

Barrier treatments for bovine serum to prevent introduction of viral contaminants into biologics

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What are barrier treatments and why are they needed for bovine serum?

Barrier treatments are serum treatments (e.g., gamma, UV, heat) intended to mitigate (not eliminate) the risk of introducing a viral contaminant into a geographical region (import concern) or a cell culture (biologics industry concern).

Regulatory Basis

European guidance document

EMA/CHMP/BWP/457920/2012 rev 1 addresses the use of bovine serum in the manufacture of human biological medicinal products, stating that “it is strongly recommended, in addition to direct testing for viruses, to inactivate the serum by a validated and efficacious treatment. The use of non-inactivated serum should be justified.”

and that “Gamma irradiation is the most commonly used method for viral inactivation of serum as a means of obtaining a safe but biologically active product.”

Regulatory Basis

European Pharmacopoeia

Monograph 2262 *Bovine Serum* states that:

“For bovine serum intended for use in immunological veterinary medicinal products, inactivation by gamma irradiation at a minimum dose of 30 kGy is to be applied, unless otherwise justified and authorized.”

Chapter 5.2.5 *Management of Extraneous Agents in Immunological Veterinary Medicinal Products* recommends that:

“[Users] apply processing treatments that remove or inactivate extraneous agents.”

Viral risk in non-barrier-treated bovine serum

In non-treated bovine serum, the risk of viral contamination is mitigated only by sourcing considerations (region of origin, immunization status of animals, regulatory oversight of abattoirs, etc.) and viral safety testing.

Sampling considerations mean that insufficient risk mitigation is afforded by viral testing alone.

What are the most commonly used barrier treatments?

Gamma irradiation (deeply frozen serum)

Heat treatment (liquid serum)

Ultraviolet C light irradiation (liquid serum)

Additional approaches include:

Electron beam irradiation (low penetrability)

X-ray treatment (intermediate penetrability)

Mechanism of action

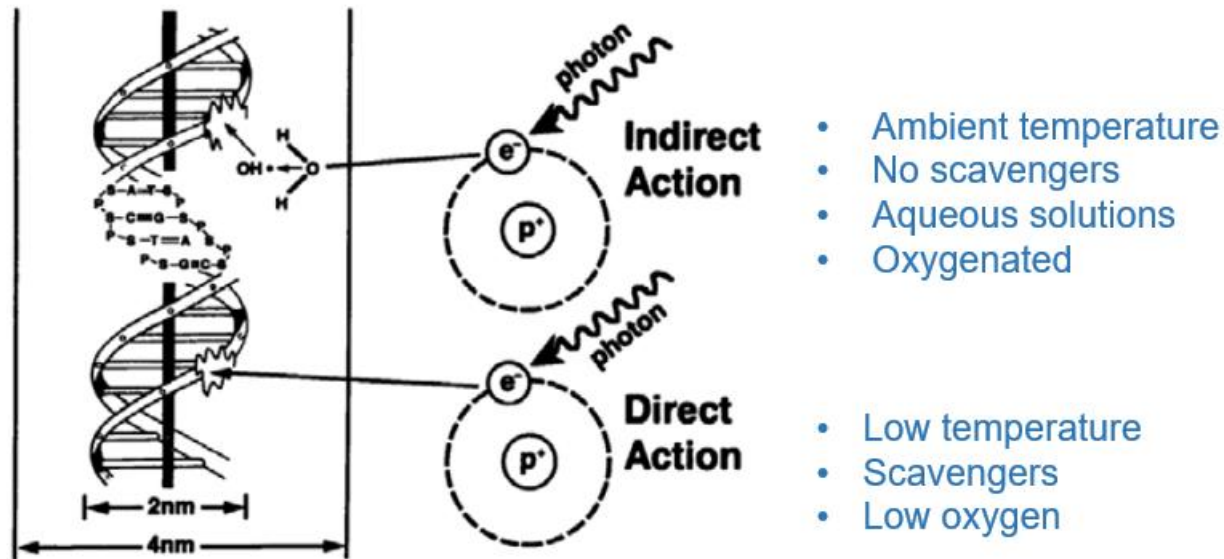
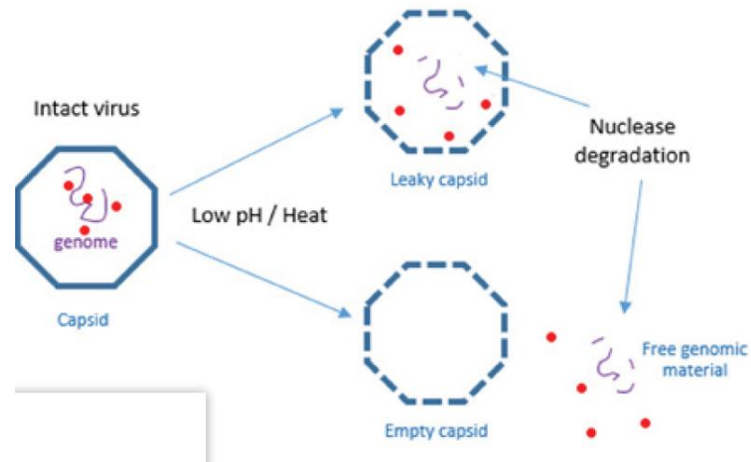


