HCP immunoassays - The Science, the Art, and the Integration of orthogonal analytical methods

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What are they and where do they come from?

Why do we care?
- Clearance demonstrates process consistency
- Potential to increase immunogenicity
- Potential to impact PK of the Drug Product
Methods to Monitor HCP Clearance

- 1D Western blot
- 2D Western blot
- Immunoassays (variations of sandwich assays to include ELISA and most automated platforms)
- Biosensors
- Mass Spectrometry
Methods to Monitor HCP Clearance

1D Western blot

- Pros:
  - Quick assessment of reactivity
  - May be able to detect abundant HCPs

- Cons:
  - Lack of sensitivity and selectivity
  - Product protein can interfere with separation
  - Non-quantitative
Methods to Monitor HCP Clearance

2D Western blot

- **Pros:**
  - Better separation from the product protein
  - Better estimation of number of abundant HCPs
- **Cons:**
  - Poor selectivity
  - Non-quantitative
  - Requires an expert to run the method
Methods to Monitor HCP Clearance

Immunoassays (ELISA)

- Pros:
  - Gold Standard for monitoring clearance and release testing
  - Semi-quantitative
  - High sensitivity and selectivity
  - Does not require special level of expertise to run
  - Effective in relatively high levels of product protein

- Cons:
  - No information about what HCPs are present
  - Requires an expert to develop the method
Methods to Monitor HCP Clearance

Biosensors

• Pros:
  • Less influenced by sample matrix
  • Semi-quantitative

• Cons:
  • Expensive instrumentation
  • Relatively slow
  • Requires expert to develop the method
  • Sensitivity not as good as ELISA
Types of HCP ELISA

**Generic:** An assay that attempts to measure HCP for a specific expression platform. Uses an antigen from a representative strain of the species for animal immunizations. Generic assay assume that a large majority of proteins will be conserved across strains of the same species.
Types of HCP ELISA

Platform: An assay that is designed to work for a specific strain of a host cell line using a standard cell culture process. An organization may establish a platform HCP ELISA to readily have analytics in place for newer projects.
Types of HCP ELISA

Cell Culture Process Specific: An HCP ELISA designed for a specific strain of host cell using a specific cell culture process. This is usually used for later stage projects with a locked cell culture process.
Types of HCP ELISA

Purification Process Specific: This type of assay uses a downstream sample as the antigen source for immunizing animals.
Critical Decisions for Development of an HCP ELISA

- Antigen Selection
- Antigen Processing
- Immunization procedure
- Animal species and number
- Antibody Purification
Critical Decisions for Development of an HCP ELISA

Antigen Selection

• First critical decision in developing an HCP ELISA
  • Source of cells to use
    • Production cell line
    • Mock transfected
    • Null cells
  • What is the source of the antigen for immunizations and assay standards
    • Harvest media
    • Lysed cell paste
    • Combination
Critical Decisions for Development of an HCP ELISA

Antigen Processing
• How to prepare the antigen for success
  • Cells cultured in protein free media
  • Removal of cellular debris
  • Removal of low molecular weight peptides
  • Modification of the antigen to increase immunogenicity??
Critical Decisions for Development of an HCP ELISA

Immunization Procedure

Immunization scheme
• Standard boost
• Cascade Immunization
• Adjuvant
• Immunization sites
Critical Decisions for Development of an HCP ELISA

Animal Species and Number to use

or
Critical Decisions for Development of an HCP ELISA

Protein A/G Purification:
- Simple way to purify antibodies
- Greater than 90% of antibody on the plate is dead with respect to the assay
- Generally produces assays with poorer sensitivity

Affinity Purification:
- Requires certain level of expertise to make the affinity column
- Almost 100% of antibodies are specific for HCP
- Allows for superior levels of sensitivity
- Less issues with poor dilutional linearity
Qualification of HCP ELISAs

- Dilutional Linearity
- Coverage
- Accuracy
- Precision
Dilutional Linearity

- Dilutional linearity demonstrates that the array of HCPs in a sample are a good fit for the array of antibodies in the ELISA.
- First step of assay qualification and has to be performed on all sample types to be tested using the ELISA.
- Also used to establish the “Minimum Required Dilution” (MRD) for each sample type. MRD is defined as the minimum dilution required that each subsequent dilution, when adjusted for dilution, yields the same result.

