# Assessment of Media Fill Performance in Vaccine Containers with Sub-Nominal Volumes

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**Abstract:**

Introduction: Packaging processes must be validated through media fill simulations to demonstrate sterility assurance. Traditionally, regulatory guidance recommends that containers used in media fill runs be filled to the nominal volume of the final drug product. However, this approach may be resource-intensive, especially in high-throughput or large-volume manufacturing. The rationale for this study was to determine whether reduced, or sub-nominal, fill volumes could be scientifically justified as a practical and equivalent alternative for aseptic process validation.

Material and Methods: This investigation assessed the feasibility and performance of sub-nominal fill volumes in media fill simulations. Tryptic Soy Broth (TSB), was dispensed into 100 mL semi-transparent plastic vaccine containers at two reduced volumes. To evaluate microbial detectability,

each container was deliberately inoculated with 10–100 colony-forming units (CFU) of defined challenge microorganisms. Growth Promotion Tests (GPT) were also performed on aliquots of the same TSB formulation to confirm medium fertility and suitability.
During a 14-day incubation period, all inoculated containers exhibited visible microbial growth, whereas negative controls showed no contamination. Microbial proliferation was readily observable through the semi-transparent container walls, even at reduced volumes, eliminating the need for sample transfer.
Results: The results indicate that reduced fill volumes do not compromise microbial detectability or the integrity of the visual inspection process.
Conclusion: These findings are consistent with the flexibility provided in FDA and EU GMP Annex 1 guidance, supporting the scientific justification for sub-nominal fills as a validated, resource-efficient, and cost-effective alternative to conventional full-volume media fills in aseptic process validation.

Keywords: Asepsis, Containers, Process Simulation, Validation.

## 1. Introduction

Sterile pharmaceutical products are categorized as either terminally sterilized or aseptically processed. In terminal sterilization, products are sterilized after filling and sealing, providing a high sterility assurance level. However, many biological formulations cannot tolerate the heat or pressure of terminal sterilization, and therefore must be manufactured under aseptic conditions. For these products, all post-sterilization steps—such as filling and packaging—must occur in highly controlled environments to prevent recontamination. Because any handling of sterile components or containers introduces contamination risk, such processes require strict validation [1].

According to Good Manufacturing Practice (GMP) guidelines, all critical steps in sterile product manufacturing must be validated to ensure consistent performance and compliance with predefined specifications. Aseptic filling and packaging are especially important because they directly determine the sterility of the final product. Validation confirms that equipment, environmental controls, personnel practices, and aseptic techniques collectively maintain sterility. Among the accepted methods for validating aseptic operations, media fill simulations are considered one of

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65 the most reliable approaches [2]. In a media fill, the drug product is replaced with a sterile medium,
66 typically TSB, and filled into the same production containers under routine conditions. After 14
67 days of incubation, any microbial growth is recorded. This test evaluates the overall performance
68 of the aseptic process, including equipment, packaging, environment, and operator handling [3].
69 All media fill parameters should closely replicate actual production [4], including container
70 number, fill volume, process duration, environment, and personnel involvement. Risk assessment
71 is crucial, as factors like manual versus automated operations, intervention complexity, and use of
72 isolators or cleanrooms determine the appropriate simulation scale [5]. Container characteristics
73 can also impact microbial detection during media fill. Industry guidelines recommend the use of
74 clear containers to facilitate visual inspection. When using semi-transparent or opaque containers,
75 alternative validated methods or equivalent clear substitutes are required to ensure contamination
76 can be reliably detected [6]. This consideration is particularly relevant in sterile product
77 manufacturing that uses plastic vials with limited optical transparency.
78 Another critical design parameter is the volume of medium filled per container. Regulatory
79 guidelines generally recommend filling to the nominal product volume. However, the FDA
80 acknowledges that alternative approaches may be acceptable, provided they are scientifically
81 justified and demonstrated to adequately simulate critical aspects of the aseptic process, such as
82 intervention duration and contamination risk [3]. The revised EU GMP Annex 1 (2022) also
83 explicitly states that the volume of medium filled should be sufficient to ensure contact with all
84 potential contamination sources and allow for the visual detection of turbidity [7]. This
85 requirement has likewise been emphasized in other authoritative regulatory and industry guidance
86 documents [6]. Furthermore, regulatory guidance allows for practical adaptations in the design and
87 scope of media fill simulations. For example, PIC/S PI 007-6 permits limiting the number of filled
88 units by running the equipment for a reasonable duration, as long as the scientific validity of the
89 test is not compromised [8]. Several scientific investigations support these regulatory flexibilities.
90 For example, Sandle (2013) argued that while the fill volume during media fill simulations should
91 ensure wetting of all internal surfaces during incubation, manufacturers often use reduced volumes
92 for larger containers, provided that the exposure time and process conditions reflect routine
93 operations [9].
94 The study evaluated the feasibility of using sub-nominal fill volumes (25% and 50%) in 100 mL
95 semi-transparent vaccine containers. The media fill runs were conducted at correspondingly

