, 2.5 μg/well will saturate a large spot 96-well surface.
- BSA (up to 5 fold excess) may be present in the coating solution. With the more limited capacity of standard plates, the presence of BSA may not work as well. It may also lead to poor results for antibodies that are in more crude matrices.
- The capacity may be lower on pre-coated plates such as Strepavidin, Avidin, and Anti-Species.

Working Volume Guidelines

Optimal Volumes			
	96 Well	384 Well	
Spotting/Coating	2-5 µL	0.5-2 µL	
Immobilization from solution/ Minimum volumes in well at any given time	20-30 µL	10-15 µL	

96 Well: Do not use volumes between 5 and 20 µL in an empty well.

384-Well: Do not use volumes between 2 and 10 µL in an empty well.

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Appropriate Signal Levels

- The SECTOR Imager instruments have signals that range from near zero to 1-2 million counts.
- Conservative estimates for the instrument noise for the SECTOR 6000 for the different plates are as follows: 10 counts for 96-well single spot; 15 counts for 96-well 4-spot; 20 counts for 96-well 7-spot; 30 counts for 96-well 10-spot.
- Low count signals are significant if the noise in the assay is well controlled. Assuming the assay is instrumentation noise limited, then in a 4-spot plate, 45 counts is 3 standard deviations of the instrument noise. Therefore, 45 counts over the background is significant.
- Always check to see if the signal ratios are comparable to western blots or another standard technology.
- Signals may be increased by directly labeling antibodies and increasing the labeling ratio.
- There can be a 3-5 fold increase in signals or sensitivity when detection antibodies are directly labeled as opposed to using an anti-species antibody.

Running Partial Plates

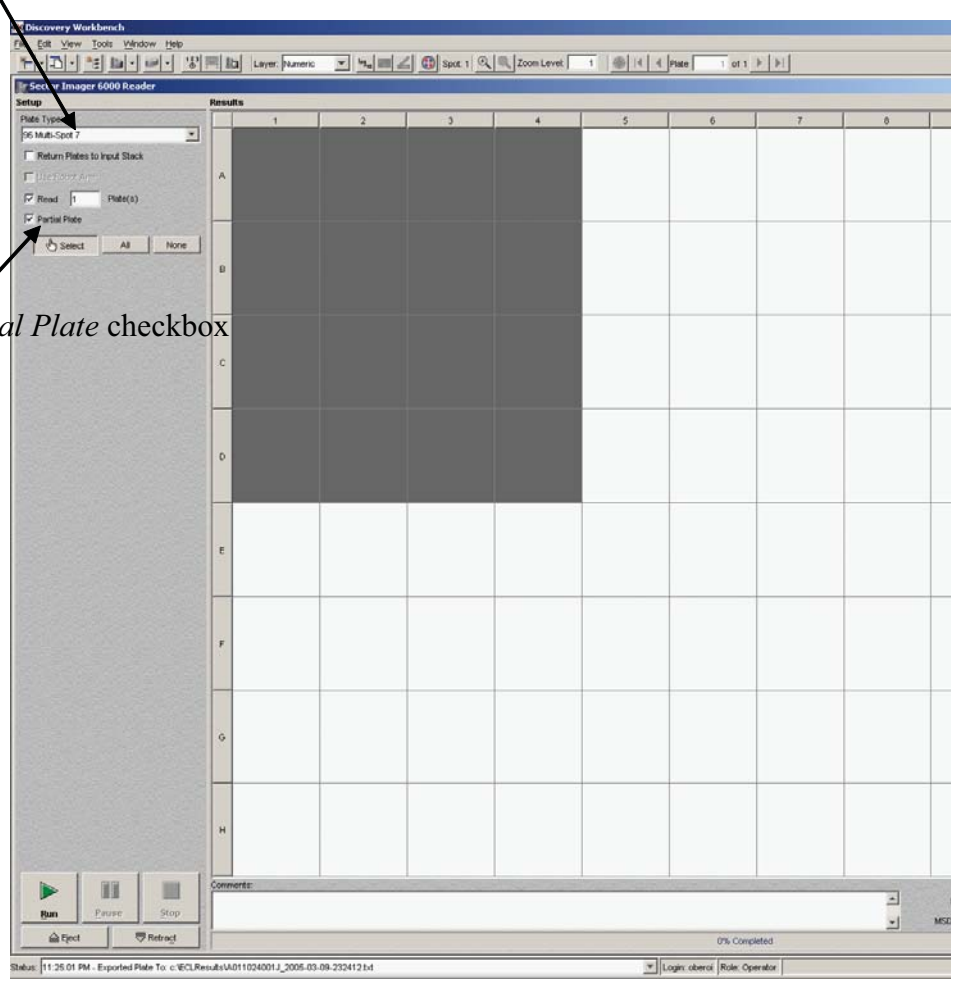
To run a partial plate:

