# BSR/PDA Standard 04-201x, Phage Retention Nomenclature Rating for Small and Large Virus-Retentive Filters

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# BSR/PDA Standard 04-201x, Phage Retention Nomenclature Rating for Small and Large Virus-Retentive Filters

## Authors

Scott Lute (Chair)
U.S. Food and Drug Administration

Duncan Low (Co Chair)
Claymore Biopharm LLC

Kurt Brorson
Parexel International

Stephen Stoltzfus
Eurofins Lancaster Laboratories, Inc.

Anthony Laccetti
Lyophilization Services of New England

Eric R. Weiss
MilliporeSigma

Brian Beck
Microbiologics

Morven McAlister
Pall Biotech

Sherri Dolan
Sartorius North America

Quanyi (Charlie) Li
Millipore Sigma

Julie Kozaili
Asahi Kasei Bioprocess America

Albert Mihranyan
Uppsala University

Lee Ineui (ILee)
Kobe University

Kavita Nanda
Biogen

Deepak Aggarwal
U.S. Food and Drug Administration

Kang Cai
AstraZeneca

Andrew Zydney
The Pennsylvania State University

Ashley Slocum
Pfizer

Sean Michael O'Donnell
Eli Lilly and Company

Astrid Schwantes
Paul Ehrlich Institute

Johannes Blümel
Paul Ehrlich Institute

Daniel Eshete
Pall Biotech

**1. Introduction** ..... **2**
**2. Scope** ..... **2**
**3. Normative References** ..... **2**
**4. Terms and Definitions** ..... **3**
**5. Acronyms /Abbreviations** ..... **5**
**6. Large Virus-Retentive Filter Test Protocols** ..... **6**
**6.1 Strategy** ..... **6**
**6.2 Model virus** ..... **6**
**6.3 Model proteins** ..... **6**
**6.3.1 Model protein acceptance criteria:** ..... **6**
**6.4 Buffer system** ..... **7**
**6.5 Scaled-down model filters** ..... **7**
**6.6 Operating parameters** ..... **7**
**6.7 Hold Control** ..... **8**
**6.7.1 Set point values for relevant operating parameters** ..... **8**
**6.8 Nomenclature acceptance criteria** ..... **8**
**6.9 Other considerations** ..... **9**
**6.10 Other acceptance criteria** ..... **9**
**7 Small Virus-Retentive Filter – Test Protocol** ..... **9**
**7.1 Strategy** ..... **9**
**7.2 Model viruses** ..... **10**
**7.3 Model protein** ..... **10**
**7.4 Scaled-down model filters** ..... **11**
**7.5 Operating parameters** ..... **11**
**7.6 Hold control** ..... **12**
**7.7 Nomenclature acceptance criteria** ..... **12**
**7.8 Other considerations** ..... **13**
**7.9 Other acceptance criteria** ..... **13**
**8 Bacteriophage Preparation Procedures** ..... **13**
**8.1 Introduction** ..... **13**
**8.2 Equipment and supplies** ..... **14**
**8.3 Procedure** ..... **14**
**8.4 Plate method** ..... **14**
**8.5 Broth method** ..... **15**
**8.6 Crude preparation** ..... **15**
**8.7 Pure concentrate** ..... **16**
**8.8 Storage** ..... **16**
**9 Procedure for Enumeration of Bacteriophage** ..... **16**
**9.1 Introduction** ..... **16**
**9.1.1 Equipment and supplies** ..... **16**
**9.2 Procedure** ..... **17**
**9.2.1 Preparation of bacteriophage host** ..... **17**
**9.2.2 Preparation of dilution tubes and soft agar for use in phage assays** ..... **17**
**9.2.3 Phage assays** ..... **17**
**10 Bibliography/ References** ..... **18**

# 1. Introduction

Virus filtration is performed as part of a manufacturer's overarching virus safety strategy. Virus filtration (size-based removal) is a complement to virus inactivation and adsorptive virus removal (e.g., chromatography), all, of which contribute to virus clearance [1, 2]. Implementation of virus clearance complements additional measures, such as control over raw materials and testing of cell culture or plasma feedstock. Collectively, these measures form the framework of a virus safety strategy [3-5].

This potential standard address virus-removal filters that retain viruses by a size-exclusion mechanism. This document should be considered as a guide; it is not intended to establish any mandatory or implied standard.

# 2. Scope

This guide is intended to provide filter suppliers and end-users with an approach to standardizing methodology and nomenclature for large and small virus retentive filters using bacteriophage as a model. The objective is to assist users/manufacturers in selecting the most appropriate filter for their specific application needs. It is intended for virus retentive filters used where a virus clearance claim is made.

This proposed American National Standard (ANS) is intended to:

- Provide detailed methods and acceptance criteria for testing bacteriophage (also referred to as phage) retention by large and small virus-retentive filters,
- Provide methods for preparing and enumerating suitable sized bacteriophage (PP7 and PR772 as models for small and large viruses, respectively) as test items, and
- Help selection of appropriately rated filters as defined by suppliers in a standardized manner using a risk-based approach.

This is not a substitute for process validation for viral clearance claims.

