

Studies to Evaluate
Disinfectant Efficacy and
Facility Disinfection
Programs



About BioReliance

BioReliance was acquired by Sigma-Aldrich Corporation, in January, 2012 to supply customers access to a powerful, single-point provider whose products and services span the drug discovery, development and commercialization pipeline. As part of the SAFC business unit, BioReliance is a leading provider of cost-effective contract services, offering more than 1,000 tests or services related to biologics safety testing and specialized toxicology. BioReliance has over 700 employees and has laboratory operations in Rockville, MD and Scotland. For more information, visit www.bioreliance.com.

About SAFC

SAFC, the custom manufacturing and services business unit of Sigma-Aldrich Corporation, is recognized as a top 10 global specialty chemicals and biologics supplier. As a trusted manufacturer for the life science and high technology industries, SAFC works closely with customers to resolve development challenges and accelerate the product pipeline utilizing its global “Centers of Excellence” and dedicated manufacturing facilities. Its rich portfolio includes high-purity inorganic materials for high technology applications, critical raw materials and extensive biologics safety testing services for biopharmaceutical manufacturing, and complex, high-potent APIs and key intermediates for pharmaceutical manufacturing. For more information, visit www.sigmaaldrich.com/safc.

BioReliance’s Key Services:

- **Custom Assay Development** to fulfill your exact requirements
- **Biosafety Testing** of biologicals for viruses, bacteria, mycoplasma, fungi
- **Cell Line Characterization** including identity testing, genetic stability, EM, sequencing
- **Final Product Testing** including biopotency testing, residual DNA, host cell proteins, cross-reactivity
- **Virus/TSE Validation Studies** for downstream processing
- **Contract GMP Production and Testing** of viral vectors and cell banks
- **Veterinary Vaccine Services** including characterization/identity, extraneous agent testing
- **Genomic Services** for advanced product safety and clinical trial testing
- **Regulatory and Consulting Services**

All work undertaken by BioReliance is in compliance with appropriate regulatory standard.

Further information is available by visiting our website at www.bioreliance.com

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Introduction

A disinfection efficacy study is part of a manufacturing facility's overall contamination control program and should include the following elements (**Figure 1**):

1. Facility controls to minimize the potential for contamination through:
 - Testing raw materials for potential contaminants (e.g., viral contaminants, bioburden)
 - Flow of personnel and materials, including controlled zones identified by garments or other visual methods
 - Air handling flow
 - Facility and equipment cleaning and disinfection
2. Monitoring the manufacturing environment to establish baseline flora
3. Trending environmental isolates and defining appropriate limits
4. Validating that the established disinfection procedures provide the expected level of disinfection
5. Verification that cleaning and disinfection procedures are documented in SOPs and that the procedures that are understood and replicated by all operators

The disinfection efficacy study will generate data to provide a high degree of assurance that the cleaning program will consistently yield results that meet pre-determined specifications. These data add a layer of product safety and generate confidence in the manufacturer's ability to deal with an unexpected contamination event.

Cleaning vs. Disinfection

Although these words are sometimes used interchangeably, cleaning is not the same as disinfection. Cleaning is concerned with removal of particulates, residue buildup and chemical cross contamination. Disinfection, on the other hand, is a component of a contamination control program. Evaluation of cleaning involves visual inspection and verification by analytical techniques such as total organic carbon (TOC), gas chromatography (GC) or high performance liquid chromatography (HPLC).

Evaluation of disinfection involves verification by assays that can detect infectious organisms like bacteria, viruses or mycoplasma. A clean surface is easier to disinfect and so the cleaning and disinfection programs complement each other. Disinfection efficacy studies are designed to be consistent with the USP General Chapter <1072>, "Disinfectants". The elements of a study to verify the efficacy of a disinfection program are discussed here.