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96 reduced volumes (25% and 50% of the nominal volume), with TSB dispensed accordingly and
97 inoculated with 10–100 CFU of challenge microorganisms. Containers were incubated under
98 standard conditions, and microbial growth was visually assessed through the semi-transparent
99 container walls. This study was evaluated within the framework of current regulatory guidance to
100 evaluate the use of sub-nominal fill volumes in media fill simulations. The results provide a
101 practical and cost-effective strategy for aseptic process validation in high-volume vaccine
102 production, without compromising test sensitivity or GMP compliance.
103

104 **2. Material and methods**

105 **2.1. Materials**

106 Tryptic Soy Broth (TSB), Tryptone, Soya peptone, Sodium chloride, Dextrose (glucose),
107 Dipotassium hydrogen phosphate, Hydrochloric acid and Sodium hydroxide from Merck
108 (Darmstadt, Germany).
109 Microorganisms (challenge strains): Staphylococcus aureus ATCC 6538, Pseudomonas
110 aeruginosa ATCC 9027, Bacillus subtilis ATCC 6633, Clostridium Sporogenes ATCC 19404,
111 Candida albicans ATCC 10231, Aspergillus brasiliensis ATCC 16404.
112

113 **2.2 Apparatus**

114 All aseptic manipulations were performed in a Class A laminar airflow cabinet (Beasat, Iran). A
115 steam autoclave (Sazgar, Iran) was used for sterilization of media, containers, and accessories. A
116 temperature-controlled incubator (Binder, Germany) was used for incubation of test samples at
117 20–25 °C and 30–35 °C.
118 For anaerobic microorganisms (*C. sporogenes*), incubation was performed in an anaerobic jar
119 (Anoxomat, Mart, Netherlands) equipped with gas-generating packs and an anaerobic indicator.

120 **2.3. Preparation of Culture Medium**

121 Tryptic Soy Broth (TSB) was prepared in-house from individual analytical-grade components
122 according to standard formulation (per liter of purified water): Tryptone (17.0 g/L), Soya peptone
123 (3.0 g/L), Sodium chloride (5.0 g/L), Dextrose (2.5 g/L), and Dipotassium hydrogen phosphate
124 (2.5 g/L). All components were accurately weighed and dissolved in purified water under
125 continuous stirring. The pH of the medium was adjusted to 7.2 ± 0.1 at 25 °C using 0.2 N HCl or

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0.25 N NaOH, as required. The medium was subsequently sterilized by autoclaving under standard conditions.

**2.4. Sterility and Growth Promotion Test (GPT)**

Following sterilization, the quality and fertility of the prepared TSB were evaluated by performing a Growth Promotion Test (GPT) in accordance with USP <71> guidelines [10].

For this purpose, 10–100 CFU/mL of each test microorganism (Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Clostridium sporogenes, Candida albicans, and Aspergillus brasiliensis) was separately inoculated into six test tubes containing 10 mL of the sterile TSB. Clostridium sporogenes was incubated in an anaerobic jar at 30–35 °C, while B. subtilis, S. aureus, and P. aeruginosa were incubated aerobically at 20–25 °C.

Candida albicans and Aspergillus brasiliensis were incubated aerobically at 30–35 °C.

All cultures were examined for visible growth after 3 days of incubation.

**2.5. Media Fill Simulation Procedure**

In this study, Tryptic Soy Broth (TSB), the standard medium for media fill operations, was dispensed into semi-transparent 100 mL bottles at volumes of 25 mL and 50 mL. Each bottle was inoculated separately with 10–100 CFU of each challenge microorganism. To ensure statistical relevance, three bottles per volume per microorganism were prepared. Additionally, four negative control bottles per volume (without microbial inoculation) were included.

In total, 44 sterile semi-transparent 100 mL bottles were used: 18 bottles with 50 mL TSB (three per microorganism), 18 bottles with 25 mL TSB (three per microorganism), 4 negative control bottles with 50 mL TSB, and 4 negative control bottles with 25 mL TSB. All bottles were incubated for 14 days, comprising 7 days at 25 °C followed by 7 days at 37 °C. Clostridium sporogenes cultures were incubated under anaerobic conditions using an anaerobic jar system (Anoximat).

Visual inspection of microbial growth was conducted on days 1, 3, 7, and 14 post-inoculation. To enhance detection, a reference marker was placed behind each semi-transparent bottle to improve contrast and facilitate identification of turbidity, sedimentation, or fungal growth.

This setup allowed the evaluation of microbial proliferation under sub-nominal fill volumes while maintaining statistical and regulatory relevance. Furthermore, the methodology enabled assessment of the visual detectability of contamination in semi-transparent containers, a critical consideration for vaccine production lines.