# 3. Normative References

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies. ASTM WK65429 (New Practice for the Process to Remove Retrovirus by a Small Virus Retentive Filter) retrovirus removal claim for small virus retentive filters should also be considered if approved

- PDA *Technical Report No. 41 (revised 2008): Virus Filtration* (specifically, the sections related to the protocols highlighted here) [6]
- *PDA Journal of Pharmaceutical Science and Technology*, Vol. 62 • No. S-4 • 20084 [6]
- ASTM E2888 (low pH virus inactivation) [5]
- E3042 (detergent virus inactivation) [7]

## 4. Terms and Definitions

- Bracketing – A demonstration of unit operation performance at high/low values of a given parameter (e.g., ionic strength, dwell time or temperature), allowing the use of any values of that parameter falling within this range.
- Bubble Point – The minimum pressure at which a wetting liquid is pressed out of the pore of a membrane by a gas (typically air or nitrogen) while forming a steady bubble chain.
- Capsule Filter – Compact, self-contained filter assembly. Generally, the whole assembly is disposable.
- Cartridge Filter – Filter elements encased in a housing. Generally, the filter elements are disposable while the housing units are multiuse. In a few cases, both filter and housings are disposable.
- Compatibility (Filter) – The ability of a filter to be used with a particular process fluid without a change in the inherent properties of the filter materials.
- Diffusion Test (or Forward Flow Test) – An integrity test in which a filter is subjected to differential gas pressures below the bubble point, and gas molecule migration through the water filled pores of a wetted membrane is measured. This behavior follows Fick's Law of Diffusion (i.e., the gas diffusional flow rate for a filter is proportional to the differential pressure and the total surface area of the filter).
- Endogenous Virus-like Particles – (e.g., Type C endogenous retroviruses) Virus-like entity whose genetic material is stably integrated into the germ line of an organism or cell line. Cell lines (notably CHO) may constitutively produce virus-like particles, which are typically noninfectious but still of safety concern. Model retroviruses are generally used as surrogates to measure virus-like particle clearance.
- Filtrate – The fluid that has passed through the membrane (also see "Permeate").
- Flux – The volumetric flow rate of fluid per unit filtration area. Flux is often expressed in $L/m^2$-h.
- Flux Decay – In the context of this Standard, and in order to provide a fair comparison between the variety of filter types, flux decay will be determined using the initial flux relative to current flux. An 80% flux decay refers to the current flux being 20% of the initial flux (80% less).
- Integrity Testing – A fundamental requirement of critical-process filtration applications that verifies the absence of leaks and defects, confirms proper installation, and assures filter performance.as per product claims.
- Limit of Detection – The lowest amount of analyte in a sample that can be distinguished from the absence of analyte.
- Log Reduction Factor (LRF) or Log Reduction Value (LRV) – The virus reduction factor of an individual purification, removal or inactivation step is defined as the $\log_{10}$ of the ratio of the virus titer or total load in the pre-process material and the virus titer or load in the post-process material which is ready for use in the next step of the manufacturing process. The viral clearance capacity of a unit operation calculated as LRV = $\log_{10}$ (virus titer or load pre-process ÷ virus titer or load– post-process).
- Multiplicity of Infection (MOI) – The average number of infectious units added per cell in an infection.
- Nominal Pore Size Rating – A filter rating with an arbitrary value, indicating a particulate size range at which the filter manufacturer claims the filter removes some percentage. Nominal ratings vary among

suppliers and may not be a suitable criterion to compare filters among manufacturers. Processing conditions, such as operating pressure and concentration of contaminant may have a significant effect on the retention efficiency of the nominally rated filters.

- Model Virus – A virus used for characterization of viral clearance capacity of a manufacturing process to remove and/or inactivate viruses.
- Permeate – The fluid which passes through a membrane (also see "Filtrate").
- PP7 – Ribonucleic acid (RNA) bacteriophage that infects *Pseudomonas aeruginosa* bacteria and that has a size of approximately 28-30 nm.
- PR772 – Double-stranded deoxyribonucleic acid (DNA) bacteriophage that infects *Escherichia coli* bacteria and that has a size of approximately 80 nm.
- Pressure Hold Test (or Leak Test) – A test for leaks and gross defects in which the system is held at a defined pressure for a defined time. Failure is indicated by the observation of a steady stream of air bubbles downstream of the filter.
- Porosity (Synonym: Void Volume) – The ratio of void volume to bulk volume of the filter media.
- Porosimetry (Gas-liquid and Liquid-liquid) – An analytical technique used to determine various quantifiable aspects of a material's porous nature, such as pore diameter, total pore volume, surface area, and bulk and absolute densities.
- Transmembrane Pressure (TMP) – The difference ($P_{feed} - P_{permeate}$) in pressure across a filter membrane.
- Ultrafiltration Membranes – Membranes that retain solutes/particles whose sizes are measured by molecular weight, with retention ranges from 1,000 to 1,000,000 Daltons.
- Viral Clearance – Reduction of a target virus by removal of viral particles or by inactivation of viral infectivity. Viral clearance capacity is determined by studies in which model viruses are used in conjunction with process parameters representative of manufacturing conditions.
- Viral Inactivation – Reduction of virus infectivity caused by chemical or physical modification.