Fig. 2.2 **Direct** versus indirect action (Hall and Giaccia, 2006).

High energy photons/electrons: gamma irradiation, electron beam, x-ray

Mechanism of action

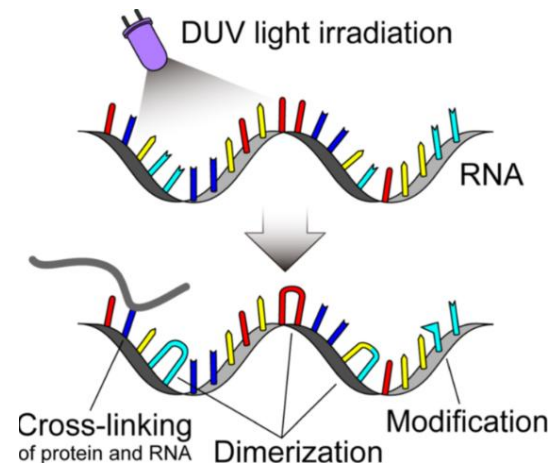
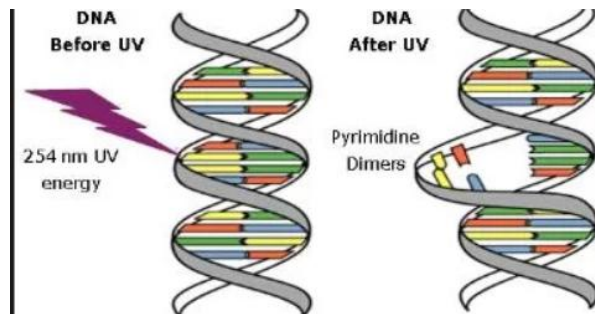
The mechanism of heat inactivation of viruses is thought to involve denaturing of capsid proteins, allowing entry of nucleases and loss of genomic material.



Heat treatment

Mechanism of action

Genomic base composition, especially the specific sequence of adjacent base pairs, determines the intrinsic sensitivity of viruses to UV C light irradiation



Ultraviolet light irradiation

Virucidal efficacy of barrier treatments

The **virucidal efficacies** of barrier treatments are determined by:

- Fluence (radiation dose = dose rate $\times$ exposure time)
 - Gamma irradiation: kGy (kiloGrays)
 - Ultraviolet C light: mJ/cm² (milliJoules per cm²)
- Temperature and time (heat inactivation)
- Inactivation matrix, in particular:
 - Presence of scavenging protein, oxygen, temperature (gamma)
 - Presence of 265 nm-absorbing materials (UVC)
 - Liquid vs. solid (heat inactivation)

Virucidal efficacy of barrier treatments

The **virucidal efficacies** of barrier treatments depend on:

- The virus being inactivated, with susceptibility dependent on:
 - Viron size (gamma irradiation)
 - Pyrimidine dinucleotide content (UV-C irradiation)
 - Capsid protein make-up (heat inactivation)
- Note that envelope status is not particularly important for determining the efficacy of these physical approaches for inactivating viruses.