1. Select the plate type from the *Plate Type* pull down menu. Once you do this, a *Partial Plate* checkbox will appear grey. If you are unsure of the plate type, please check the plate packaging for the appropriate plate type.
2. Select the number of plates to activate the *Partial Plate* checkbox.
3. Check the *Partial Plate* checkbox, and then select the Sectors to be read by highlighting them in the plate display.
4. Hit the run button (only the selected sectors will be read).

Warning: If the wrong plate type is selected, the entire plate will be read and coated wells that were not used as part of the experiment could be damaged.

Plate Type pull down menu

Partial Plate checkbox



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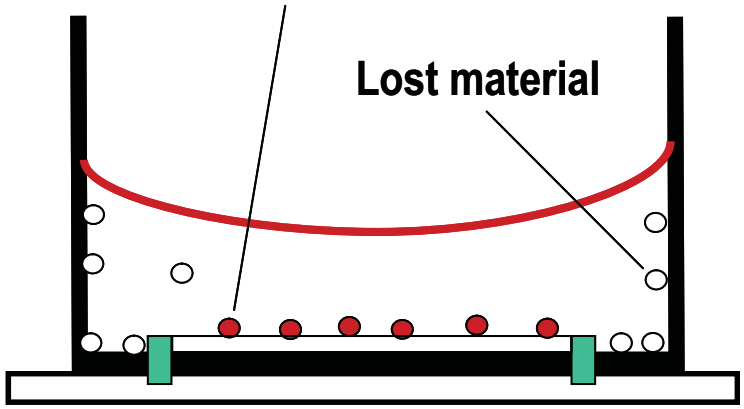
Blocking Strategies

- Blocking reduces non-specific binding to carbon surfaces. High backgrounds may result from something in the sample binding to the carbon surface. For example, if looking for a specific antibody in serum, other antibodies in the same serum could bind to the carbon surface.
- Blocking also prevents the loss of materials to non-productive areas of the well, thereby increasing signal.

Material that will be read
(bound at working electrode)



Material that will be read
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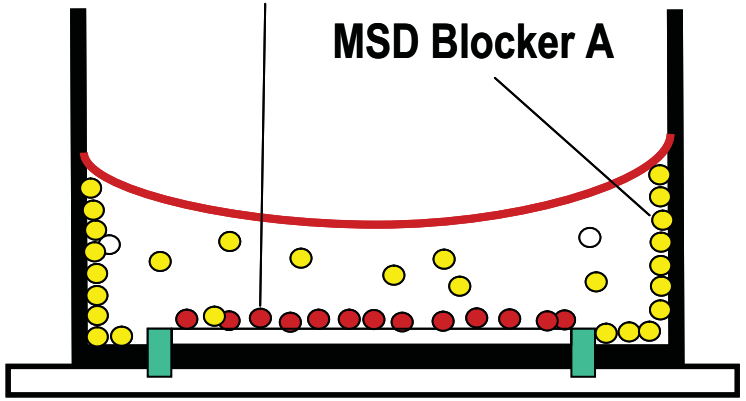