## 5. Acronyms /Abbreviations

| | |
|---|---|
| ANS | American National Standard |
| ASTM | American Society for Testing and Materials |
| BSA | Bovine Serum Albumin |
| CHO | Chinese Hamster Ovary |
| CoA | Certificate of Analysis |
| DFF | Direct Flow Filtration |
| HPLC | High Performance Liquid Chromatography |
| ICH | International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use |
| IVIG | IntraVenous ImmunoGlobulin |
| LOD | Limit of Detection |
| LAF | Laminar Air Flow |
| LRV | Log Reduction Value |
| MOI | Multiplicity of Infection |
| NLT | Not Less Than |
| NMT | Not More Than |
| NTE | Not to Exceed |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PFU | Plaque Forming Units |
| RT | Room Temperature |
| TFF | Tangential Flow Filtration |
| TMP | Transmembrane Pressure |
| TNC | Tris, NaCl, $CaCl_2$ buffer (10 mM Tris, 1.17 M NaCl, 1 mM $CaCl_2$, pH 7.5) |

# 6. Large Virus-Retentive Filter Test Protocols

## 6.1 Strategy

This study will evaluate virus filters designated as clearing large viruses (65-85 nm or greater). Prospective filter manufacturer-specific protocols are chosen based on prespecified ranges outlined in this Standard. Filter-specific parameter set points (+/− reasonable limits) are chosen from bracketed acceptable operating ranges that were set based on industry practice, published literature, development testing by the PDA Virus Filter Task Force [6], and filter manufacturer recommendations.

Please note that sterile techniques, the use of sterile equipment, sterile media, and the use of laboratory safety measures are all to be adhered to throughout.

## 6.2 Model virus

1. PR772 is used as the model challenge virus for rating purposes.
2. PR772 and its host organism should be obtained from a reputable standard reference collection. Methods for propagating and quantifying PR772 are found in the section titled **Bacteriophage Preparation Procedures** and the section titled **Procedure for Enumeration of Bacteriophage.**
3. Phage/BSA preparation is prefiltered not to exceed (NTE) 2–4 hours before use (as a means to reduce phage aggregate load in the feed).
    - Prefilters (e.g., nominal 0.1 µm rated) should be used to eliminate potential phage aggregates.
    - Inspection of the manufacturer certificate of analysis (CoA) is sufficient to fulfill qualification of prefilters.
    - A test should be carried out to demonstrate that sufficient PR772 remains after the prefiltration step to conduct phage-retention testing at the appropriate challenge titer.
    - Phage preparations should be monodispersed [8].
4. Input feedstock is not less than (NLT) $1 \times 10^8$ PFU/mL after prefiltration.
5. Phage spike NTE 2% of total volume.

## 6.3 Model proteins

1. Phage retention evaluation
    - Bovine serum albumin, BSA (Fraction V, commercially available), 15 mg/mL
2. Protein transmission evaluation
    - Human IgG (IVIG), 1-50 mg/mL

### 6.3.1 Model protein acceptance criteria:

1. IVIG
    - Clinical grade
2. BSA
    - Beige to off-white powder based on visual exam
    - NLT 95% purity (based on gel electrophoresis or an examination of the CoA)

- Not more than (NMT) 1% aggregated protein by size exclusion-high performance liquid chromatography (SE-HPLC) or an examination of the CoA
- Moisture NMT 8% (based on loss on drying or examination of the CoA)

## 6.4 Buffer system

1. Phage retention: phosphate buffered saline (PBS)*, pH 7.4
2. Protein transmission evaluation: high purity $H_2O$ or IVIG-compatible buffer (e.g., 200 mM glycine, pH 4.2); pH should be checked before and after dilution. It is critical to maintain the same formulation pH to avoid IVIG aggregation.
3. Process temperature: 22 ºC ± 3 ºC

*PBS without EDTA throughout

## 6.5 Scaled-down model filters

1. Model filters reflect those recommended for commercial-process validation studies. The membrane lots must be intended to be for process-scale manufacturing. Each lot will be used for both protein-passage and phage-retention studies.
2. Filter devices should be integrity- or installation-testable.
3. Filters that fail integrity or installation testing are retested once. If integrity or installation testing identifies a failed filter, two additional filters are tested. Should a filter failure occur due to air locking or any other non-filter related issue, only one additional filter is to be tested.
4. Use only a scalable process.
5. Data demonstrating the equivalence of a membrane disc or small-scale test device (cartridge/capsule), process-scale device retention, and other characteristics should be available.
6. Protein-passage operating conditions are comparable to phage-retention conditions (except buffer and model protein).

## 6.6 Operating parameters

1. Filter manufacturers pick parameter set points from bracketed acceptable operating ranges in this Standard. These ranges were set based on industry practices, published literature, and supplier recommendations **(Table I)**.
2. Manufacturer may prospectively choose to run in DFF or TFF mode.
3. Manufacturer may prospectively choose to run in constant-pressure or constant-flow mode.
4. The following parameters are monitored and recorded to evaluate the study acceptability:
    - Pressure and cumulative volume at appropriate intervals and overall time to ensure the process stays within prospectively defined set point limits.
    - Total throughput and wash volumes at the end of the study.
5. Collect adequate volumes for initial testing and potential retesting of starting material (after prefiltration) and pooled effluent.
6. Collect adequate volumes for initial testing and potential retesting of in-process intermediate at appropriate intervals.
7. Extreme care is warranted to avoid potential carryover.

## 6.7 Hold Control

### 6.7.1 Set point values for relevant operating parameters

Hold load solution (pressurized flow only) or run through same pumping system (without virus filtration) at room temperature. Samples are titered for the presence of infectivity at the beginning and at the end of the virus filtration process. If the phage $\log_{10}$ reduction value (LRV) of the hold control exceeds 1 $\log_{10}$, a different pumping system should be used. This control is important to make sure that virus infectivity is not reduced by factors other than size exclusion.

