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Viral and Prion Clearance Studies

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A major concern for medicinal products of biological origin is the potential risk of contamination with known or unknown viruses. Manufacturers of biopharmaceutical products (such as recombinant proteins and monoclonal antibodies, or products derived from blood, tissue, or urine) are required to assess the ability of their manufacturing process to produce a product that is safe for use in humans.

Three complementary approaches have evolved to control the potential risk of viral contamination of biopharmaceuticals:

- Selecting and testing source material including media components for the absence of undesirable viruses
- Testing the product at appropriate steps during production for the absence of contaminating infectious viruses
- Assessing the capacity of the production processes to inactivate or remove viruses

The latter approach is referred to as a viral clearance study and plays an important role in establishing the safety of biopharmaceuticals. Another term that is often used is viral validation study, which is partially misleading because it is not the virus that is validated, but the manufacturing process itself. The regulatory definition of a viral clearance study is:

"A documented programme, which provides a high degree of assurance that a specific process will consistently manufacture a product, meeting predetermined specifications."[1]

A viral clearance study should reflect the viral risks associated with the product. It should include a selection of viruses that varies in size, shape, genome, and structure with varying degrees of resistance to various methods of physico-chemical inactivation procedures. A minimum of two distinct process steps is normally evaluated for viral clearance and at least one of the steps should be effective against non-enveloped viruses.[2] The steps should be complementary in their mode of action, e.g, one inactivation and one separation step should be evaluated. A viral clearance study determines the overall level of viral clearance obtained by the manufacturing process. This is expressed as the logarithmic reduction factor (LRF) for the individual viruses.

The same approach is used to determine the ability of a manufacturing process to remove or inactivate prion material. If the product is at any time exposed during its manufacture to human or animal tissues that could be potentially infected with a transmissible spongiform encephalopathy (TSE) agent or prions, the manufacturing process may have to be evaluated for its ability to remove or inactivate prions in addition to viruses.[3]

Concept of Viral and Prion Clearance Evaluation

The principle of viral and prion clearance studies is different from studies searching for traces of contaminants such as mycoplasma, endotoxins, residual DNA, or host cell proteins in process samples or final products. The underlying aim is a risk assessment for the worst case of a potential virus or prion contamination of the manufacturing

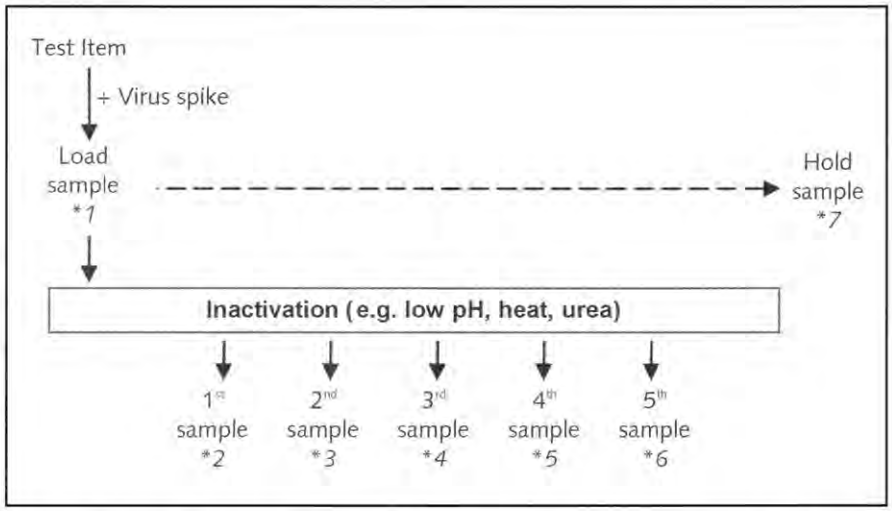

Figure 1. Sample scheme for a process step based on virus inactivation. The samples *1 to *7 will be withdrawn from the "virus-spiked" down-scaled process step and tested for residual infectivity on cell culture by endpoint-titration. The virus spike serves as positive control. The LRF results from the titer differences between hold sample (*7) and the last time point (sample *6).