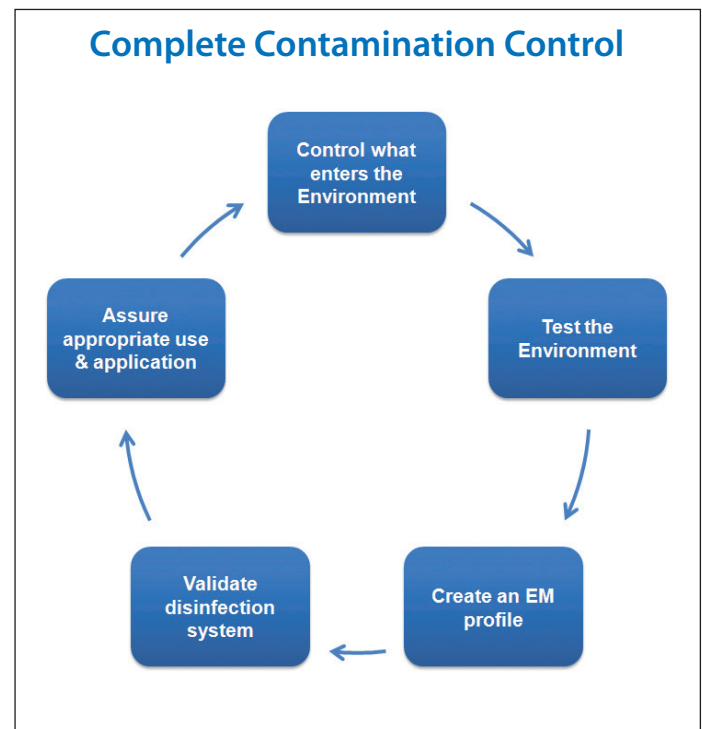


Figure 1. Contamination Control Process

Overview of Disinfection Efficacy Studies

Typical disinfection efficacy studies involve replication of the surface disinfection procedure at small scale to verify the clearance of spiked infectious agents (Figure 2). An infectious agent (e.g., bacteria, spore, mycoplasma, virus, etc.) is dried onto a small coupon of a surface that is representative of surfaces in the manufacturing facility. The disinfectant is applied to the coupon mimicking the procedure used in the facility, and any remaining infectious agent is recovered and quantitated using an infectivity assay. Things to consider when designing a disinfection efficacy study are detailed in the sections below.

Considerations for Disinfectant Efficacy Studies

Pre-Studies

Prior to the initiation of the challenge experiments, it is essential to demonstrate whether the samples to be tested in the disinfectant efficacy study interfere with the detection of the challenge agents. These data verify that any decrease in the challenge agent is due to the disinfection procedure and is not the result of the disinfectant interfering with the endpoint assay used to detect the challenge agent.

Microbial (i.e. bacterial, fungal, or mycoplasma) stasis studies are performed to demonstrate whether samples to be tested in the disinfectant efficacy study inhibit the recovery of the microorganisms. For stasis studies, mock recovery solutions are prepared using each disinfectant and D/E neutralizing broth (or equivalent). Individual samples of each mock recovery solution are spiked separately with less than or equal to 100 CFU of each challenge organism and then plated on an appropriate growth medium. Upon the satisfactory completion of the stasis study, an appropriate microbial recovery procedure will have been established.

Cytotoxicity and viral interference studies are performed to demonstrate whether samples to be tested in the disinfectant efficacy study are toxic to the detector cells used in the virus detection assay or interfere with the ability of the virus to infect the detector cells. These experiments are performed for every virus detection assay used in the challenge study. Mock recovery solutions are prepared that contain virus recovery buffer and a volume of disinfectant