**Table I Filtration operating parameters**

| Parameter | Direct flow | Tangential flow |
|---|---|---|
| Total throughput volume | PR772 phage retention: 200 L/m$^2$ or 60–75% decay in flow rate, whichever comes first | |
| | Protein passage: NLT 10% of throughput volume of phage-retention study. The first three downstream holdup volumes are discarded. | |
| Transmembrane pressure (constant pressure mode) | Test at a pressure recommended by the manufacturer, with a reasonable tolerance range (± 10-15%). Manufacturer documentation of the recommended operating pressure range should be available. Appropriate rationale should be provided for selecting a specific test pressure. | |
| Flux (constant flow mode) | Filter-specific appropriate range (L/m$^2$-h) | Retentate: no limit<br>Permeate: filter-specific appropriate range (L/m$^2$-h) |
| Wash volume (optional) | Phage retention: 10–15% of total throughput volume | |
| | Protein passage: none | |

## 6.8 Nomenclature acceptance criteria

1. Primary rating: TR-41 Large Virus-Retentive Filter: PR772-LRV6, based on PR772 titer reduction of at least 6 logs by 3 membrane lots of virus-retentive filters [8].
2. The initial acceptance criteria for the passing/failing of an individual membrane lot of filter should be based on achieving an LRV of >6 $\log_{10}$ calculated from titers of phage content of starting material and in the pooled effluent, or input versus output after a few holdup volumes (in case of DFF) if appropriate.
3. For the intermediate samples,
    - Test results are defined as “conforming” when the individual LRVs are >6 $\log_{10}$
    - For “nonconforming” results, retesting of the intermediate sample(s) should be conducted to determine the root cause. Additionally, two new filters from the same membrane lot should be tested with monitoring of starting material and pooled effluent only. If both retests pass (based on pooled effluent and starting material), the filter lot passes.
4. If all three membrane lots from a given manufacturer pass, the large virus-retentive filter series is rated PR772-LRV6. The rating description includes a listing of operating conditions of testing (e.g., flux, transmembrane pressure, protein concentration, or total throughput volume). This information can be provided in an equation.

### 6.9 Other considerations

1. **TFF mode:** Phage titer is measured in samples from starting material, permeate, and retentate (to make sure operation does not kill phage). Note that retentate value will diminish over time. There should be no expectation that this will be constant. Mass balance of phage between starting material and final retentate is not a required element of this protocol. Test appropriate intervals and the pooled effluent at the end of the filtration (including the wash).
2. **DFF mode:** Phage titer is measured on starting material and pass-through intermediate. The pass-through intermediate samples are online, sufficient grab sample volume for testing (and retest as needed) taken at appropriate intervals. The pooled effluent is tested at the end of the filtration (including any wash) and is the primary sample used to calculate the overall process LRV.
3. **Sample size:** Phage enumeration of input and output samples is performed with a sufficient sample size (based on ICH guidance) to assure adequate precision; NLT five samples of starting material, hold controls, and pooled effluent should be used. Phage enumeration of intermediate samples is performed with duplicate samples (1 mL and/or 100 µL). For the starting material and hold control, appropriate dilutions should be done to ensure accurate plaque enumeration. For the pool filtrate samples, undiluted aliquots ranging from 1mL to 10 µL, set on a case-specific basis, should be used. Only plates with 10–300 plaques should be counted and used for titer enumeration, when feasible.
4. **Retesting:** A retest procedure should be prospectively defined for outlier phage titer results. Retesting is warranted when results differ by $\geq 1$ $\log_{10}$ higher or lower in cases where (1) dilution corrected samples have one plate that is higher or lower than other plates from the same sample, or (2) an intermediate sample is higher or lower than the other intermediate or pooled filtrate samples.

### 6.10 Other acceptance criteria

1. All virus filters pass specified integrity or installation testing recommended by a supplier, a visual examination, and an agreed-upon out-of-specifications investigation procedure.
2. Protein permeability is NLT 95%. This is performed in a separate experiment using IVIG diluted with $H_2O$ or suitable buffer, with transmembrane pressure the same as in the phage-removal study. The protein passage is defined as the percentage of the protein concentration sent through the filter present in a pooled eluate NLT 10% of throughput volume of phage retention study, after discarding the first three holdup volumes. UV-spectrometry ($A_{280}$), or other appropriate techniques, can be used to measure this value.
3. Operation stays within set point limits for the duration of the experiment.

## 7 Small Virus-Retentive Filter – Test Protocol

### 7.1 Strategy

This study will evaluate virus filters designated as clearing smaller virus (28-30 nm or greater). Prospective filter manufacturer-specific protocols are chosen based on prespecified ranges outlined in this Standard. Filter-specific parameter set points (+/− reasonable limits) are chosen from bracketed acceptable operating ranges that were set based on industry practice, published literature, development testing by the PDA Virus Filter Task Force 28-30 and filter manufacturer recommendations.