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Coverage Analysis

• Performed to demonstrate that the antibodies used in the ELISA are broadly reactive to the HCPs in the expression platform

• Common methods:
  • 2D Western blot compared to silver stain
  • Antibody Affinity Extraction (AAE)
    • Silver stain detection
    • DIGE detection

• Specification:
  • Reactive with the majority of spots found on the silver stain gel
  • Must have spots in each quadrant of the gel where spots are found on the silver stain.
Large Format 2D Silver Stain
Accuracy:

• Accuracy is test to assess the ELISAs ability to recover known amounts of HCP in the presence of sample matrix.
• Performed by assessing spike recovery in all sample types to be tested in the assay
• How to determine accuracy???
Accuracy

ICH Q2(R1):
• Testing: A minimum of 9 determinations at 3 concentration levels covering the analytical range of the assay (3 replicates at 3 concentrations)
• Specifications: Not stated

US FDA:
• Testing: A minimum of 5 determinations at 3 concentration levels covering the analytical range of the assay (5 replicates at 3 concentrations)
• Specifications: The mean value should be within 20% of the expected value except at the LLOQ, where the mean value should be within 25% of the expected value

EMA:
• Testing: A minimum of 5 determinations at 4 concentrations (at the LLOQ, within 3X the LLOQ (low QC), 30-50% of the curve (medium QC), at least 75% of the upper curve (high QC). (5 replicates at 4 concentrations)
• Specifications: The mean value should be within 15% of the expected value except at the LLOQ, where the mean value should be within 20% of the expected value
Precision:

- Precision is the closeness of agreement between multiple measurements.
- For qualification assessing Repeatability is sufficient.
- How to test for repeatability???
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- **Testing:** A minimum of 5 determinations at 3 concentration levels covering the analytical range of the assay (5 replicates at 3 concentrations)
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- **Testing:** A minimum of 5 determinations at 4 concentrations (at the LLOQ, within 3X the LLOQ (low QC), 30-50% of the curve (medium QC), at least 75% of the upper curve (high QC). (5 replicates at 4 concentrations)
- **Specifications:** The within-run and between run CV value should not 15% for the QC samples and an allowance of 20% at the LLOQ.
Common Problems and How to Correct Them:

**Problem:** Observed Low OD values

**Diagnosis:**
- Incorrect plate shaker and/or plate shaking speed
- Low incubation temperature
- Stability of the reagents
Common Problems and How to Correct Them:

**Problem:** High CVs

**Diagnosis:**
- Assay contamination
- Strong plate washing
- Pipetting inconsistency
Common Problems and How to Correct Them:

**Problem:** Poor Dilutional Linearity

**Diagnosis:**
- Have not achieved the MRD
- Hook Effect
- Lack of antibody excess

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## Hook Effect and MRD

### Hook Effect (OD)

![Graph showing Hook Effect (OD)]

### MRD

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Avoiding Common Mistakes in Developing HCP ELISAs
Mistake # 1 – Use of inappropriate antigens to generate antibody and as calibrators

Common misconception - we must make antibody to entire proteome

Goal of an HCP ELISA is not to detect the entire proteome but rather to detect those HCPs that persist through your purification

This leads to mistaken belief you should add materials like lysate or cell debris to mock harvest material (CCM) to get even broader coverage

How can an assay that uses non-process-specific antigen be termed “Process-Specific”?

Qualitative detection of entire proteome and quantitative detection of downstream HCP are often exclusive of each other
Mistake # 2 – Relying on Western blot to drive Ab & ELISA development

Western blot has long been the “Gold Standard for determination of coverage of an HCP antibody to a set array of HCPs

Limitations of Western blot:
- Poor Specificity
- Poor Sensitivity
- Physical alignment of fixed gel and Western blot
- Destruction of conformational epitopes
- Harsh chemical treatment of the proteins

These limitations make 2D Western blot a poor predictor of the performance of the antibodies in an HCP ELISA
Case Study: A controlled experiment of coverage analysis by 2D Western blot and Antibody Affinity Extraction

Determination of coverage of the Cygnus 3G CHO (Cat# F550) kit antibodies to null harvest media

- Western blot
  - Probed with Cygnus 3G CHO HCP kit antibody
  - Probed with Cygnus *E. coli* HCP kit antibody

- AAE
  - CHO null harvest extracted on CHO AAE column
  - *E. coli* HCP extracted on a CHO AAE column

- AAE with 2D-DIGE Detection
  - CHO null harvest extracted on CHO AAE column
  - Starting sample and AAE elution fraction labeled with Cy3 and Cy5 mixed and run on the same gel
Western Blot Probed with 3G CHO Antibody