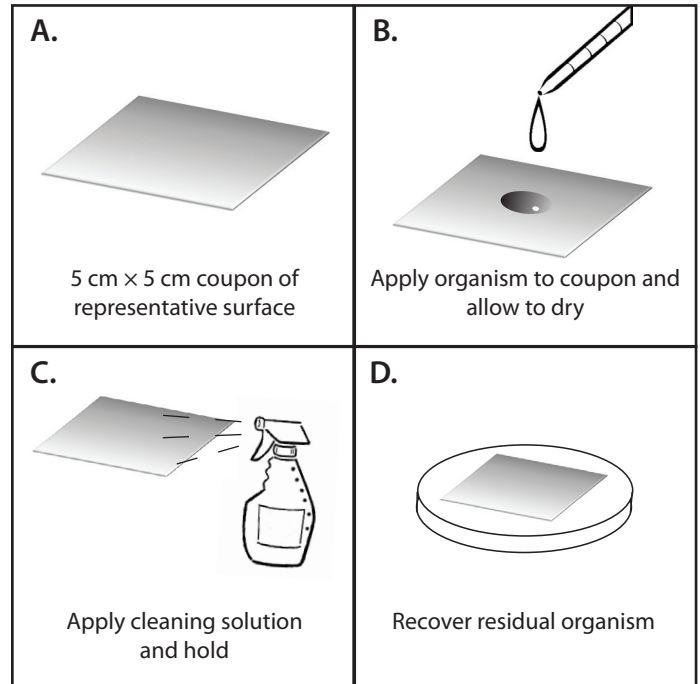


Figure 2. Overview of steps in a disinfectant efficacy study: A.) obtaining coupons representative of the surfaces that are disinfected; B.) drying challenge agent onto the coupon surface (with or without representative “soil”); C.) accurately mimicking the cleaning procedure using worst-case processing conditions; D.) recovery of any residual challenge agent.

that has been experimentally determined to represent the residual volume of disinfectant remaining after the coupon disinfection procedure. For cytotoxicity studies, dilutions of the mock recovery solution are inoculated onto detector cell monolayers in the absence of virus to determine the dilution of the recovery solution that is non-cytotoxic to the cells. For viral interference studies, the mock recovery solution is used as a diluent to quantitate virus. A control is prepared in which virus buffer is used as the diluent, and the resulting titers are compared. If the titer of the virus diluted in mock recovery solution is similar to the titer of the virus diluted in buffer, then there is no viral interference with the assay. If viral interference is detected, then the mock recovery solution is diluted until no interference is detected. The cytotoxicity and viral interference studies will establish the dilutions that will be required before the recovery solutions from the spiking study can be tested in the virus detection assays.

Surfaces

5cm × 5cm (2" × 2") coupons of representative facility surfaces are used in disinfection efficacy studies. It is important that the coupons are representative of the surfaces in the facility. The types of surfaces as well as the condition of the surfaces should be representative. For example, new stainless steel does not represent the same disinfection challenge as well as 'used' stainless steel, which can be pitted and can be more difficult to disinfect. Typical surfaces include:

- Stainless Steel
- Glass
- Vinyl flooring
- Epoxy coated wallboard
- Fiberglass
- Lexan (plexiglass)
- Vinyl curtain
- Tyvek
- Terrazzo tiles
- Plastic, polycarbonate

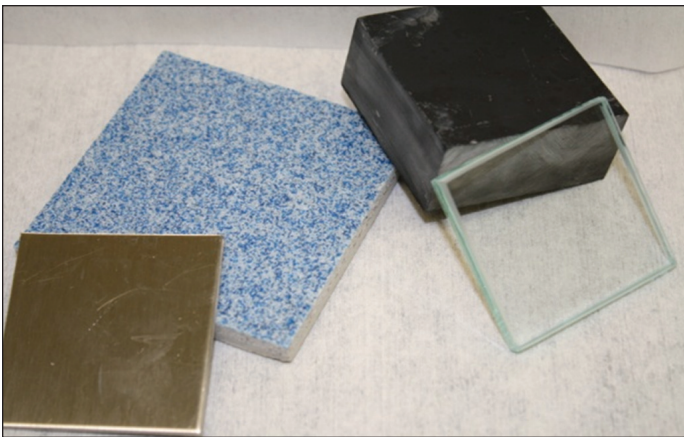


Figure 3. Coupons of surfaces representing the type and condition of those present in a manufacturing facility are used for the disinfectant efficacy study.