## 7.2 Model viruses

1. PP7 phage is used as the model challenge virus for rating purposes.
2. PP7 as well as its *Pseudomonas aeruginosa* host should be obtained from a standard reference collection. Methods for propagating and quantifying PP7 are found in section titled **Bacteriophage Preparation Procedures** and section titled **Procedure for Enumeration of Bacteriophage.**
3. PR772, is expected to be completely retained by a non-defective small virus-retentive filter. Retention of PR772 may therefore be used, at the user's discretion, as an internal control for membrane defects or inadvertent bypass of the filter. When co-spiked with PP7, its appearance downstream in the filtrate may provide diagnostic information if a test filter is giving unexpectedly high passage of PP7 (i.e., low LRV). If co-spiking is desired, the suggested level of PR772 is not to exceed 1% of the spiking level of PP7, but enough to be detectable in the filtrate of a compromised filter (consider the 10-30 PFU/mL limit of quantitation of the plaque assay used to analyze filtrate). If desired, PR772 and its host bacteria should be obtained from a standard reference collection (see section titled **Bacteriophage Preparation Procedures**). Methods for propagating and quantifying PR772 are provided in section titled **Bacteriophage Preparation Procedures** and section titled **Procedure for Enumeration of Bacteriophage)**
4. Phage/BSA preparation is prepared and prefiltered fresh (NTE 2 hours before use) as a means to reduce phage aggregating in in the feed prior to use.
5. Appropriate prefilters (e.g., nominal 0.1 µm rated) should be used to reduce phage aggregates
    - Titer losses of PP7 and PR772 after prefiltration typically do not exceed 1 log10 of infectivity
    - Inspection of vendor CoA is sufficient to fulfill qualification of prefilters.
6. A suitable method should confirm monodispersion of phage preparations in the buffer and/or protein solution intended for the filter evaluation. Suitable techniques such as sizing using appropriately rated filters or assessment by light scattering can be considered. When prepared as described (see section titled **Bacteriophage Preparation Procedures**) PP7 can be expected to be >90% monodispersed. The choice of technique or approach should be justified. These studies can be contracted to a qualified third party to demonstrate in advance that the chosen buffer system and phage preparation procedure is adequate for use during the actual filter study.
7. Input feedstock is NLT $1 \times 10^6$ PFU/mL PP7 and sufficient PR772 to provide a meaningful challenge as described above. Note: Use of greater titers ($10^8$ PFU/mL and above) has previously shown to overload filters [9].
8. Total combined spike NTE 2% of fluid volume.

## 7.3 Model protein

1. Phage retention evaluation – BSA (Fraction V, commercially available)
    - Protein concentration 1 mg/mL
2. Protein transmission evaluation – human IgG (IVIG)
    - Protein concentration 1–10 mg/mL
    - Model protein acceptance criteria:
        a) Human IVIG (clinical grade)
            i. If approved by regulatory authorities for clinical use can be assumed to contain minimal aggregates.
3. BSA
    - Beige to off-white powder based on visual exam
    - NLT 99% purity (based on agarose gel electrophoresis or examination of CoA)

- NMT 1% aggregated protein by size exclusion high performance liquid chromatography (SE-HPLC) or examination of CoA
- Moisture NMT 8% (based on loss on drying or examination of CoA)
- Impact on filtration: BSA lots should be carefully prescreened prior to use in this method to eliminate lots that foul filters. Where feasible, the BSA batch should be of sufficient quality (e.g., minimal aggregation and impurities) to not foul the filter type being tested beyond 15–20% flux decay when filtered at 1 mg/mL after 50 $L/m^2$ (relative to initial product flux). When not feasible, see **Table II** below.

4. Buffer system

- Water: High quality (e.g., 0.2 µm filtered deionized water or water for injection)
- Phage retention: 1 × PBS, pH 7.0–7.4
- Protein Transmission Evaluation: H2O for dilution of IVIG or the formulation buffer of the IVIG (if known, e.g., 0.2 M glycine, pH 4.2)

5. Process temperature: RT

## 7.4 Scaled-down model filters

1. Model filters reflect those recommended for commercial-process validation studies. The membrane lots must be intended to be for process-scale manufacturing. Each lot will be used for both protein-passage and phage-retention studies.
2. Filter devices should be integrity- or installation-testable.
3. In addition to vendor-recommended testing, retention of PR772 is considered to be an internal control for the purposes of this nomenclature method. Acceptance criteria for PR772 passage is no detectable infectious phage (minimum of 0.5 mL sample) in filtrate.
4. If a filter fails physical integrity, installation, or in-process control testing, testing of a new filter is allowed on a one-time basis. If the second filter fails, the test assembly is not considered to be suitable.
5. Use only a scalable process.
6. Data demonstrating the equivalence of a membrane disc or small-scale test device (cartridge/capsule), process-scale device retention, and other characteristics should be available.
7. Protein-passage operating conditions are comparable to phage-retention conditions (except buffer and model protein).

## 7.5 Operating parameters

1. Parameter set points (+/− reasonable limits) are prospectively chosen from bracketed acceptable operating ranges based on known attributes of the filters **(Table II)**. This information is supported by industry practice, published literature, and/or vendor recommendations.
2. Manufacturer may prospectively choose to run in DFF or TFF mode.
3. Manufacturer may prospectively choose to run in constant-pressure or constant-flow mode.
4. The following parameters are controlled, monitored and recorded to evaluate the study acceptability:
    - Pressure and cumulative volume at appropriate intervals and overall time to ensure the process stays within prospectively defined set point limits.
    - Total throughput and wash volumes (if applicable) at the end of the study.
5. Collect samples of starting material and pooled effluent (NLT 50$L/m^2$) of sufficient volume to allow phage titer enumeration with a limit of detection (LOD) of 1–5 PFU/mL.
6. Collect samples of in-process intermediate (“grab samples”) at NLT 25 $L/m^2$.
7. Extreme care is warranted to avoid potential carryover.