Silver Stain of Harvest sample

Western blot with 3G CHO Antibody

The 3G CHO HCP recognized 717 of the 1293 total spots for 55% coverage
Western Blot Probed with *E. coli* Antibody

The *E. coli* HCP antibody recognized 571 of the 1191 total spots for 48% coverage.
AAE Analysis of CHO Harvest Sample by CHO AAE column

Silver stain of starting sample

Silver stain of AAE elution fraction

827 of the 1138 total spots were found in the AAE elution fraction for a coverage of 73%
AAE Analysis of E. coli HCP by CHO AAE Column

68.4 mg of E. coli HCP was extracted over the CHO AAE column

• 1.7 micrograms of E. coli HCP was non-specifically bound as detected by the F410 E. coli HCP ELSIA
  • 0.002% of the total E. coli protein

• Demonstrates the poor specificity of the 2D Western and the superiority of the AAE extraction
2D-DIGE detection of AAE

Cy3 Labeled CHO Harvest Sample  Cy5 Labeled AAE Elution Fraction

896 of the total 976 spots are found in the AAE elution fraction for coverage of 92%
Conclusions of the Coverage Analysis case study:

• 2D Western blot is not recommended for determining coverage due to poor sensitivity and specificity.

• AAE with silver stain detection is a superior method in that the issues of sensitivity and specificity are corrected. However, gel to gel variability may still cause an underestimation of coverage.

• AAE with DIGE detection is the superior method of determining coverage.
Misconception #3

Generic and platform assays have the potential to miss certain process-specific HCPs & therefore process-specific assays are superior.

- Literature suggests that there is little difference in HCP profiles regardless of strain, time of culture, or viability at harvest.

- The proof of any HCP antibody or assay is not in terminology we use but rather in the qualification and validation of the assay.
Misconception #4
ELISAs tend to under-estimate HCP & the major reason for this is lack of antibody coverage

- The major reason for under-quantitation of "total HCP" is lack of antibody excess

- ELISA has about the same tendency to over-estimate total HCPs as to under-estimate

  Even a good ELISA is only semi-quantitative with a quantitative range of uncertainty of about 4 fold
Mistake # 5 – Failure to understand ELISA stoichiometry

• Many labs see high HCP results and immediately think it must be cross reactivity to the product

• For ELISA to be quantitative for each HCP there must be an excess of Ab to that HCP
  – Average Coating Ab: ~1 ug/well
  – Average Detection Ab: <100 ng/well

• Implications for cross reactivity
• Arguments for affinity purification
Mistake #6 – Failure to correctly perform and analyze sample Dilutional Linearity data

- Dilutional linearity demonstrates that the array of HCPs in a sample are a good fit for the array of antibodies in the ELISA.
- First step of assay qualification and has to be performed on all sample types to be tested using the ELISA.
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Mistake #6 – Failure to correctly perform and analyze sample Dilutional Linearity data

Information from properly performed dilutional linearity analysis:

- Determine the Minimum Required Dilution (MRD) for each sample type.
- Determine if the ELISA being used is a good fit for the array of HCPs in the samples.
- Assist in determining if single or small group of HCPs are being enriched in the purification process.
- Help rule out cross reactivity.
Mistake 7
Using Western Blot to conclude there is cross reactivity of the anti-HCP antibody to your drug substance

• True DS immunological cross reactivity is very rare

• Even when there is cross reactivity it will most often not manifest as false HCP levels due to lack of sufficient excess of the cross reacting Ab
Mistake 8
-Failure to Affinity Purify the HCP Antibody

- Anti-HCP antibodies in goat plasma account for 1% – 10% of total IgG
  - If coating with Protein G purified antibody, 90% - 99% of IgG is dead with respect to the ELISA
    - Leads to poor sensitivity
    - Can cause specificity problems
    - Can make dilutional linearity a problem

- If titer is high enough you may get away with not doing Ag affinity purification
Integration of Orthogonal Methods
ELISA is an excellent method to monitor HCP clearance through the purification process and for release testing final drug substance but it cannot tell the entire story alone.