Disinfectants

The disinfectants evaluated in a disinfectant efficacy study must represent those that are in use in your facility. They may include formulated and ready-to-use agents. For a disinfectant efficacy study, formulated disinfectants will be prepared as they are routinely prepared in your facility, using a similar quality of water and/or following similar sterile filtration procedures.

In order to represent a "worst-case", as is typically recommended for efficacy studies, BioReliance suggests aging disinfectants to just beyond their expiration date before use in the study. For example, pre-formulated disinfectant would be held until its expiration and then used to prepare the formulated disinfectant. The formulated disinfectant would also be held until its expiration before use.

Below is a list of agents that are commonly used in facilities for disinfection:

- Cavicide®
- Conflikt®
- Decon-Cycle®
- Decon-Phene®
- Decon-Spore 100®
- Decon-Spore 200 Plus®
- Ethanol
- Exspor®
- Hypochlorite
- Isopropanol
- LpH®st
- Septihol®
- Spor-Klenz®
- Vesphene® II st
- Vespore



Figure 4. The disinfectants used in a disinfectant efficacy study should represent those used in your facility.

Cleaning Procedures

During the disinfectant efficacy study, disinfectant is applied to the surface coupon in a manner that mimics a worst-case interpretation of the procedure that is used in your facility and detailed in your current SOPs. Application typically involves saturation of the surface with the disinfectant by spraying, mopping or another method, allowing the disinfectant to remain in contact with the surface for a specified time and then removing excess solution by rinsing, wiping or other means. Although SOPs may describe the procedure, there are often aspects of it that could be open to interpretation by operators and therefore not reproducibly performed. Wiping is a good example of a procedure that is very difficult to reproduce. The pressure used during wiping, direction of wipes, and whether or not wipes should overlap must be considered. This reproducibility is just as important during routine facility disinfection as it is during a disinfection efficacy study, when procedures used in the facility are mimicked. Procedures like this are difficult to describe in an SOP, yet it is critical that they are described accurately so that the procedure can be performed reproducibly.

It is very useful to consult with the operators who perform your facility cleaning to understand how disinfection procedures are performed. They can provide insights into areas that are easily missed or not thoroughly disinfected. These individuals are essential for ensuring that disinfectant efficacy studies are designed to accurately mimic facility cleaning procedures.

Establish Worst-Case Conditions for Cleaning Procedures

A disinfectant efficacy study, like other clearance studies, must mimic the worst-case limits for processing parameters. Typical critical processing parameters for a disinfectant efficacy study include contact time, temperature, disinfectant expiration limits, and surface soil. BioReliance recommends that during a study, reduced contact times are used and that temperatures are at the lowest end of the parameter range. For surfaces in which residue removal is part of the disinfection procedure, such as during cleaning of a piece of processing equipment, the challenge spike is incorporated into a representative "soil" substance prior to drying the spike onto the coupon. For example, serum can be used to represent a protein-rich product residue. As discussed in the Disinfectants section, BioReliance recommends using disinfectants at the limit or just beyond their expiration.

Acceptance Criteria

Since microorganisms vary in their susceptibility to disinfection procedures, BioReliance recommends an expectation of 3 log₁₀ of reduction for enveloped viruses and vegetative bacteria and ≥2 log₁₀ of reduction for non-enveloped viruses and bacterial spores. This expectation is consistent with USP <1072> "Disinfectants and Antiseptics".