**Table II Filtration operating parameters**

| Parameter | Direct Flow | Tangential flow |
|---|---|---|
| Total throughput volume | Phage retention: NLT 50 L/m2 or 25% decay in flow rate, whichever comes first. Initial flow rate is determined based on initial product flow rate. Protein passage: Sufficient volume to allow stabilization of protein concentration (e.g., 25 L/m2, or after two or more holdup volumes) | |
| Transmembrane pressure (constant pressure mode) | Test at a pressure recommended by the manufacturer, with a reasonable tolerance range (± 10-15%). Manufacturer documentation of the recommended operating pressure range should be available. Appropriate rationale should be provided for selecting a specific test pressure. | |
| Flux (constant flow mode) | Filter-specific appropriate range (L/m2-h) | Retentate: no limit<br>Permeate: filter-specific appropriate range (L/m²-h) |
| Post-filtration wash volume (optional) | Phage retention: 10–15% of total throughput volume<br>Protein passage: none | |

**NOTE:** Monitored parameters are temperature, flow rate (constant pressure mode), and transmembrane pressure (constant flow mode).

## 7.6 Hold control

Hold controls should be performed as mentioned in **Section 6.7**.

## 7.7 Nomenclature acceptance criteria

1. Primary rating: Small Virus-Retentive Filter: PP7-LRV4, based on PP7 titer reduction of at least 4 $\log_{10}$ after throughput NLT 50 L/m² by three membrane lots of virus filters, three filter samples tested per lot.
2. The initial acceptance criteria for an individual filter should be based on achieving an LRV (i.e., Ri as defined in Appendix 4 of ICH Q5A, 1998) [3]) of NLT 4 $\log_{10}$ calculated from the total (e.g., volume-adjusted) phage content of starting material and the pooled effluent.
3. Test results of intermediate samples are defined as "conforming" when the individual LRVs are NLT 4 $\log_{10}$.
4. If an intermediate sample is "nonconforming," retest the sample.
5. If the sample retest is still "nonconforming," retest two new filters from the same membrane lot but monitor starting material and pooled effluent only.
6. If both retests pass (based on pooled effluent and starting material), the membrane filter lot passes.
7. If all samples from a given manufacturer pass, the filter series is rated as a Small Virus- Retentive Filter: PP7-LRV4. The rating includes a listing of operating conditions of testing (e.g., flux, transmembrane pressure, protein concentration, or total throughput volume). This information can be provided in a footnote. The format of the nomenclature and test reporting should conform to the precedent large virus-retentive filter format [6].

## 7.8 Other considerations

1. **TFF mode**-phage titer is measured at starting material, permeate (filter-specific appropriate range (L/m²-h as indicated in **Table II**)), and retentate (i.e., to ensure operation does not kill phage). Test after an appropriate throughput (i.e. NLT 25 L/$m^2$) and the pooled effluent at the end of the filtration (including the wash, if any).
2. **Direct flow**-phage titer is measured in the starting material, the final pooled filtrate, and one pass-through intermediate. The pass-through intermediate sample is an online grab sample taken at NLT 25 L/$m^2$. The pass-through intermediate sample should be of sufficient volume to allow an assay LOD of 1–5 PFU/mL. The pooled filtrate is tested at the end of the filtration (NLT 50 L/$m^2$ of load with an additional 10–15% volume buffer wash; see **Table II**) and is the primary sample used to calculate the overall process LRV.
3. **Sample volume**: Phage enumeration of input and output samples is performed with a sufficient sample volume to assure adequate precision to support statistical confidence for measurement of an LRV of 4 $\log_{10}$. NLT five samples of pooled effluent and starting material of appropriate dilution should be quantified to accurately measure titer (i.e., 10–300 plaques/plate). Dilutions within a 10–100-fold range are acceptable. Phage enumeration of intermediate samples is performed with duplicate samples.
4. **Retesting**: A defined retest procedure should be defined for outlier phage titer results. Retesting is restricted to the following cases:
    - One plate of duplicate or quintuplicate is 1.0 $\log_{10}$ higher or lower than others from the same sample.

      Note: For small-virus-retentive filters, phage breakthrough may occur throughout the filtration process. There is no expectation that the breakthrough will be evenly dispersed in the intermediate and the pool samples, resulting in differences in ≥ 1 $\log_{10}$ between samples; in this case retesting may not be necessary

## 7.9 Other acceptance criteria

1. All virus filters pass prespecified integrity or installation testing recommended by a vendor, a visual examination, and PR772 retention, if performed. A pre-specified out-of-specification investigation procedure is followed in cases of integrity or installation testing.
2. Protein passage or transmission is NLT 90%. This is performed in a separate experiment using IVIG diluted with $H_2O$ or an IVIG formulation buffer (to maintain pH), but all other operational parameters except total volumetric throughput. The protein passage is defined as the percentage of the protein concentration in a post-filtration "grab sample" relative to the load protein concentration after a sufficient volume is processed to allow the stabilization of protein concentration in the filtrate. UV-spectrometry (A280) can be used to measure this value.
3. Operation stays with set point limits for duration of experiment.

