CELL LINE CHARACTERISATION
Viral Safety from the Start for Biologicals Manufactured using CHO Cells
Key Regulatory Guidance Documents – Drives Industry Testing

- Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993)
- Points to Consider in the Manufacturing and Testing of Monoclonal Antibody Products for Human Use (1997)

- Q5A (R1): Quality of biotechnological products: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin
- Q5B: Quality of biotechnological products: Analysis of the expression construct in cells used for production of rDNA derived protein products
- Q5D: Derivation and characterization of cell substrates used for production of biotechnological / biological products


- WHO Recommendations for the Evaluation of Animal Cell Cultures as Substrates for the Manufacture of Biological Medicinal Products and for the characterisation of Cell Banks. Final, 2011
Biologics manufacturing process:  
Overview of testing requirements

Upstream Bioprocessing

- Research Cell Line
- Master Cell bank Production
- Working cell bank Production

Downstream Bioprocessing

- Unprocessed Bulk Product
- Purified Bulk Substance
- Final Biological Product

Bioreactor

Raw Material Testing

Cell line characterization

- Master cell bank
- Working cell bank
- End of production cells

Materials characterization

- Media components

Lot release testing:

- Unprocessed bulk
- Purified Bulk
- Final product

Clearance Testing:

- Evaluation of downstream purification process performed once process is defined
- Repeated if changes to process are made
**Pre-bank Testing for Research Cell Lines**

**Minimum Testing for Release into cGMP Banking Facility**
- Sterility
- Bacteriostasis and Fungistasis
- Mycoplasma (28-day culture method or mycoplasma PCR)

**Additional Testing**
*Dependent on History of Cells; Worker Safety Considerations*

- *In vitro* virus screening assay
- Identity

*“Showstoppers” for a cell line: Positive sterility or positive Mycoplasma results or cross cell contamination (15% rate outside of regulated industry)*
Cell Line Characterization in Brief

Identity
Verify species of cell

Genetic Stability
Expression Construct

Purity
Bacteria, Fungi
Mycoplasma (Spiroplasma)
Viruses (endogenous and adventitious)
Cell Line Characterization

Identity
- Verify species of cell

Genetic Stability
- Expression Construct

Purity
- Bacteria, Fungi
- Mycoplasma (Spiroplasma)
- Viruses (endogenous and adventitious)
Identity testing of Cell Banks

Required to confirm the species origin of cell lines

Genotypic approaches:
- DNA fingerprinting
- Cytochrome c oxidase subunit I (CO1) PCR and barcoding

Phenotypic approaches:
- Isoenzyme testing has been used extensively previously however due to unreliable supply issues, this service is no longer available

Karyology
- Recommended for new cell lines and for diploid cell lines, but not necessary for well characterized cell lines such as CHO, Sp2/0, NS0

One of the above methods is performed on MCB and EOP/CAL
**CO1 Barcode Assay**

- Conserved mitochondrial coding region
  - Lack of introns
  - Limited exposure to recombination
  - Haploid inheritance

- Universal primers for this gene are robust, enabling recovery of its 5’ end from all animal phyla

- CO1 possesses a greater range of phylogenetic signal than any other mitochondrial gene, allowing for clear species identification

- CO1 analysis is the method of choice for taxonomic identity and for cell line identity at cell culture collections

- Greater number of reference species, minimal subjectivity relative to isozyme analysis
Cell Line Characterization

Identity

- Verify species of cell

Genetic Stability

- Express Construct

Purity

- Bacteria, Fungi
- Mycoplasma (Spiroplasma)
- Viruses (endogenous and adventitious)
Genetic Stability Testing

ICH Q5B:

Analysis of the Expression Construct in Cells used for Production of rDNA Derived Protein Products

- Typically performed in Phase 3 using cells from MCB and EPC/CAL
- Verifies that expression system has not undergone any changes that would impact integrity of the product
- Molecular studies required to verify
  - Correct sequence made and incorporated into host cell
  - Structure and copy # maintained to end of production
Requirements for Genetic Stability

- Sequence analysis of the expressed gene*
- Sequence analysis of 5’ and 3’ flanking regulatory regions of recombinant gene*
- Copy number determination (qPCR)*
- Restriction enzyme digest analysis*
- Number of integration sites (by Fluorescence In Situ Hybridization; FISH)
- Analysis of the expressed gene mRNA size and abundance (e.g., northern blotting); typically only required for less well established cell lines