The 9 CFR 133.53 list of bovine viruses of concern

A list of bovine viruses of concern is provided in 9 CFR 113.47 *Detection of extraneous viruses by the fluorescent antibody technique.*

- Bovine viral diarrhea virus (enveloped, medium sized)
- Reovirus (non-enveloped, medium sized)
- Rabies virus (enveloped, large sized)
- Bluetongue virus (non-enveloped, medium sized)
- Bovine adenoviruses (non-enveloped, medium sized)
- Bovine parvovirus (non-enveloped, small sized)
- Bovine respiratory syncytial virus (enveloped, large sized)

Additional viruses are tested for in the “modified 9 CFR bovine virus test”

From World Health Organization:

- **Infectious bovine rhinotracheitis virus** (enveloped, large sized)
- **Parainfluenza type 3** (enveloped, large sized)
- **Vesicular stomatitis virus** (enveloped, large sized)

Annex 3

**Recommendations for the evaluation of animal cell cultures
as substrates for the manufacture of biological medicinal
products and for the characterization of cell banks**

Replacement of Annex 1 of WHO Technical Report Series, No. 878|

Viruses of concern from the biologics manufacturing point of view

Viral Contaminant	Family	9CFR113 virus?
Epizootic hemorrhagic disease virus	Reovirus	no
Reovirus type 2	Reovirus	yes
Bluetongue virus	Reovirus	yes
Vesivirus 2117	Calicivirus	no
Cache valley virus	Bunyavirus	no

Viruses of Concern per USP <1024>

In addition to the viruses listed above, other viruses can be causative agents of disease and may require testing in various bovine serum applications. The serum end user is responsible for determining whether full 9 CFR testing is sufficient, and if other specific viral agents should be tested for. Examples of specific viruses not covered by the current virus testing guide may include akabane, bovine herpesvirus 1 (BHV-1), Parainfluenza-3 virus (PI-3), bovine leukemia, bovine rotavirus, bovine circovirus, bovine polyomavirus, coronavirus, torovirus, bovine enterovirus, bovine astrovirus, foot-and-mouth disease virus (FMDV), and rinderpest. *Appendix 2* provides a general description of some of these viruses as well as the ones for which testing is required. A serum end user's thorough risk analysis should determine the scope of testing and serum treatment options.

What does the biologics regulatory guidance tell us?

Test serum for the 9 CFR 113.53 viruses, but also....

7.3.5. Other bovine viruses

Methods based on the detection of viral DNA suggest that bovine polyoma virus (BPyV) is a common contaminant of bovine serum. Serum manufacturers and users are encouraged to apply infectivity assays for BPyV and to investigate methods for the inactivation/removal of BPyV in order to limit or eliminate infectious virus from batches of serum¹.

Serum suppliers and users should also be aware of emerging bovine viruses and are similarly encouraged to investigate the presence of such agents in bovine serum and to take appropriate action to eliminate or reduce the presence of any novel virus in serum.

30 May 2013
EMA/CHMP/BWP/457920/2012 rev 1
Committee for Medicinal Products for Human Use (CHMP)

Guideline on the use of bovine serum in the manufacture of human biological medicinal products

Viruses of current importation concern

Viruses listed in the 9 CFR 113 plus:

- Infectious bovine rhinotracheitis virus (enveloped, large sized).
- Bovine coronavirus (enveloped, large sized)
- Bovine rotavirus (non-enveloped, medium sized)
- Bovine leukosis virus (enveloped, large sized)
- Schmallenberg virus (enveloped, large sized)

Comparative efficacy of different barrier treatments

Data to be shown for gamma irradiation are primarily for serum irradiated while deeply frozen, while data for heat and UV are for a variety of liquid matrices.

Limited data are available for electron beam and x-ray irradiation. Due to penetrability issues, these treatments may not be useful for frozen serum packaged in 500-mL or 1-L serum bottles. Electron beam has been used for powdered trypsin and for thin tissue sterilization.

Inactivation by physical barrier treatments is typically first order

Gamma, UV, and heat inactivation are considered first-order processes. This means that if a treatment dose yields 1 $\log_{10}$ inactivation, a 2 $\times$ dose is expected to yield 2 $\log_{10}$ inactivation.