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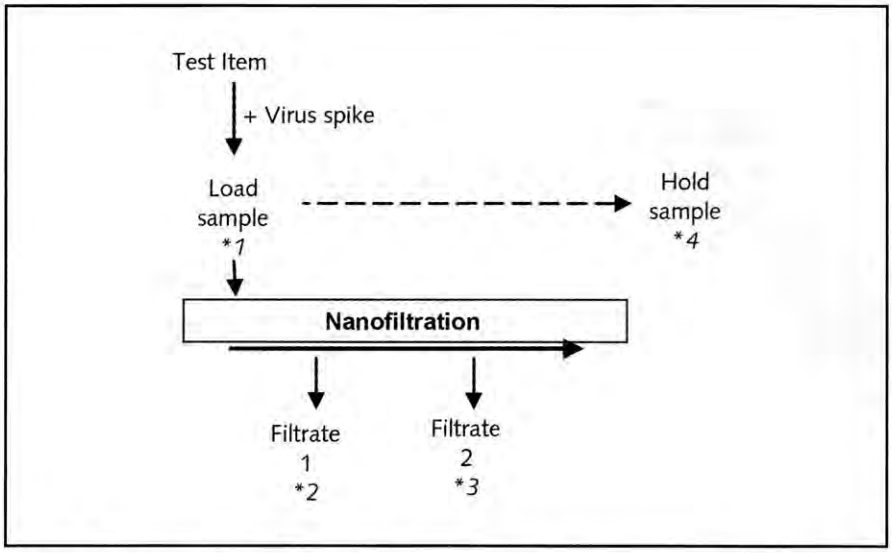

Figure 2. Sample scheme for a process step based on virus removal (nanofiltration). The samples *1 to *4 will be withdrawn from the "virus-spiked" down-scaled process step and tested for residual infectivity on cell culture by endpoint-titration. The virus spike serves as positive control. The LRF results from the titer differences between hold sample (*4) and the filtrate (sample *3).

process. Specific steps in downstream processing are evaluated regarding their capacity to inactivate or remove viruses or prions. The purpose of the viral studies is not only to determine a step's ability to inactivate or remove viruses, but also to characterize the robustness of a step's ability to achieve this reduction. The manufacturers are encouraged to continue their investigations by identifying steps or processes that would be effective in removing or inactivating prions.[3]

Viral clearance studies employ cell culture-based assays to determine the inactivation kinetic or separation capacity of a certain process step expressed as a LRF. Prion clearance studies generally use Western blot analyses for the initial investigation. The steps that exhibit the greatest promise for removing or inactivating prions are then normally re-tested in hamster or mouse studies.

Figures 1 and 2 show typical sample schemes for process steps based on inactivation and removal, respectively. The marked samples (*1 to *n) are tested for their residual infectivity by end-point titration. Examples of results obtained during an inactivation kinetics study as well as removal of porcine parvovirus (PPV) by nanofiltration are depicted in Figures 3 and 4. To evaluate the clearance capacity for the total process, the LRFs for the individual test viruses are added up. Table 1 shows the results for various viruses including PPV, MuLV, and HIV which have been tested in the common steps of downstream processes that are used in biopharmaceutical production. Each individual downstream process must be evaluated for its ability to remove or inactivate viruses because generic LRFs for a downstream process are not, with a few exceptions, accepted by the authorities.

The overall viral safety is reflected by the clearance capacity for all test viruses in conjunction with the robustness of the manufacturing process. Other factors contributing to the viral safety of a biopharmaceutical include the nature of the source material, the extent of cell bank characterization and qualification, culture medium constituents, culture methods, and facility and equipment design.

General Laboratory, Technical, and Formal Requirements

A prerequisite for performing viral clearance studies is the existence of a laboratory with at least a biosafety two level. As suggested by the guidelines, the laboratory and the studies should be certified according to the principles of Good Laboratory Practice.