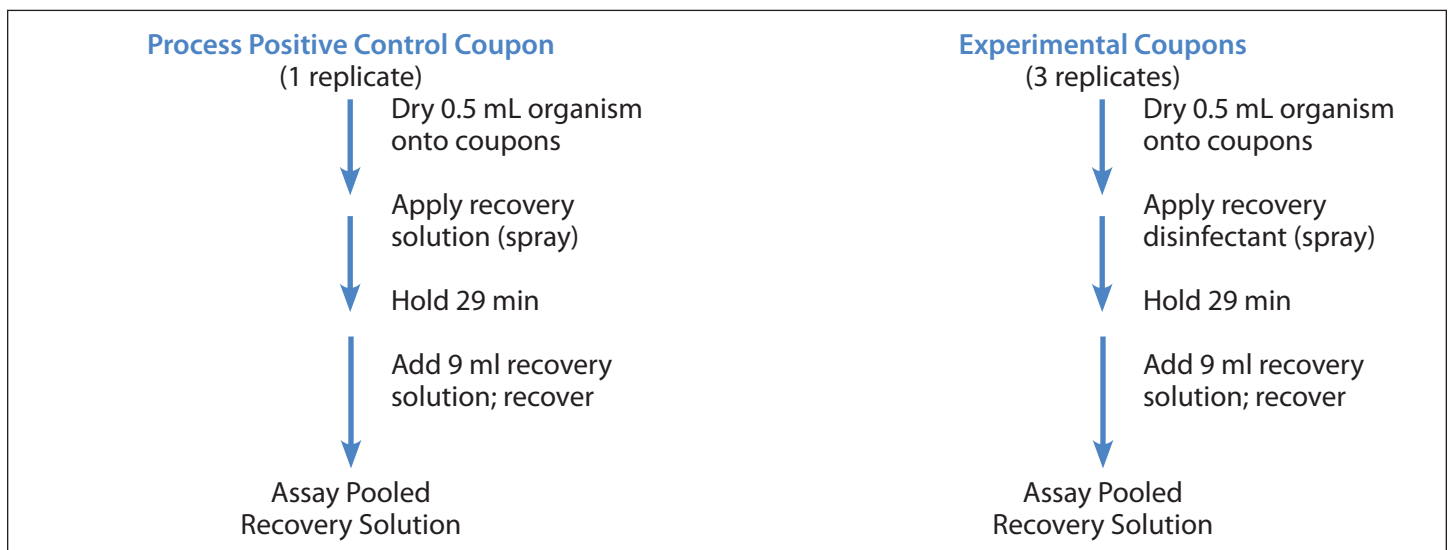



Figure 5. Example of a coupon disinfectant efficacy study.

Prions	Scrapie, Chronic Wasting Disease, Creutzfeld-Jacob Disease	MOST RESISTANT  LEAST RESISTANT
Bacterial Spores	<i>Bacillus, Clostridium</i>	
Mycobacteria	<i>Mycobacterium tuberculosis</i>	
Small, Non-Enveloped Viruses	Parvoviruses, Circoviruses	
Fungal Spores	<i>Aspergillus, Penicillium</i>	
Gram Negative Fungi	<i>Pseudomonas, Esherichia</i>	
Vegetative Fungi	<i>Aspergillus, Candida</i>	
Large, Non-Enveloped Viruses	Adenoviruses, Rotaviruses	
Gram Positive Bacteria	<i>Staphylococcus, Streptococcus, Enterococcus</i>	
Enveloped Viruses	HIV, Hepatitis B Virus, XMuLV	

From McDonnell G. *Antisepsis, disinfection and sterilization: types, action and resistance*. Washington, DE: ASM Press: 2007.

Figure 6. Relative resistance of microorganisms, viruses and prions to disinfectants.

Challenge Spike

Microorganisms display varying levels of susceptibility to disinfection, and some require high level disinfectants for inactivation. **Figure 6** illustrates a general hierarchy of disinfection; however, this is not an absolute representation. There are instances where a generally less resistant microorganism may display uncharacteristic resistance under certain disinfection conditions. Environmental isolates are typically more resistant to disinfection than the related laboratory strains. Some disinfectants are unlikely to be effective against resistant microorganisms.