# 8 Bacteriophage Preparation Procedures

## 8.1 Introduction

Bacteriophage stocks may be prepared either in broth cultures or on the surface of agar plates (agar overlay method). Crude preparations should be filter-sterilized, and then, used as is or further purified and concentrated using CsCl density gradient ultracentrifugation. This method is adapted from *The Structure and Infective Process of a Pseudomonas Aeruginosa Bacteriophage containing Ribonucleic Acid* [10] and

*Properties of R Plasmid R772 and the Corresponding Pilus-specific Phage PR772* [11]. Alternative methods are acceptable, but the pure concentrate method below should yield titers in excess of $10^{12}$ PFU/mL.

## 8.2 Equipment and supplies

1. Bacteriophage (PR772) and homologous bacterial host (*E. coli* K-12 J-53-1).
    - Tryptic soy agar (TSA) plates (150 mm)
    - Tryptic soy broth (TSB)
2. Bacteriophage (PP7) and homologous bacterial host (*P. aeruginosa*) [12]
    - Nutrient agar (NA) 1.5% plates (150 mm)
    - Nutrient broth (NB)
3. Phosphate buffer saline (PBS) or TNC buffer (10 mM Tris, 1.17 M NaCl, 1 mM $CaCl_2$, pH 7.5)
4. Top soft agar (TSB or NB with 0.7% electrophoresis grade agarose)
5. Tabletop-style centrifuge
6. Sterile centrifuge tubes (50 mL) and bottles (250 mL)
7. Incubator
8. Sterile glassware/plasticware as required
9. Sterile pipettes
10. Water bath
11. Vortex
12. Inoculating loops
13. Cesium chloride (CsCl)
14. Ultracentrifuge swinging bucket rotor (NLT 100,000 g) and appropriate tubes
15. Ultracentrifuge vertical rotor (NLT 350,000 g) and appropriate tubes for CsCl banding
16. 19-gauge needles
17. 3 mL and 6 mL syringes
18. Sterile 0.1 µm, 0.2 µm, and 0.45 µm filter
19. Spectrophotometer that can measure absorbance of visible light

## 8.3 Procedure

The procedure for PR772 and PP7 are the same except for the host bacteria, the culture broth, and the target CsCl density for ultra-purified preps. *P. aeruginosa* is a Biosafety Level-2 (BSL-2) organism. Consult the joint Centers for Disease Control and National Institutes of Health publication, *Biosafety in Microbiological and Biomedical Laboratories* [12] for specific guidance regarding operating a BSL-2 lab. When feasible, work in a biosafety cabinet.

## 8.4 Plate method

1. Prepare the host suspension (*E. coli* K-12 J-53-1 or *P. aeruginosa*) using the following method:
    - Prepare a streak plate of host bacteria from the frozen stock. Incubate overnight at 37 °C. The streak plate can be used for about 1 month if stored at 2–8 °C and overnight cultures for NMT 1 to 1-½ weeks.
    - Start a broth culture of host bacteria by inoculating broth with one colony from a streak plate of the bacterial host. Incubate overnight (12–18 hours) in a 37 °C shaker. The overnight culture can be used for about 1 to 1-½ weeks if stored at 2–8 °C.

2. Start an appropriate host bacterial broth culture in proper media with ~1/100th volume of the overnight culture. Allow it to grow in a shaker at 37 °C until the culture is in mid-log phase (Target OD550: 0.3–0.6). This may take approximately 2 hours to incubation.
   **NOTE:** Actively growing bacterial cultures (i.e., those in mid-log phase) MUST be used to provide a bacterial host lawn for phage assays. DO NOT use an overnight bacterial culture for the phage enumeration assay.
3. Calculate the appropriate dilution of stock bacteriophage required to give semi-confluent lysis on the bacterial lawn for one plate. [For example, on the surface of a 150 mm plate, use a dilution of bacteriophage stock that will yield $10^5$ PFU/ml or per plate since 1 ml of phage was added.]
4. Make dilutions of the phage stock in 1 mL broth, PBS or TNC buffer, as required for the number of lysis plates that will be made (10–20 plates should yield ~$10^{12}$–$10^{13}$ total PFU of phage).
5. The bacterial host culture should be in mid-log phase and used within a short period of time (Target OD550: 0.3–0.6).
6. For each plate, add 1 mL of the specific dilution of the bacteriophage and 2 mL of the bacterial host in a sterile disposable tube. Let sit 5 minutes at room temperature (RT).
7. Add 9 mL of warm, soft agar (approximately 50 °C). Mix by pipetting up and down twice. Pour the mixture onto the surface of a 150 mm agar plate. Swirl the plate to ensure mixing and complete coverage of the plate. Allow the plate to solidify at RT (NLT 10 minutes). Transfer and invert. Return the inverted plates to the incubator for overnight incubation.
8. Following incubation, the plates should demonstrate semi-confluent to confluent lysis. Harvest the plates using broth, PBS or TNC buffer, covering each plate. Pipette 10 mL of buffer on top of the plates; incubate for 4 hours at RT or overnight at 4 °C with gentle agitation on an orbital shaker. Pool the wash from all the plates into 250 mL centrifuge bottles. Rinse each plate with an additional 5 mL of broth or buffer.

## 8.5 Broth method

1. Calculate the appropriate volume of a spike of stock bacteriophage required to give a reasonable spike, for example, targeting a multiplicity of infection (MOI) of 1 to 10.
2. Grow the bacterial host culture to mid-log phase (Target OD550: 0.3–0.6). This can be accomplished by seeding broth with ~1/100th volume of the overnight culture and growing for about 2 hours at 37 °C.
3. In a sterile tube, add a small volume (1 to 2 mL) of bacteriophage to 1mL of the bacterial host. Incubate 5 minutes on the benchtop.
4. Add bacteria/phage mixture to 250 mL broth, grow overnight at 37 °C with vigorous agitation.