Test performed on MCB AND EOP/CAL
Cell Line Characterization

Identity
- Verify species of cell

Genetic Stability
- Expression Construct

Purity
- Bacteria, Fungi
- Mycoplasma (Spiroplasma)
- Viruses (endogenous and adventitious)
Compendial Sterility Test

- Methods are harmonized globally
  - Direct Inoculation
  - Membrane Filtration

- Media
  - TSB media (total aerobes) – Incubated at 20-25°C
  - THIO media (microaerophiles/anaerobes) – Incubated at 30-35°C

- Observed for microbial growth on day 3, 4 or 5, day 7 or 8, and day 14.

- Assay is performed on 1% of total bank, but not less than 2 vials

Sterility positive rate <1% with no false positives across >1000 samples tested.
Bacteriostasis and Fungistasis

- Low level of challenge microbe spiked into sample and monitored for growth
- B&F is recommended prior to performing sterility assay to determine that the sample is free of any inhibiting factors that may give a false positive result

Sterility assay is performed on MCB, WCB and EOP/CAL

B&F is performed on MCB and EOP/CAL
Cell Line Characterization

Identity
- Verify species of cell

Genetic Stability
- Expression Construct

Purity
- Bacteria, Fungi
- Mycoplasma (Spiroplasma)
- Viruses (endogenous and adventitious)
Mycoplasma Testing

Culture based method is 28 days in duration

Culture based include three components:

- Indicator culture cells (3-7 days duration)
- Direct cultivation onto agar plates (14 days duration)
- Incubation in broth followed by agar subculture (28 days duration)

Qualification (Mycoplasmastasis) – Mycoplasma spiked into test sample and observed for any inhibition of mycoplasma outgrowth.

USP/EP requirement and good scientific practice

Mycoplasma positive results at BioReliance for past three years were <0.1%
Mycoplasma Testing – Governing Regulatory Guidelines

<table>
<thead>
<tr>
<th>Source</th>
<th>Scope</th>
</tr>
</thead>
<tbody>
<tr>
<td>21CFR Part 610.30, FDA, CBER, Guidance 2010</td>
<td>Live and inactivated virus vaccines</td>
</tr>
<tr>
<td>FDA, CBER, Points To Consider, 1993</td>
<td>Licensed biological products e.g. viral vaccines, monoclonal antibodies, immunological modulators, interferon and other cytokines, erythropoietin, growth factors and similar products</td>
</tr>
<tr>
<td>European Pharmacopoeia, section 2.6.7</td>
<td>All biotechnological products and associated materials</td>
</tr>
<tr>
<td>Japanese Pharmacopoeia XV, section 14 – Supplement 2</td>
<td>Cell substrates used for manufacture of biotechnological/biological products</td>
</tr>
<tr>
<td>United States Pharmacopeia, &lt;63&gt;</td>
<td>All biotechnological products and associated materials</td>
</tr>
</tbody>
</table>

Worldwide regulatory documents
Regulatory View of NATs For Mycoplasma

European Pharmacopoeia
- Direction on application of validated NAT methods for detection of mycoplasma
- Guidelines on validation expectations
  - Specificity
  - Detection limit
  - Robustness
  - Comparability testing

United States Pharmacopoeia
- Validated NAT test may be applied if shown to be comparable to culture methods

Japanese Pharmacopoeia
- Recent update is more in line with EP
## Cell Line Characterization in Brief

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<td>Viruses (endogenous and adventitious)</td>
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Rationale for virus assays

To detect unknown and a wide range of possible contaminants must utilize a number of different assays

- **Broad specificity assays**
  - *in vitro* and *in vivo* virus assays, electron microscopy

- **Assays to detect contaminants associated with specific species**
  - Rodent, bovine, porcine, human viruses

- **Assays to detect retroviruses**
  - Infectivity assays
  - Molecular biology assays (PCR)
  - Biochemical assays (reverse transcriptase)
  - Morphological assays (electron microscopy)

- **Assays to detect specific viruses**
  - Molecular biology assays (PCR)
In Vitro *Adventitious Virus Assay*