Typical challenge spikes include mycoplasma, bacteria, fungi, spores, viruses and transmissible spongiform encephalopathies (TSEs), but the selection of agents to use as a challenge for your cleaning procedure should be tailored to your product and your manufacturing facility. The selection should be based on the nature and origin of the raw materials used in your manufacturing process and their potential contaminants. In addition, any operator (human) derived or environmental potential or known contaminants should be represented. **Tables 1-3** list representative agents that could be included in a disinfectant efficacy study.

Spiking agents need to be grown to high titers and must be detectable in reliable and sensitive infectivity assays. At times it is necessary to use a surrogate organism that models the contaminant, yet readily grows in culture and can be easily detected in an infectivity assay.

Bacteria and Fungi

BioReliance recommends that clients use a typical United States Pharmacopoeia (USP) panel of bacterial and fungal agents which includes the following types of organisms, also listed in **Table 1**:

1. Gram positive bacteria
2. Gram negative bacteria
3. Gram positive, spore-forming bacteria
4. Fungi, mold spores, yeast

Environmental isolates specific to your facility can complement this list or be used as a substitute for a similar type of organism. Environmental isolates represent a realistic and often worst-case challenge to your cleaning procedures, and should be included in your study.

Table 1. Representative Microbial Spiking Agents

Microorganism	Characteristics
Gram positive bacteria	Plasma membranes with peptidoglycan wall. Firmacutes: Examples: <i>Streptococcus sp.</i> , <i>Staphylococcus sp.</i>
Gram positive, spore forming bacteria	Firmacutes. Endospore formation during sub lethal or environmental stress conditions. Demonstrate resistance to ultraviolet, gamma irradiation, desiccation, extremes of temperature, and chemical disinfectants. Show sensitivity to Alkylating agents e.g., Ethylene oxide. Commonly found in soil and water. Examples: <i>Clostridium sp.</i> & <i>Bacillus sp.</i>
Gram negative bacteria	Triple layer (plasma membrane, peptidoglycan wall and outermost layer containing lipopolysaccharide). Outer membrane may prevent uptake of disinfectant. Proteobacteria Examples: <i>E. coli</i> & <i>Pseudomonas sp.</i>
Fungi, mold spores, yeast	Mechanisms of resistance include exclusion and phenotypic modulation. Examples: <i>Candida sp.</i> & <i>Aspergillus sp.</i> , <i>Aspergillus brasiliensis</i> may be used as the worse case fungi.

Note: It is recommended that representatives of the four major groups of microbial agents are included in the study.

Mycoplasma

Mycoplasma that are commonly used in disinfectant efficacy studies include the organisms listed in **Table 2**. It is recommended that a Mycoplasma species relevant to your product and manufacturing process or raw material source is included in a disinfectant efficacy study.

Table 2. Representative Mycoplasma that have been used as Spiking Agents

Mycoplasma	Model/Host
<i>Mycoplasma orale</i>	Human/arginine hydrolyzer. Recommended for vaccines for human and veterinary use.
<i>Mycoplasma pneumoniae</i> or <i>Mycoplasma fermentans</i>	Human/dextrose fermenter. Recommended for vaccines or cell banks for human use.
<i>Mycoplasma synoviae</i>	Avian. Recommended when avian material has been used during production or when the vaccine or cell bank is intended for use in poultry.
<i>Mycoplasma gallisepticum</i>	Avian. Recommended when avian material has been used during production or when the vaccine or cell bank is intended for use in poultry.
<i>Spiroplasma citri</i>	Insect/Plant. Recommended when insect cell lines or plant-derived raw materials are used.
<i>Spiroplasma melliferum</i>	Insect/Plant. Recommended when insect cell lines or plant-derived raw materials are used.

Viruses

A list of viruses that are commonly used in disinfectant efficacy studies is provided in **Table 3**. As a minimum, BioReliance recommends including a resistant enveloped virus, such as BVDV and a resistant non-enveloped virus, such as a parvovirus, in your study. Any constructs or isolates that are relevant to your product and manufacturing process should also be included. These may be substituted for the recommended viruses.