The test system for viral clearance studies consists of the target cells and the model viruses that induce cytopathic effects in the cells. The cell lines and viruses used in viral clearance studies can be obtained from national and international cell bank organizations such as Robert-Koch Institute, Berlin (RKI), German Collection of Microorganisms and Cell Culture, Braunschweig, Germany (DSMZ), Federal Research Center for Viral Diseases of Animals, Insel Riems, Germany (BFAV), European Collection

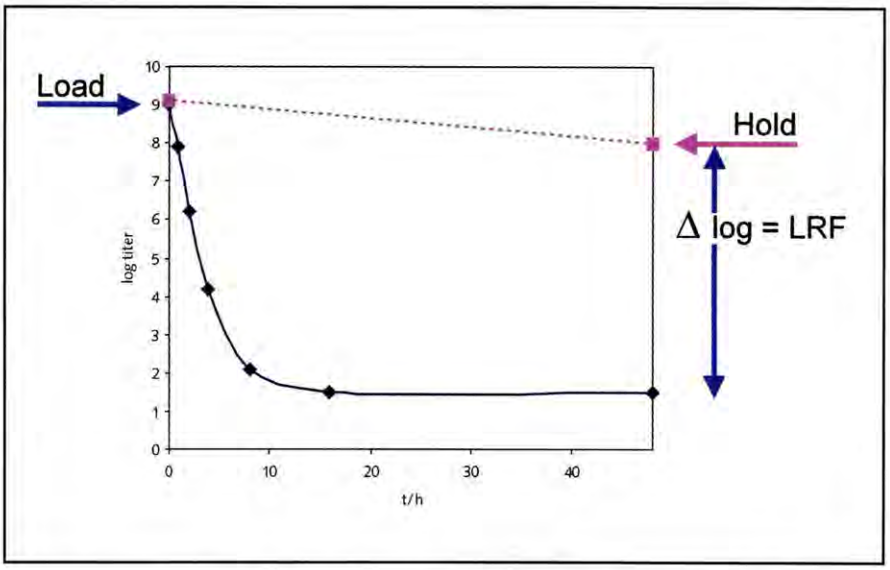

Figure 3. Inactivation kinetic of porcine parvovirus at pH 2. The difference in virus titer between the last time point and the hold sample corresponds to the logarithmic reduction factor (LRF).

of Cell Cultures, Salisbury, UK (ECACC), or American Type Culture Collections, Rockville, MD (ATCC). All new cell lines and single lots of expanded cell lines should be tested for mycoplasma prior to use. Mycoplasma-free cell lines can then be verified for identity by isoenzyme analysis on a routine basis. High titer virus suspensions are prepared from cell culture supernatants for spiking of the individual steps within the downstream process. The test systems should be validated for precision and accuracy according to the corresponding International Conference on Harmonization (ICH) guideline.[4]

In order to comply with GLP requirements, a study plan for the viral clearance study must be prepared and signed by both the test facility and the sponsor prior to initiating experimental work. The study plan describes in detail how the entire spiking, inactivation or elimination and detection procedure is to be performed. The sponsor must also demonstrate, prior to the start of the study and as precisely as possible, that the scaled-down version of the process step used for virus removal in the viral clearance study is representative of the production procedure. Furthermore, the rationale has to be defined for the schedule of sampling as well as the selection of the model viruses that will be used in the clearance study.

The Extent of Viral Clearance Studies

The country where the clinical trials will be performed and the development phase of the biopharmaceutical determine the number of viruses required in the study as well as the number of steps to be investigated. The requirements may vary. Often for clinical phases I and II, viral clearance is demonstrated in two steps for a parvovirus or a retrovirus [such as murine leukemia virus (MuLV)] when the product is derived from a rodent cell line, such as CHO cells. The two steps include one inactivation and one separation step. However, there may be conditions that require evaluations of more viruses or additional steps. The sponsor must discuss these issues with the authorities prior to starting the study.