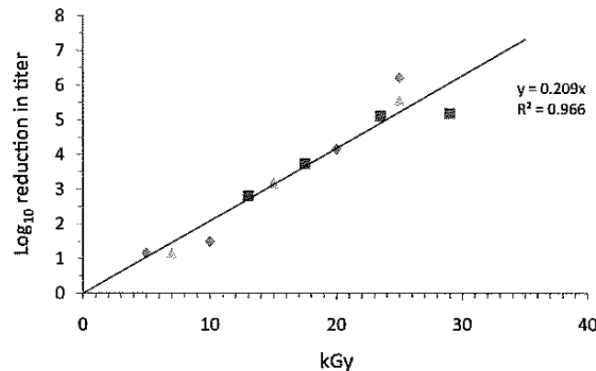


Fig. 1. Relationship between gamma irradiation dose (kGy) and inactivation ($\log_{10}$ reduction in viral titer) for parainfluenza virus type 3 (PI3) spiked into fetal bovine serum, from Ref. [14] ($\diamond$) Ref. [13], ($\blacksquare$), and Ref. [12] ($\blacktriangle$). The line equation and R^2 value for the linear trend line are shown.

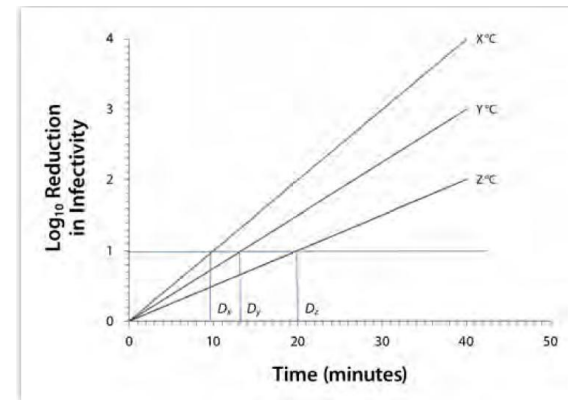


FIGURE 2. Theoretical inactivation vs. time curves for a virus at three temperatures ($X^{\circ}\text{C}$, $Y^{\circ}\text{C}$, and $Z^{\circ}\text{C}$). A graphical representation of the D value (decimal reduction value, or the time in minutes for a 1 $\log_{10}$ reduction in viral titer) at each temperature is also shown.

Comparative efficacy of different barrier treatments

Bovine virus of concern	Virus family	Log ₁₀ inactivation expected following barrier treatment		
		Gamma irradiation (25 kGy)*	Heat (56°C, 30 minutes)	UVC (50 mJ/cm ²)
Enveloped (large sized)				
Schmallenberg virus	bunyavirus	4.0 (Akabane, Aino virus)	2.6 (Rift Valley fever virus)	2.4 (Cache Valley virus)
Cache Valley virus	bunyavirus	4.0 (Akabane, Aino virus)	2.6 (Rift Valley fever virus)	2.4
Infectious bovine rhinotracheitis virus	herpesvirus	7.8	4.1 (herpes simplex 1)*	3.0
Bovine coronavirus	coronavirus	>10 (SARS-CoV-2)	6.2 (SARS-CoV-2)	>10
Bovine leukosis virus	retrovirus	3.3 (feline leukemia virus)	>10 (HTLV III)	2.0 (murine leukemia virus)
Rabies virus	rhabdovirus	8.5 (bovine ephemeral fever virus)	>10	>10 (vesicular stomatitis virus)
Vesicular stomatitis virus	rhabdovirus	8.5 (bovine ephemeral fever virus)	>10	>10
Parainfluenza type 3	paramyxovirus	5.2	9.7 (Newcastle disease virus, Nipah virus)	3.5
Bovine respiratory syncytial virus	paramyxovirus	5.2 (parainfluenza type 3 virus)	9.7 (Newcastle disease virus, Nipah virus)	3.5 (parainfluenza type 3)
Enveloped (medium sized)				
Bovine viral diarrhea virus	flavivirus	5.0	6.0	4.1
West Nile virus	flavivirus	5.0 (bovine viral diarrhea virus)	6.0 (bovine viral diarrhea virus)	9.1
Non-enveloped (large sized)				
Bovine adenovirus	adenovirus	5.1 (canine adenovirus)	2.1 (human adenovirus 5)*	1.2 (adenovirus)
Non-enveloped (medium sized)				
Reovirus	reovirus	4.9	<1*	2.2
Bluetongue virus	reovirus	3.0	<1 (reovirus 3, bovine rotavirus)	2.2 (reovirus)
Bovine rotavirus	reovirus	3.9 (reovirus, bluetongue virus)	2	2.2 (reovirus)
Epizootic hemorrhagic disease virus	reovirus	3.9 (reovirus, bluetongue virus)	1.5 (reovirus 3, bovine rotavirus)	2.2 (reovirus)
Non-enveloped (small sized)				
Bovine polyomavirus	polyomavirus	1.4 (SV40)	0.5 (SV40)	0.7 (SV40, mouse polyoma virus)
Seneca Valley virus	picornavirus	4.8 (foot and mouth disease virus)	3.0 (foot and mouth disease virus)	2.3 (foot and mouth disease virus)
Foot and mouth disease virus	picornavirus	4.8	3.0	2.3
Vesivirus 2117	calicivirus	Not tested	7.5 (feline calicivirus)	6.5 (feline calicivirus)
Bovine parvovirus	parvovirus	1.4 (porcine parvovirus, mouse minute virus)	<1.0	4.0 (porcine parvovirus)