Table 3. Representative Viruses that have been used as Spiking Agents

Virus	Family	Structure/ Genome	Size	Physico-Chemical Resistance	Model/Host
Ad-2 <i>Human Adenovirus 2</i>	<i>Adenoviridae</i>	Non-enveloped, Double Stranded DNA	70-90 nm	High	Model for avian adenoviruses. Since adenoviruses are resistant to physico-chemical inactivation they provide a rigorous challenge to a purification/inactivation process.
BACV <i>Autographa californica multicapsid nucleopolyhedro-virus</i>	<i>Baculoviridae</i>	Enveloped, Double Stranded DNA	30-60 x 250-300 nm	Low	A model insect virus and considered a host agent for baculovirus expression systems (BESV).
BVDV <i>Bovine viral diarrhea virus</i>	<i>Flaviviridae</i>	Enveloped, Single Stranded RNA	40-60 nm	Low to medium	Model for potential togavirus or flavivirus contaminants. BVD is the preferred model for <i>Hepatitis C virus</i> in human blood and plasma derivatives. Alternatively <i>Sindbis virus</i> may be used.
CVV <i>Cache Valley Virus</i>	<i>Bunyaviridae</i>	Enveloped, Single Stranded RNA	80-120 nm	Low to Medium	Model for arboviruses, which are transmitted by mosquitoes and are also a potential contaminant of ovine derived material. The virus infects livestock and may be a potential contaminant of bovine serum.
Reo 3 <i>Reovirus type 3</i>	<i>Reoviridae</i>	Non-enveloped, Double Stranded RNA	60-80 nm	Medium to high	Infects human and animal cells, potential contaminant of hybridoma and recombinant cell lines. Model for orbiviruses and rotaviruses, model for <i>Bovine blue tongue virus</i> .
MMV <i>Mouse minute virus</i>	<i>Parvoviridae</i>	Non-enveloped, Single Stranded DNA	18-25 nm	High	Model for <i>Human parvovirus B19</i> , representing a severe test of the downstream process. Parvoviruses are known contaminants of CHO cell fermenters, and are also potential contaminants of rodent derived biopharmaceuticals.
PCV-2 <i>Porcine circovirus type 2</i>	<i>Circoviridae</i>	Non-enveloped, Single Stranded DNA	17 nm	High	Model for porcine circoviruses, known to infect most pig herds. Smallest viruses known to replicate autonomously in eukaryotic cells; a severe test of purification processes.
PPV <i>Porcine parvovirus</i>	<i>Parvoviridae</i>	Non-enveloped, Single Stranded DNA	18-25 nm	High	Model for <i>Human parvovirus B19</i> , representing a severe test of the downstream process. Parvoviruses are known contaminants of CHO cell fermenters, and are also potential contaminants of rodent derived biopharmaceuticals.
XMuLV <i>Xenotropic Murine Leukaemia Virus</i>	<i>Retroviridae</i>	Enveloped, Single Stranded RNA	70-100 nm	Low	Represents a non-defective C type retrovirus. Mandatory for biological products derived from CHO cell lines and monoclonal antibody products.

TSE Agents

Due to the risk of Transmissible Spongiform Encephalopathies (TSE) from bovine products, these agents represent potential contaminants for some biological products. While the study design for disinfectant efficacy studies involving TSEs is similar to that involving microbial agents or viruses, the endpoint assay is quite different. Direct testing methodologies for TSEs do not exist. There are two approaches for detection of TSEs: an animal bioassay or a western blot assay. Two rodent-adapted scrapie strains are used as model agents for TSE bioassays, mouse adapted scrapie strain ME7 and hamster adapted 263K strain (Table 4). The hamster adapted 263K strain appears to be an especially good model for BSE, CJD and other TSEs due to its well known incubation period and a well characterized brain histopathology. Positive identification of TSE can be seen through vacuolized lesions from the brain tissue. BioReliance has an exclusive license on a unique TSE western blot assay that is fully validated and GLP compliant. This sensitive, specific assay is semi-quantitative over a 5.0 log₁₀ range and can be used to rapidly determine TSE removal by a disinfection procedure. A comparison of the bioassay to the western blot assay is provided in Table 5.