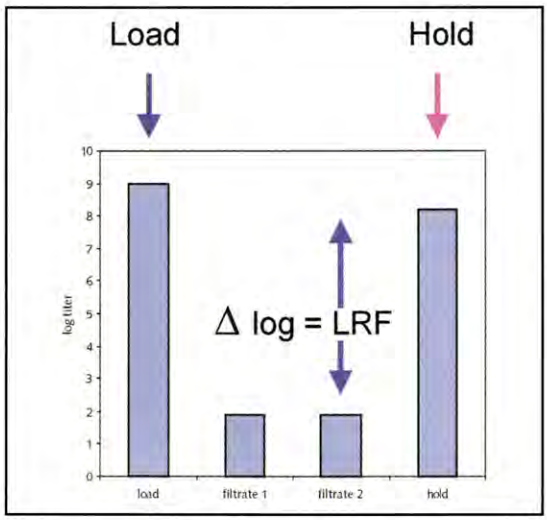

Figure 4. Separation of porcine parvovirus by nanofiltration in dead-end mode. The difference in virus titer between the filtrate and the hold sample corresponds to the logarithmic reduction factor (LRF).

Predictability of LRFs

Viral clearance should be addressed in the early phase of downstream process development. Timely planning will avoid time-consuming and costly redesign of the downstream process. It is also advisable to include one specific step in the downstream for viral removal. In designing the downstream, however, it is nearly impossible to predict the viral clearance that will be achieved. Available data on viral clearance via chromatography shows no uniform behavior of viruses or classes of viruses regarding their removal. This is true for anion and cation exchange chromatography. Even with size exclusion chromatography, the viral clearance remains unpredictable. Although nanofiltration normally results in good viral clearance, actual values are still impossible to predict.

With inactivation procedures such as heat, pH or solvent/detergent treatment, it may be easier to provide an estimate regarding the expected clearance. However, it is still impossible to provide a true prediction. This is due to the fact that the inactivation can be affected by many different variants, such as protein concentration or temperature of inactivation that may have a protective effect on the virus during the inactivation period.

The Choice of Viruses for a Viral Clearance Study

When selecting viruses for a clearance study, it is important to distinguish between evaluating processes for their ability to clear viruses that are known to be present, and estimating the robustness of the process by characterizing the

Table 1. Typical LRFs for test viruses in common downstream process steps. For the evaluation of the viral clearance capacity of the manufacturing process, the individual LRFs are added up.

Process steps typical for recombinant proteins	Typical LRF for PPV	Process steps typical for monoclonal antibodies	Typical LRF for MuLV	Process steps typical for plasma products	Typical LRF for HIV
Affinity Chromatography	3 - 6	Low pH	4 - 5	Dry heat	5 - 7
Gel Filtration	2 - 4	Ion exchange	2 - 5	Solvent/Detergent or Pasteurization	4 - 7
Nanofiltration	5 - 7	Nanofiltration	4 - 5	Nanofiltration	4 - 5
Total process log reduction	10 - 17	Total process log reduction	10 - 15	Total process log reduction	13 - 19

clearance of model viruses.

In those cases where no virus, virus-like particles or retrovirus-like particles have been demonstrated in the cells, virus removal and inactivation studies should be performed using model viruses. The model virus used, however, should be relevant when possible, i.e., BVDV as a model for hepatitis C virus.

In the case of rodent cells, where only rodent viruses or non-pathogenic retrovirus-like particles such as types A and R are present, the process evaluation should be performed using relevant species-specific viruses such as a MuLV. Other standard, non-specific viruses for the characterization of viral clearance are commonly used. The set of viruses used in a virus safety study should include a variety of viral characteristics such as RNA and DNA viruses, and enveloped and non-enveloped viruses. The size and the resistance to physico-chemical treatments must also be considered. Examples of useful model viruses used in our laboratories include: parainfluenza virus (PI, RNA/enveloped), bovine viral diarrhea virus (BVDV, RNA/enveloped), pseudorabies virus (PRV, DNA/enveloped), Encephalomyocarditis Virus (EMC, RNA/non-enveloped), Reovirus 3 (Reo-3, RNA/non-enveloped), SV-40 (DNA/non-enveloped), and porcine parvovirus (PPV, DNA/non-enveloped).[5]

Test viruses used in studies for human plasma-derived products should include potentially contaminating viruses such as HIV and HAV. These can be produced to high virus titer stocks via cell culture methods, and are normally used in viral clearance studies.