Test Article

Day 14
HA/HAD
Passage conditioned medium

Day 28
HA/HAD
End of Assay

Cell Monolayer

Passaging of cells

Common Detector Cells

- MRC-5 (Human Diploid)
- Vero (Simian)
- Same Species and Tissue - SP2/0, BHK, CHO, NS0 etc.
Detection of Adventitious Viruses Using the In Vitro Adventitious Virus Assay

20 Years of BioReliance (US) Testing – Summary

Cell Lines (MCB, WCB, EPC): No viruses detected
For non-CHO cell production: No viruses detected

The following viruses were detected in CHO unprocessed bulk:
- Reovirus – two positive studies; attributed to serum*
- Cache Valley virus – four positive studies; attributed to serum*
- Vesivirus 2117 – two positive studies

For the past 15 years of BioReliance testing (over 15,000 in vitro viral assays), there were only eight positive studies for adventitious viruses, which represent 0.05% of assays performed.

*Elimination of fetal bovine serum from the manufacturing process or use of gamma-irradiated serum mitigates the risk of experiencing an adventitious viral contamination.
**Broad specificity in vitro virus assays**

*In vitro virus assays*

- Use selected cell lines permissive to a wide variety of viruses
- Endpoints for viral detection may be
  - Cytopathic effects (CPE)
  - Hemagglutination or hemadsorption
  - IFA

**Advantages:**

- Screens for a broad range of viruses
- Low Limit of Detection (LOD), approx 1 TCID$_{50}$

**Limitations:**

- Only detect viruses that
  - Can grow in culture
  - Cause CPE
- Cultures susceptible to toxicity as well as viral effects
- Practical limitations in the number of detector cells lines used
In Vivo Adventitious Virus Screening Assay

- *In vivo* adventitious virus assay – General screen (28 day) using suckling and adult mice and embryonated eggs to reveal viruses that cannot grow in cell cultures- additional species may be used depending on nature and source of cell line (Guinea pigs frequently used for FDA submissions)

- Required for viral vaccines and at least one time on UPB for other biologics

- Endpoint = morbidity and mortality

*In over 20 years of testing (thousands of studies) no adventitious virus was detected in this in vivo viral screen.*
Viruses Detected Using In Vivo Assay

- **Suckling mice:**
  - *Human viruses*: alphaviruses, arboviruses, arenaviruses, bunyaviruses, coxsackieviruses types A and B, echoviruses, flaviviruses, herpesviruses, polioviruses, rabies

- **Adult mice:**
  - *Human viruses*: Coxsackieviruses types A and B, flaviviruses, rabies virus
  - *Murine viruses*: Lymphocytic choriomeningitis virus (LCMV)

- **Guinea pigs:**
  - Filoviruses, paramyxoviruses, reoviruses
  - *Mycobacterium tuberculosis*

- **Embryonated eggs:**
  - *Allantoic route*: alphaviruses, orthomyxoviruses, paramyxoviruses, vesiculoviruses
  - *Yolk sac route*: Herpesviruses, poxviruses, rhabdoviruses
In Vivo *Species-Specific Assays – Antibody Production Tests*

Mouse viruses screened in MAP
- Ectromelia
- GDVII
- Lactate Dehydrogenase Virus (LDV)
- Lymphocytic Choriomeningitis
- Hantaan Virus
- Mouse Minute Virus (MMV)
- Mouse Parvovirus (MPV)
- Mouse Adenovirus
- Mouse Hepatitis Virus (MHV)
- Pneumonia Virus of Mice (PVM)
- Polyoma
- Sendai
- Epizootic Diarrhea of Infant Mice (EDIM)
- Mouse Salivary Gland Virus (Mouse Cytomegalovirus) (MCMV)
- Reovirus Type 3
- Mouse K Virus
- Mouse Thymic Virus (MTV)

Historical Note: The MAP assay was used back in the late 90’s at BREL to confirm MMV for Genentech’s CHO reactor “crashes”.
**Broad specificity in vivo virus assays**

**In vivo virus assays**
- Inoculate mice (adult/suckling), guinea pigs, embryonated eggs
  - Viruses detected by appearance of disease symptoms (animals), loss of viability (eggs) or by hemagglutination assay (egg fluids)
- Antibody production assays in mice/rat/hamster
  - Serum screened by ELISA for production of virus-specific Abs

**Advantages**
- Broad specificity methods that can detect viruses that don’t grow in culture
- Sensitive

**Limitations**
- Ethical concerns
- Toxic effects of test material
- Lengthy assay
In Vitro 9CFR Bovine and Porcine Virus Assay

