Validation of Antibodies on Protein Arrays

The Antibody Society Webcast series – Antibody Validation #8

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Antibody validation: problem and solution

• Use of antibodies depends on target specificity and selectivity. But they often cross-react

• This leads to misleading or erroneous data

• Validation of specificity is therefore critical for antibody reagents and therapeutics

• A solution for anti-protein antibodies: screen for binding to the widest range of proteins possible

• Efficient and economical using protein arrays
What can be learned from protein arrays for antibody validation?

Protein Arrays can address essential questions for validation of **antibody specificity and selectivity**:

Does the antibody:

- Recognize its nominal target?
- Cross-react with other proteins. If so, what is its relative binding to them?
- Working concentration affect its cross reactivity?
- Recognize native (conformational), denatured (linear) or modified epitopes?
Features of protein arrays

- Miniaturised solid phase binding assays: with hundreds or thousands of surface-immobilised proteins
- Highly multiplexed, parallel screening of interactions
- Content expandable to proteome scale
- Low antibody consumption; high sensitivity
- Extensive data from single experiments
Screening applications of protein arrays

- Antibody and binder validation: format-independent definition of specificity and cross-reactivity (mAbs; recombinants; nanobodies; scFv; polyclonals; alternative scaffolds)
- Autoantibodies (plasma, sera): identify novel targets
- Protein-protein interactions
- Protein-DNA, -RNA, -small molecule interactions
- Protein target modifications
- Epitope mapping on peptide arrays

Note: Biomarker screens require antibody arrays (not covered in this presentation)
# Protein array technology issues

<table>
<thead>
<tr>
<th>Issue</th>
<th>Steps and variables</th>
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</thead>
<tbody>
<tr>
<td>Protein production</td>
<td>Prokaryotic, eukaryotic, cell free; tags, modifications; native, denatured; soluble, membrane</td>
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<tr>
<td>Surface</td>
<td>Functionalised glass, nitrocellulose, beads, hydrogel</td>
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<tr>
<td>Attachment chemistry</td>
<td>Covalent (epoxy), adsorption (nitrocellulose), tagged</td>
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<tr>
<td>Spotting</td>
<td>Inkjet, contact, spot dimensions, concentration</td>
</tr>
<tr>
<td>Incubation conditions</td>
<td>Native, denatured</td>
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<tr>
<td>Detection</td>
<td>Secondary Ab, streptavidin, directly labelled</td>
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<tr>
<td>Scanning</td>
<td>Fluorescence (10µm resolution), 2 or more channels</td>
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<tr>
<td>Data analysis</td>
<td>Grid alignment, numerical data extraction, processing (e.g. R, Excel), filtering, z-scores, ranking</td>
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Construction of a human proteome array

Jeong J S et al. Mol Cell Proteomics 2012
Workflow for antibody validation on protein arrays

1. Incubate test antibody on array
2. Antibody binds to specific and x-reactive spots
3. Incubate, wash
4. Binding revealed with labelled 2ry reagent
5. Scan image

<table>
<thead>
<tr>
<th>Rank</th>
<th>Name</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protein ABC</td>
<td>55,000</td>
</tr>
<tr>
<td>2</td>
<td>Protein XYZ</td>
<td>3,000</td>
</tr>
</tbody>
</table>
Protein array data analysis: z score

Log₂ fluorescence intensity distribution

- $z \geq 3$
  - $P = 0.001$
  - 32 in 23,800

- $z \geq 3.5$
  - $P = 0.0002$
  - 6 in 23,800

- $z \geq 4.0$
  - $P = 0.00003$
  - 0.8 in 23,800

- $z \geq 5.0$
  - $P = 2.9 \times 10^{-7}$
  - 0.007 in 23,800

Antibody validation screening examples

Specific antibody

Cross-reactive antibody

Broadly reactive (sticky) antibody

Ab 1

Ab 2

Ab 3

Target circled
Antibody screening on denatured arrays

- Arrayed proteins can be denatured by treatment with 8M urea/DTT, or heat
- Antibodies are tested on native and denatured arrays in parallel
- Results can be compared with WB or IHC to identify cross-reactive bands or off-target staining

Diagram:
- **Antibody screening**
  - ~ 5 µg/ml
- **Denature**
  - 1 Array: Urea / DTT
  - 2 Array:
    - Test antibody + 2nd Ab
    - Test antibody + 2nd Ab

2 Protein Arrays
Antibody screening on denatured arrays: examples

- Anti-SMAD4 binding after array denaturation is consistent with linear epitope
- Anti-ACO2 binding to a conformational epitope is lost by array denaturation

Hu et al., CDI Laboratories & Johns Hopkins School of Medicine
Benefits of antibody validation on protein arrays

- Sensitive screening of binding against hundreds or thousands of proteins in parallel
- Confirmation of primary antibody target specificity
- Identification of cross-reactive and off-target protein binding with ranking by relative binding strength
- Detection of linear and conformational epitopes
- Data to facilitate decision on use of antibody reagents in particular applications
- Data to guide use of a therapeutic Ab
Questions?