PPV is a good virus to use in the initial testing of certain process steps, especially those involving inactivation or nanofiltration. PPV is a very small virus of 18–24 nm in diameter and is very resistant to physico-chemical inactivation. If PPV can be inactivated or physically removed, the step will most probably inactivate or remove most other test viruses as well.

The Scope of a Viral Clearance Study

The scope of a viral clearance study consists of different sections that must be addressed sequentially:

- Verification of the scaled-down process step
- Determination of cytotoxicity of the test items on the indicator cells
- Determination of the existence of viral interference
- Performance of the process steps in the presence of virus spike and initiation of end-point titration on the same day
- Evaluation of the cytopathic effects (CPE) and statistical analyses
- Final report

Verification of the Scaled-Down Process Step

The scalability of the process step must be determined by the sponsor and not by the testing facility. Scalability is especially important for viral removal steps as the scaled-down step must exactly mimic that of the production scale. All relevant process parameters such as flow rates, volume/area ratios, pressure, temperatures, doses, concentrations, etc. must be comparable at both scales. Proof of scalability must be well documented.

Cytotoxicity

The test item or process sample must not have a cytotoxic effect on the indicator cells. Therefore, any cytotoxic effect induced by the test item on the indicator cells must be prevented. The test item may be toxic to the indicator cells and the degree of cytotoxicity differs for the various indicator cell lines. If the test item is cytotoxic, it must be diluted to the point where no more cytotoxicity is observed. However, if the dilutions are too high, this can result in a test item that is no longer similar to the process sample itself. This must be considered when designing the study. In addition, the higher the dilution, the smaller the dynamic range for the LRF.

The test item's cytotoxicity is evaluated using an MTT-test whereby viable and proliferating cells convert yellow tetrazolium salt into purple formazan by the activity of their mitochondrial dehydrogenases.[6] The resultant product is quantified photometrically (A_{550}). Test item concentrations that result in less than 75% of the proliferative activity of the control are considered cytotoxic for the indicator cells. Additionally, a microscopic evaluation of the indicator cells is performed. Both parameters (enzymatic and microscopic evaluation) determine the final dilution for the end-point titration.

Viral Interference

The test item must not interfere with the capability of the virus to replicate. Therefore, each test item must be tested for its possible interference in viral replication. Non-interference is demonstrated using a titration experiment in which the virus replicates in both the presence as well as the absence of the test item. The resulting virus titer should be comparable. If this is not the case, the test item must be diluted so that it no longer interferes with the virus' ability to replicate. When the test item contains large protein molecules, it does not adhere to the virus and, thereby, cannot make the virus inactive. This would be detectable during the course of the interference testing.

Spiking Experiment and End-Point Titration

The spiking experiment can be performed after the necessary dilution of the test item has been defined and no viral interference could be observed in the presence of the test item. A volume of serum-free media containing the high titer virus is added to the process. The volume used is usually a 1:10 or 1:20 dilution of the process media. A sample of the spiked virus, known as the hold sample, is held untreated throughout the length of the process. The hold sample is used to determine what viral loss occurs over the time of experimentation versus what viral loss occurs due to the process step itself. In an inactivation study, samples are withdrawn at various time points. In a study

of a filtration step or a chromatography step, the sample points are defined by the process itself.

The titer of the virus stock and the samples withdrawn during the study are determined by endpoint titration. Serial dilutions of the aliquots are made with cell culture medium and added in eight-fold replicates to the wells of 96-well microtiter plates containing the indicator cells. After the appropriate incubation period, which is virus-cell line dependent, the indicator cells are inspected microscopically for virus-induced changes in cell morphology, loss of cell adherence, syncytia formation, and cell death. These are generally referred to as cytopathic effects, or CPE.

Evaluation of the Cytopathic Effects and Statistical Analyses

The virus titer is calculated as $TCID_{50}$ according to the methods of Spearman and Kärber, and as outlined by the Paul-Ehrlich-Institut.[7–9] It should be recognized that virus inactivation is not a simple, first order reaction but is usually more complex with an initial fast phase followed by a slow phase two kinetic, which should be reflected in the inactivation curve. In the case of virus removal, the distribution of the virus load in the different fractions should be investigated.