## 8.6 Crude preparation

1. Spin harvest (either from plate method or broth method) in tabletop centrifuge at approximately >2000 *x g* for 20 minutes (4 °C) to remove debris. Decant the supernatant into sterile centrifuge tubes. Repeat centrifugation step under the same conditions, and decant the supernatant into a sterile container.
2. Perform sterilizing filtration on the supernatant prior to storage. Aliquot the bacteriophage stock, label, and store at 4 °C or frozen for long-term storage at -70 °C.
3. Determine the stock bacteriophage titer of the newly prepared stock using the agar overlay method and record the stock titer.

### 8.7 Pure concentrate

***Note: This procedure will not work with low titer-starting materials.***

1. Centrifuge crude preparation for 15 minutes at 10,000 *x g* (4 °C) using an ultracentrifuge with a swinging bucket rotor to remove large particles and debris.
2. Save supernatant for further processing.
3. Centrifuge supernatant 2 hours at 90,000 *x g* (4 °C) using ultracentrifuge and a swinging bucket rotor.
4. Discard supernatant; phage will be in pellet.
5. Resuspend phage pellet overnight in PBS buffer (4 °C). Mild vortexing and/or pipetting the liquid up and down will facilitate the resuspension process. This preparation can be used for filter studies as a "phage concentrate."
6. If further purification is desired, add enough CsCl to bring density to 1.3 g/mL for PR772 or 1.4 ± 0.1 g/mL for PP7. This should be ~3–3.2 g CsCl for every 7 mL of liquid.
7. Confirm density of solution by measuring mass of 10–100 µL on a balance.
8. Spin 20 hours at 300,000 *x g* (20 °C) using an ultracentrifuge and a vertical or fixed angle rotor.
9. Phage band should be clearly visible if sufficient phage existed in the starting material. Draw out band with a needle and syringe. If two bands are evident, both may be drawn separately and determined to see which has the highest titer of live phage. For PR772, the top band typically has the highest titer; for PP7, a red band should appear near the bottom of the gradient.
10. Dialyze overnight against three changes of PBS (4 °C).
11. Perform sterilizing filtration prior to storage. To further minimize PP7 aggregates, highly purified PP7 can be filtered through a 0.1 µm or "large virus" filter.
12. Determine the stock bacteriophage titer of the newly prepared stock using the agar overlay method and record the stock titer.

### 8.8 Storage

1. Phage: Aliquot the bacteriophage stock, label, and store at 4 °C for short-term use or -70 °C for long-term banking.
2. Host: Mix overnight culture with filter-sterilized glycerol and aliquot 0.5–1.0 mL fractions into screw-cap tubes. Store at -70 °C.

## 9 Procedure for Enumeration of Bacteriophage

### 9.1 Introduction

This method estimates bacteriophage concentration by enumerating the number of plaque forming units per milliliter of sample (PFU/mL).

#### 9.1.1 Equipment and supplies

1. TSA plates (100 mm) for PR772
2. NA 1.5% plates (100 mm) for PP7
3. Sterile diluent (TSB, NB, PBS, or TNC buffer)
4. Sterile disposable test tubes
5. Sterile soft-top agar for overlay (TSB or NB with 0.7% electrophoresis-grade agarose)

6. Bacterial host (*E. coli* K-12 J-53-1 and *P. aeruginosa*)
7. Sterile pipettes
8. Incubators
9. 37 °C shaker (+/- 2 °C)
10. Water bath (45–50 °C)
11. Inoculating loop
12. Sterile glassware/plasticware as required

## 9.2 Procedure

### 9.2.1 Preparation of bacteriophage host

Prepare the host suspension as listed above in Section 8.4, Step 2.

### 9.2.2 Preparation of dilution tubes and soft agar for use in phage assays

1. Working in an LAF hood will mitigate against risk-compromised results due to contamination.
2. Prepare broth, PBS or TNC buffer; dispense aseptically 1 mL (or 1.1 mL, first tube) into sterile disposable dilution tubes. Keep covered until use.
3. Prepare soft agar; keep molten in a 45–50 °C water bath.

### 9.2.3 Phage assays

1. Make serial ten-fold dilutions for the test samples as required (in broth, PBS, or TNC buffer) in the above tubes, making sure to change pipette tips between dilutions to avoid cross-contamination.
2. Aliquot 1 mL bacteria into separate sterile tubes. Dispense phage test articles into the bacteria tubes; incubate at RT for 5 minutes.
3. Add 4.5 mL of warm, soft agar to the phage-bacteria mixture. Mix by pipetting up and down once or twice. Pipette onto surface of 100 mm agar plate. Swirl plate gently to ensure that entire surface is covered.
4. Following solidification of the plates (NLT 10 minutes), invert and incubate overnight at 37 °C.
5. Score the plates for the number of PFU. Count dilution plates that have between 10 and 300 visible plaques. A light box may be useful for this procedure. Calculate PFU/mL of the original test article based on the dilution and count. Record the titer.
6. For very low titer samples, (e.g., <10 plaques on the lowest dilution plate), a plaque enumeration can still be recorded. The resulting titer determination will not be as precise as when 10 – 300 plaques can be counted, but it still may yield useful information.

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