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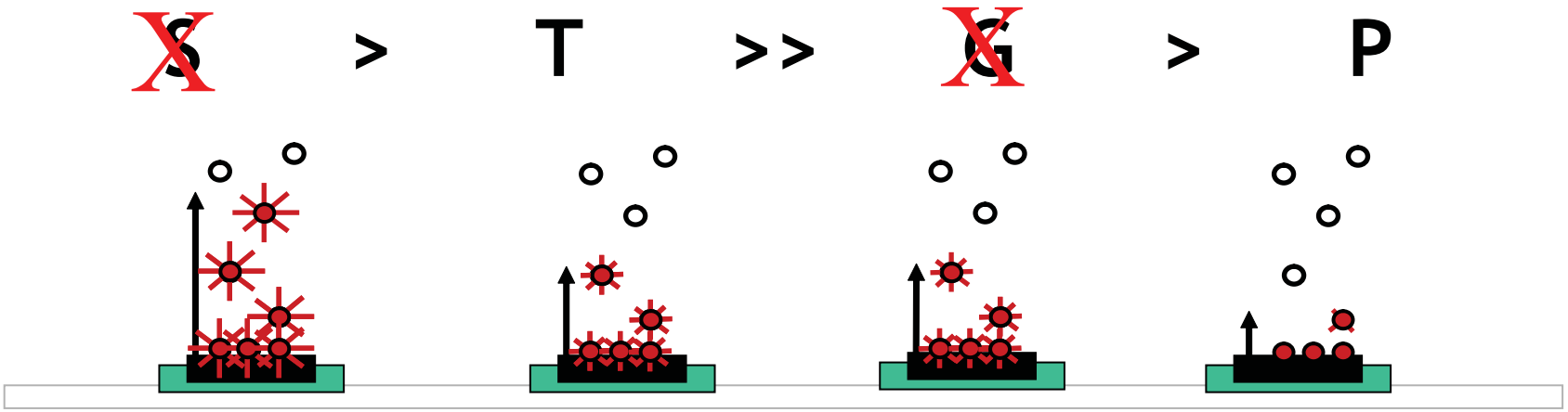
Other Blocking Agents

- Carbon surfaces have a high capacity to adsorb all sizes of molecules. It is import to make sure the blocking agent contains the desired type of molecules.
- Serum based assays may need additional IgG, IgM, and IgAs. Animal serums can be used as blocking agents to mimic serum matrix effects and non-specific binding to carbon surfaces.
- Peptide-based assays may require random peptide sequences for blocking plates.
- Some assays have been shown to work better with a casein or milk-based blocker solution, or Blocker B.
- Special coated plates with PEI have been shown to reduce the loss of ligands to the plastic in many receptor-ligand assays.

MSD Read Buffers

- Read Buffer supplements the assay with the necessary co-reactant for electrochemiluminescence
- Can be incorporated into assay buffer and added prior to analysis
- Available surfactant-free for intact cell or isolated membrane-based applications
- Each impart unique electrochemiluminescent *distance* and signal *efficiency*:



Highest Signal,
Highest Background

Lowest Signal,
Lowest Background

Meso Scale Discovery
A division of Meso Scale Diagnostics, LLC.
www.mesoscale.com

MSD Read Buffers: Optimal Imaging Volume

- MSD SECTOR Instruments read at a fixed focal length
- Focal plane set at meniscus:
  - 96 well: 150 μL
  - 384 well: 35 μL
- Difference between assay or reaction volume and optimal imaging volume is usually made up with appropriately concentrated Read Buffer
- Some assays can be read in more or less volume without major effect
- Read Buffers supplied as a concentrated 4X stock
- Dilute with deionized water
- Sensitive optics require that no bubbles are present in the wells when reading a plate