The Amounts of Process Material Required for a Viral Clearance Study

The total volume of process samples needed for a viral clearance study depends on the number and type of process steps. Chromatography steps normally require more intermediate process material than inactivation or nanofiltration steps. The following numbers provide rough estimates of the volumes required for a viral clearance study with four test viruses performed in duplicate including cytotoxicity and viral interference testing:

- Inactivation steps — 150 ml
- Nanofiltration — 100–200 ml, depending on the membrane size
- Chromatography steps — highly dependent on the degree of down-scale, but can possibly require as much material as produced in a 100 L fermentation run, if further down-scaling is not possible.

Interpretations and Limitations of Viral Clearance Studies

The dynamic range needed to determine the LRF depends on the initial titer of the virus stock, the dilution required to remove cytotoxic effects, and the amount of material that was spiked. Table 2 shows an example of the maximum LRF that can be expressed if the two starting parameters (virus stock titer and cytotoxicity) differ in the described fashion.

The objective of assessing virus inactivation/removal is to evaluate and characterize process steps that can be considered effective in inactivating/removing viruses, and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. To ensure an appropriate level of safety for the final product, there should be an excess capacity for viral clearance built into the purification process. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus present in unprocessed bulk, which provides a worst-case estimate.

An overall reduction factor is generally expressed as the sum of each step's reduction logarithm. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction in virus titer of the order of one log_{10} or less is considered insignificant. Furthermore, reduction values achieved by repetition of procedures that use the same mechanism to remove or inactivate a virus should not be included unless justified.

Clearance mechanisms may differ between virus classes. To judge the data that support the effectiveness of virus inactivation or removal, the following factors must be considered:

- the appropriateness of the test viruses used
- the design of the clearance studies
- the reduction achieved (in log_{10})
- the time-dependence of inactivation
- the potential effects of variation in process parameters on virus inactivation/removal
- the limits of assay sensitivities
- the possible selectivity of inactivation/removal procedures for certain classes of viruses

Table 2. The maximum LRF that can be expressed depends on the initial titer of the virus stock, the amount of spike volume, and the cytotoxicity of the test item.

log_{10} $TCID_{50}$/ml			Examples PPV	Examples MuLV
	10			
	9	Titer of virus stock	9.0	7.6
	8	Titer of the load/hold (10% spike)	8.0	6.6
	7	Cytotoxicity dilution factor	1:10	
	6	Cytotoxicity dilution factor		1:100
	5			
	4	LRF_{max}	> 5.7	> 3.6
	3			
	2	Titer of the diluted, processed test item (LOD)	< 2.3	< 3.0
	1			
	0			

The Scope of a Prion Clearance Study

The general principles of a viral clearance study apply to prion clearance studies as well. There is still no cell-culture based assay available that can be used for the determination of a prion LRF. A prion clearance study with the infective prion material produced in either hamster or mouse can use two options to detect prion infectivity:

- Western blot analysis by detecting proteinase K resistance PrP^{Sc}
- Animal studies using hamster or mouse

A correlation has been shown by Lee that the results obtained by Western blot analyses are comparable to those in animal studies (Table 3).[10] The Western blot is useful in determining which procedures or methods are most effective in removing or inactivating prions. Once this is known, these steps can be re-tested in animal studies to determine the actual total log reduction. This approach minimizes the use of animals to generate data.

Special attention has to be drawn to the preparation of the prion spike.

Table 3. Comparison of LRFs for various process steps generated by western blot and bioassay. Abbreviations: BH, Brain homogenate, SHa (263K), Syrian hamster, isolate 263K.[10]

Process step	Source	Spike	LRF (WB)	LRF (Bioassay)
Cryoprecipitation	SHa (263K)	BH	1.0	1.0
Cryoprecipitation/3% PEG			3.0	2.2
Fraction II/III			> 4.7	6.0
Fraction III			> 4.3	5.3
Fraction IV1			> 4.2	3.7
Fraction IV1 prec./11% PEG			> 4.9	> 5.4
Fraction IV4			> 4.1	4.6

Several types of prion material from hamster or mouse have already been used in prion clearance studies:

- Brain homogenate
- Brain homogenate, treated with solvents/detergent and filtered
- Microsomal fractions
- Caveolae-like domains (CLDs)
- Purified PrP^{Sc} or prion rods

For NaOH inactivation studies, brain homogenate is suitable as spike material. If nanofiltration or chromatography is to be tested, prions must be in a more soluble form usually using a detergent treatment followed by filtration. It is believed that prion rods behave differently from other infective prion fractions and should not be used for clearance studies. Figure 5 shows a typical Western blot from a process step for the inactivation of hamster infective prion material. It clearly demonstrates that this reduction has a resolution of half-log steps.