- ELISA estimates total HCP in a sample in a semi-quantitative manner
- ELISA does not give information regarding the HCPs present, which HCPs are being recognized, or if the assay is broadly reactive
- Need additional orthogonal methods to get the complete story
Orthogonal Methods to HCP ELISA

Methods that work to complete the HCP picture

• Antibody Affinity Extraction
• 2D SDS-PAGE Silver stain
• 2D Western blot
• 2D-HPLC
• Mass Spec
Antibody Affinity Extraction:

- Method developed as a superior alternative to 2D Western blot
- Polyclonal anti-HCP antibodies are covalently immobilized onto a chromatography support
- After conditioning, null or mock transfected harvest material is cycled over the column until nothing is being removed from the sample
- HCP eluted from the affinity support are then compared to HCP in the initial sample by large format 2D gel (detected by silver stain or DIGE)

- Advantages over Western blot:
  - Column run under more natural conditions so conformational epitopes are conserved.
  - More predictive of how the antibodies will perform in the ELISA
  - Increased sensitivity and specificity
Uses for AAE in Completing the HCP Picture

- Coverage Analysis: performed to demonstrate that the ELISA is fit for purpose
- Analyze HCPs that persist though the purification process
- Sample preparation
  - Mass spec identification of HCP in the process
  - Loading SDS-PAGE gel to cut spot and identify a specific protein
Example AAE Data for Coverage Analysis

Initial total HCP

AAE Elution Fraction
Example of AAE in Sample Preparation

Anion Exchange Pre-AAE

Anion Exchange Post AAE

Figure 9: Oriole Fluorescent Gel Stained image of the initial AEX Flowthrough. 2D SDS-PAGE markers are indicated by blue circles.

Figure 10: AAE elution fraction of the Oriole Fluorescent Gel Stained AEX Flowthrough. 2D SDS-PAGE markers are indicated by blue circles.

19 HCP Detected by Mass Spec

118 HCP Detected by Mass Spec
Large Format 2D SDS-PAGE Stain

Separates complex protein mixture by size and isoelectric point
Uses for 2D SDS-PAGE Stain in Completing the HCP Picture

- **Antigen screening prior to animal immunizations**
  - Ensure broad array of HCP in immunogen
  - Ensure no loss during antigen processing
- **Separate proteins for:**
  - AAE Analysis
  - Spot picking for MS identification
  - Investigation of proteins that may have been enriched during the purification process (ie: sample with poor dilutional linearity)
2D Western Blot

- Traditionally the gold standard for demonstrating coverage analysis
- Compares spots detected by the antibody on the Western blot to the total protein spots on a stained gel.
2D Western Blot

Uses of 2D Western blot
• Coverage Analysis
• Lot Release of Drug Substance

• Limitations of Western blot
• Poor sensitivity
• Poor selectivity
• Destruction of conformational epitopes
• In no way predictive of how antibodies will perform in an ELISA
• Use of Western blot to make a choice on antigen and antibody selection can lead to a poor assay despite good coverage data
2D-HPLC

• Fractionates proteins by charge and hydrophobicity
• Can split sample into greater than 1000 fractions so most contain 0 – 1 HCP

• Uses for 2D-HPLC
  • Test Fractions in ELISA for coverage analysis
  • Gives estimated coverage by mass
  • Fractions of interest can be analyzed by mass spec for identification
Mass Spectrometry

Quickly becoming a very valuable tool in HCP analytics

• Can be used to identify HCP using LC-MSMS
  • As many as 1000 individual HCPs when coupled to AAE
• Semi-quantitative in Discovery mode
• MRM methods allow for tracking specific HCPs throughout the purification process

• For the first time we have the ability to monitor which HCPs are persisting through the purification process
Uses of Mass Spectrometry in Completing the HCP Picture

• Second level of coverage analysis
  • Not particularly useful

• Identification of HCPs that persist through the purification process
  • Ideal for purification process development and process validation

• Can identify problematic proteins that are enriched by the purification process
  • Spot picking from a 2D silver stained gel
Comprehensive Strategy for HCP Analytics

Antigen Selection

Reference ELISA

Antibody Purification & Characterization

ELISA Development & Validation

2D PAGE

AAE

HPLC

MS

Patient Serology
Acknowledgements:

- Ken Hoffman
- John Locklear

Reference Documents

- USP Chapter 1132 Residual Host Cell Protein Measurements in Biopharmaceuticals
- ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology
- US FDA: Bioanalytical Method Validation
- EDQM: Host Cell Protein Assays
- EMA: Guideline on Bioanalytical Method Validation
- Cygnus Technologies: Antibody Affinity Extraction, A Superior Method to Western blot