Bold values:
Apply to the actual virus

Unbolded values:
Apply to other family members
(shown)

Pink highlight:
Weak inactivation responses

Some comments about the comparative efficacy table

- The efficacy shown for gamma irradiation was assessed at the low end of the typical serum irradiation dose (25 kGy). The greater the fluence applied, the greater the expected efficacy. Per USP <1024> *Serum treatment by gamma irradiation is very common and one of the most effective methods of virus inactivation.*
- Not very effective for parvoviruses or circoviruses, even at 45 kGy. Polyomavirus susceptibility is not well understood.

Some comments about the comparative efficacy table

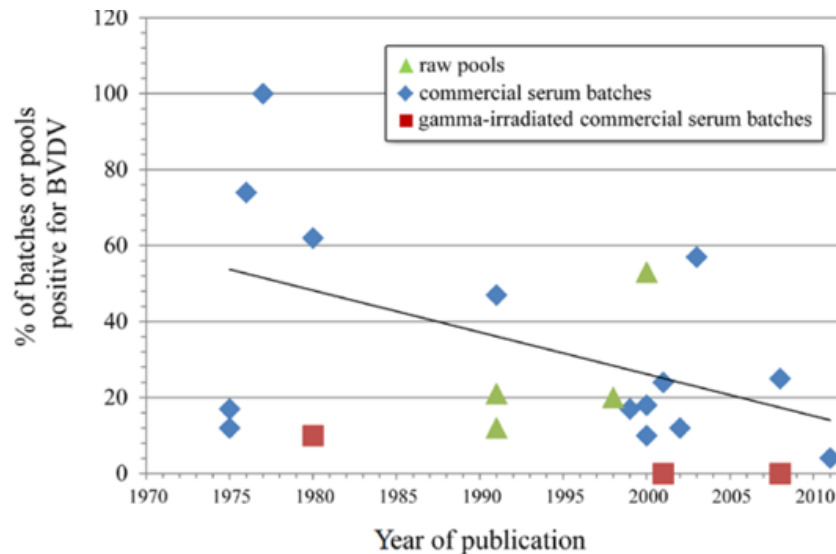
- The efficacy of heat is provided at conditions typically used for heat-inactivating serum (56°C for 30 minutes). Per USP <1024> *Heat inactivation provides significantly less assurance of virus inactivation than does irradiation*. The data shown are for heating in liquids, but not serum, so extrapolation is to be made with caution.
- Not very effective for: polyomaviruses, parvoviruses, circoviruses, reoviruses, adenoviruses, bunyaviruses

Some comments about the comparative efficacy table

- UVC irradiation is rarely used as a barrier treatment for serum. The efficacy of UVC assumes that the fluence delivered to the virus is 50 mJ/cm², a mid-range fluence for inactivating viruses in serum. Per USP <1024> *Serum manufacturers may use UV treatment to inactivate viruses, mycoplasma, and bacteria, but manufacturers must validate the process to demonstrate its efficacy.*
- Only marginally more effective than heat based on the data generated thus far.

BVDV, a viral contaminant of special concern?

Is BVDV a common contaminant of FBS? **Certainly.** The non-cytopathic biotype is found in FBS tested with the 9 CFR 113.53 assay. Frequency of detection varies.



BVDV, a viral contaminant of special concern?

Why can you get a negative results from one 9 CFR assay and a positive result with another for the same serum lot? Here are some reasons:

- Assay differences: BT cells are known to be more sensitive to infection by BVDV than MDBK cells.
- Sampling issues: testing 1 bottle of serum out of a lot of up to 3000 bottles.
- The 9 CFR 113.53 assay utilized may or may not have been characterized for limit of detection.