Table 4. Model agents for TSE studies as detected by bioassay

Model Agent	Inoculation Technique	Result	Titer
Mouse adapted scrapie strain ME7	Intracranial injection into C57 BL mice	Infected mice show symptoms from days 160-450	10 ⁷ – 10 ⁸ LD ₈₀ units/mL
Hamster adapted 263K strain	Intracranial injection into Syrian golden hamsters	Infected hamsters show symptoms from days 70-200	10 ⁷ – 10 ⁸ LD ₅₀ units/mL

Table 5. Comparison of TSE detection assays: bioassay and western blot

	Bioassay	Western Blot
Advantages	<ul style="list-style-type: none"> Measures both inactivation and removal Generally accepted by all regulatory agencies Good sensitivity 	<ul style="list-style-type: none"> Does not require the use of experimental animals Involves less time and expense
Disadvantages	<ul style="list-style-type: none"> Requires experimental animal use Involves greater time and expense 	<ul style="list-style-type: none"> Assay only measures removal Less likely to gain acceptance by regulatory agencies Lower sensitivity

Conclusions

Regulatory agencies are showing increased interest in data supporting the efficacy of manufacturing facilities' disinfection procedures. Disinfection efficacy studies must be customized to each manufacturer's facility and procedures, and these studies can quickly become large and overwhelming. BioReliance has performed many disinfection efficacy studies, and the data we have generated have been reviewed and found acceptable by regulatory bodies. We can help you streamline and optimize a study to generate definitive data to support your disinfection regime. These data will provide a further layer of product safety specifically providing confidence in your ability to handle an unexpected contamination event in your facility.

Regulations

The design of the surface disinfectant efficacy study takes account of:

- EMEA CPMP Note for Guidance on Virus Validation Studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses (CPMP/BWP/268/95)
- FDA Validation of Cleaning Processes (7/93)
- AOAC Germicidal Spray Products Testing
- European Committee for Standardization: EN 13697 Chemical Disinfectants and Antiseptics - Quantitative Non-Porous Surface Test for the Evaluation of Bactericidal and/or Fungicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and Institutional Areas
- United State Pharmacopeia <1072> Disinfectants and Antiseptics

BioReliance Corp.

14920 Broschart Road
Rockville, Maryland 20850
Tel: 800.553.5372
Fax: 301.610.2590
Email: info@bioreliance.com

BioReliance Ltd.

Todd Campus
West of Scotland Science Park
Glasgow, Scotland G20 0XA
Tel: 44 (0) 141 946.9999
Fax: 44 (0) 141 946.0000
Email: info@bioreliance.com

BioReliance Ltd.

Innovation Park
Hillfoots Road
Stirling, Scotland FK9 4NF
Tel: 44 (0) 141 946.9999
Fax: 44 (0) 141 946.0000
Email: info@bioreliance.com

BioReliance, K.K.

c/o Sigma-Aldrich Japan K.K.
Tennoz Central Tower 4F
2-2-24 Higashi-Shinagawa
Shinagawa-ku
Tokyo 140-0002, Japan
Tel: +81 (0)3 5796 7430
Fax: +81 (0)3 5796 7435
Email: jpinfo@bioreliance.com

BioReliance Ltd.

c/o Sigma-Aldrich Chemicals Pvt Ltd
102 Alpha Building
Hiranandani Gardens
Powai, Mumbai 400076
Tel: +91 22 40872364
Fax: +91 22 25797589
Email: indiainfo@bioreliance.com

www.bioreliance.com

North America Toll Free: 800 553 5372 Tel: 301 738 1000
Europe & International Tel: +44 (0)141 946 9999 • Japan Tel: +03 5796 7430
Email: info@bioreliance.com

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