Summary of Virus and Prion Clearance Studies

The design and outcome of a virus/prion clearance study depend on various factors, which might have an important

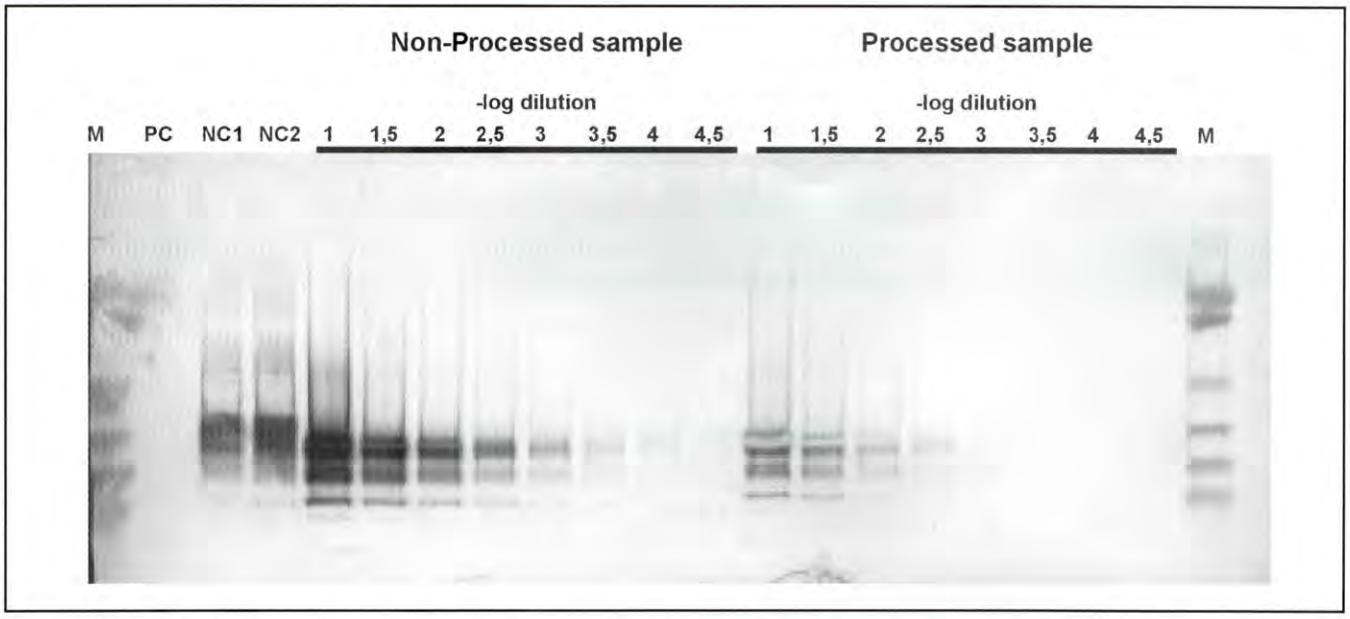

Figure 5. Western blot analysis of a process step for the inactivation of prion infectivity (hamster). Comparison of the non-processed samples (7 lanes detectable) with the processed sample (4 lanes detectable) in 0.5 log dilution series.

impact on the process development, and ultimately on the time frame required to bring the biopharmaceutical to market:

- Status of drug development (clinical phases I to III, BLA)
- Country where the clinical studies are performed
- Natural load of retroviruses
- Validated, scaled-down process
- Stability of intermediate processes or final product
- Time constraints for performing virus and prion clearance studies

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