BVDV, a viral contaminant of special concern?

Mitigation risk:

- The good news: Gamma, UVC, and heat each display $>4\text{-log}_{10}$ efficacy for inactivating BVDV at typically applied doses.
- The bad news: Positive nucleic acid results may still be obtained when testing irradiated serum. The sequences detected by the NAT assay may not have been fully degraded. Why test irradiated serum using this method? It is infectivity that is important.

BVDV, a viral contaminant of special concern?

Mitigation risk

- Why the worry about BVDV antibodies?
- The concern should not be simply the presence/absence of antibody, rather the titer of the antibody and whether or not it is neutralizing.
- Neutralizing antibodies reflect the ability to inactivate serum

Conclusions

Gamma irradiation is relied on to mitigate the risk of most of the viruses of concern from the USDA, World Health Organization, and biologics manufacturing points of view. It is not perfect, though.

Few remaining viruses that will likely survive gamma irradiation are the **parvoviruses** (common contaminants, but low likelihood of human pathology), **circoviruses** (low occurrence, low likelihood of human pathology), and possibly **bovine polyomavirus** (low occurrence, low infectivity for humans).

Conclusions

Heat inactivation at 56°C, 30 min. (protocol used for inactivating complement) mitigates risk for certain viruses of concern from the USDA, World Health Organization, and biologics manufacturing points of view. The viruses of concern that may survive heat treatment are **bovine circoviruses** (low occurrence, low likelihood of human pathology), **bovine polyomavirus** (low occurrence, low infectivity for humans), **bovine parvovirus** (low likelihood of human pathology) and **bovine adenoviruses**, **reoviruses** (cause animal and human pathology), and **bunyaviruses** (cause animal and human pathology).

Conclusions

UVC irradiation could mitigate the risk of certain of the viruses of concern from the USDA, World Health Organization, and biologics manufacturing points of view. The limited work that has been done to date to assess the efficacy of UV-C for inactivating viruses in FBS or plasma is inconsistent. More work must be done before this treatment can be considered useful. Specialized equipment is required to irradiate serum, and dosimetry may be challenging due to lamp degradation and matrix absorbance.

Conclusions

What to make of all of this?

Each of the barrier treatments is expected to be very effective for BVDV. Gamma irradiation is the preferred barrier treatment for serum. UVC is complementary to gamma irradiation in that it seems capable of inactivating parvoviruses and circoviruses, but is less useful for adenoviruses and retroviruses, so is not a good replacement. Heat is more effective than most people believe but is the least effective of the three. When done in conjunction with gamma and UVC, it does not resolve the efficacy gaps for those barrier treatments.

References

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2. Plavsic M, et al. Gamma irradiation of animal serum: Validation of efficacy for pathogen reduction and assessment of impacts on serum performance. *BioProcess J* 15:12-21, 2016.
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References

7. USP <1024> Bovine Serum
8. Meunier SM, et al. Evaluating ultraviolet sensitivity of adventitious agents in biopharmaceutical manufacturing. J Ind Microbiol Biotechnol 44:893-909 (2017).
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Efficacy Comparison: Gamma and Electron Beam

Virus (strain)	Matrix (temperature)	gamma irradiation		electron beam		Ref.
		$K (\log_{10}/\text{kGy})$	kGy for $4 \log_{10}$	$K (\log_{10}/\text{kGy})$	kGy for $4 \log_{10}$	
herpes simplex virus (Thea)	Hank's balanced salt solution (frozen)	0.368	11	0.141	28	7
herpes simplex virus (Müller)	Hank's balanced salt solution (frozen)	0.307	13	0.141	28	7
Rauscher leukemia virus	Hank's solution (frozen)	0.722, 0.198*	20	0.361, 0.117*	34	7
bacteriophage Φ X174	tap water (ambient)	2.94	1.4	1.43	2.8	12
bacteriophage MS2	tap water (ambient)	10.0	0.4	10.0	0.4	12
bacteriophage B40-8	tap water (ambient)	22.2	0.2	50.0	0.1	12

*Biphasic kinetics were attributed to two targets, Rauscher leukemia virus itself, and a defective helper virus present in the virus stock (spleen focus-forming virus). The kGy for $4 \log_{10}$ inactivation is based on the lowest K value.

Table 4: Direct comparison of viral inactivation by gamma and electron beam irradiation.