Basis

- Detection of wide variety of bovine and porcine viruses based upon development of cytopathology, hemadsorption of red blood cells, and specific immunofluorescent staining

Procedure

- Inoculate serum or clarified cell lysate into indicator bovine turbinate (BT) or porcine testicular (PT) cells and VERO cells
- Monitor microscopically for 21 days, subculturing twice
- Test for hemadsorption
- Stain fixed cells with anti-bovine or anti-porcine virus fluorescein-labeled antibodies

During the past three years at BioReliance approximately 50% of the 9CFR bovine virus screening assays performed on bovine serum samples yielded positive IFA results for non-cytopathic BVDV (Bovine Viral Diarrhea Virus)
Bovine and Porcine Viruses Specifically Screened for in the 9CFR Assays

- Bovine viral diarrhea virus (BVDV)
- Bovine adenovirus type 5 (BAV5)
- Bovine parvovirus (BPV)
- Bluetongue virus (BTV)
- Bovine respiratory syncytial virus (BRSV)
- Reovirus type 3 (REO)
- Rabies virus
- Infectious bovine rhinotracheitis virus (IBR)
- Porcine parvovirus (PPV)
- Transmissible gastroenteritis virus (TGEV)
- Porcine adenovirus (PAV)
- Bovine parainfluenza virus type 3 (PI3)
Rodent Cells and Retroviral Particles

- Rodent cell lines produce endogenous retrovirus-like (RV) particles (types A and C).
- Mouse cells are inherently capable of producing infectious mouse RV. Positive testing results may be obtained with mouse cell lines (e.g., NS0).
- CHO cell lines express defective RV particles. To date, it has not been shown that hamster cell lines can express infectious RV.
- There is a risk of unknown endogenous retroviruses that might be present.
- A variety of assays are used, depending on the cell substrate and the associated risk of RV.
Retrovirus Assays

Infectivity assay

Infectious retrovirus detected through a cell culture assay

Electron Microscopy

Retroviral particles are visualized and enumerated using transmission electron microscopy

Reverse transcriptase assays

Detection of reverse transcriptase activity using tritium incorporation into a template or through product amplification assays (PERT)
Virus-Specific Assays (PCRs)

- PCR for MMV (mouse parvovirus) for CHO cell lines
- PCR for Vesivirus 2117 for CHO cell lines
- Bovine polyoma virus (PCR) expected by EP reviewers; Bovine polyoma infects human and simian cell lines; no evidence for infecting CHO
- Human virus panel
## Viral Safety Testing in US and EU

<table>
<thead>
<tr>
<th>Stage</th>
<th>IMP in EU, Phase 1, 2 &amp; 3</th>
<th>IND in US (Standard Products)</th>
<th>IND in US (Orphan &amp; S/LT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCB</td>
<td>Full characterization as per ICH Q5A</td>
<td>Full characterization as per ICH Q5A; can prioritize assays (e.g., postpone IVV, but not IVT &amp; TEM)</td>
<td>Reduced: Sterility, mycoplasma, IVT (28 d)</td>
</tr>
<tr>
<td>WCB</td>
<td>Minimal Testing: e.g., identity, sterility, mycoplasma, IVT (28d)</td>
<td>Minimal Testing: e.g., identity, sterility, mycoplasma, IVT (28d)</td>
<td>N/A</td>
</tr>
<tr>
<td>EOP/CAL</td>
<td>Not required if BH tested as below</td>
<td>Not required for phase 1; required for phase 3</td>
<td>Not required</td>
</tr>
<tr>
<td>BH</td>
<td>Bioburden, mycoplasma, in vitro, MMV, TEM (3 lots)</td>
<td>Bioburden, mycoplasma, IVT, PCR for at risk viruses, TEM (3 lots, but can initiate IND with 1 lot)</td>
<td>Bioburden, mycoplasma, IVT not required, TEM?</td>
</tr>
</tbody>
</table>
Conclusion

- Companies need to review their testing plan carefully to assure it meets regulatory requirements and expectations.

- There is variation with reviewers’ opinions on testing. Since the reviewer has the final say, we recommend obtaining reviewer approval of the testing plan early in a company’s program. The pre-IND meeting is a good time for that discussion.
谢谢 - Xièxiè