**2.6. Analysis**

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157 Data were analyzed to evaluate the detectability of microbial growth in semi-transparent bottles at
158 sub-nominal fill volumes (25% and 50% of the nominal 100 mL volume). As the outcome measure
159 was qualitative (presence or absence of visible growth), descriptive statistics were applied.
160 Growth detection rates were assessed for each microorganism and fill volume at each observation
161 time point (24 h, 3, 7, and 14 days). Comparisons between samples and controls were performed
162 to confirm that sub-nominal fill volumes did not compromise the ability to detect microbial
163 contamination.

164 **2.7. Validation Criteria**

165 The validation of the aseptic process via media fill simulation was considered successful if the
166 following criteria were met:
167 Visual detectability in equivalent volumes: All aerobic and fungal microorganisms should exhibit
168 visible growth in bottles filled with sub-nominal volumes (25% and 50% of the nominal volume)
169 by the end of the 14-day incubation period, which is clearly distinguishable from negative controls,
170 confirming suitability for process simulation.
171 Anaerobic growth: Growth of Clostridium sporogenes must be detectable under anaerobic
172 conditions.
173 Control performance: Negative controls (without inoculum) must remain free from contamination
174 throughout the 14-day incubation period.

175 **3. Results**

176 **3.1. Growth Promotion Test (GPT) Results**

177 All six challenge microorganisms (Staphylococcus aureus, Bacillus subtilis, Pseudomonas
178 aeruginosa, Clostridium sporogenes, Candida albicans, and Aspergillus brasiliensis) exhibited
179 clear and consistent growth in GPT tubes containing 10 mL of Tryptic Soy Broth (TSB). Microbial
180 proliferation was observable within three days, confirming both the fertility of the medium and its
181 suitability for supporting the detection of microbial contamination in the media fill simulations.
182 These results also validate the proper preparation and integrity of the TSB used in this study.

183 **3.2. Microbial Growth Evaluation at Sub-nominal Fill Volumes**

184 Media fill containers, filled with either 25 mL or 50 mL of Tryptic Soy Broth (TSB) and inoculated
185 with challenge microorganisms (10–100 CFU/mL), were monitored over 14 days. Growth of
186 aerobic bacteria and fungi was visible as early as 24 hours post-inoculation and became more
187 pronounced at 3, 7, and 14 days.

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188 Anaerobic bacteria (Clostridium sporogenes) and fungal isolates (Candida albicans and
189 Aspergillus brasiliensis) exhibited delayed growth, which became detectable after 14 days under
190 their respective incubation conditions. No growth was observed in negative control bottles,
191 confirming the validity of the inoculation and incubation procedures.
192 These results (table1) indicate that using 25% of the nominal fill volume is sufficient for reliable
193 detection of microbial contamination, offering a cost-effective alternative to full-volume media fill
194 simulations.
195
196 Table 1. Microbial Growth Detectability at Reduced Media Fill Volumes in Containers

| Microorganism | Fill Volume (mL) | Growth Observed | | | |
|---|---|---|---|---|---|
| | | 24 hours | Day 3 | Day 7 | Day 14 |
| Staphylococcus aureus | 25 | + | + | ++ | ++ |
| | 50 | + | + | ++ | ++ |
| Bacillus subtilis | 25 | + | + | ++ | ++ |
| | 50 | + | + | ++ | ++ |
| Pseudomonas aeruginosa | 25 | + | + | ++ | ++ |
| | 50 | + | + | ++ | ++ |
| Clostridium sporogenes | 25 | - | - | + | ++ |
| | 50 | - | - | + | ++ |
| Candida albicans | 25 | - | - | + | ++ |
| | 50 | - | - | + | ++ |
| Aspergillus brasiliensis | 25 | - | - | + | ++ |
| | 50 | - | - | + | ++ |
| Negative control bottles | 25 | - | - | - | - |
| | 50 | - | - | - | - |

197 -: No growth
198 +: Slight turbidity
199 ++: Luxuriant
200
201 **3.3. Visual Clarity in Semi-Translucent Containers**

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202 Despite the semi-translucent nature of the vaccine bottles, visual inspection of microbial growth
203 was both feasible and reliable. Fungal contamination, particularly by Aspergillus brasiliensis, was
204 observed as floating mycelial masses and color changes in the medium. Bacterial contamination
205 produced turbidity and sedimentation at the bottom of the bottles. Observations were consistent
206 across all replicate bottles, with no false positives or ambiguous results.
207 To enhance detection, a simple visual aid (e.g., a backlit ruler or indicator placed behind the
208 container) was employed to assess turbidity and microbial growth. This approach allowed
209 consistent comparison with control bottles, ensuring accurate identification of contamination even
210 in semi-translucent containers.

211 **3-4. Observational Considerations**

212 The study highlighted several key factors for reliable visual detection. Visual inspection should be
213 performed under adequate lighting, by trained personnel, and always compared against positive
214 and negative control bottles.
215 Variability in bottle transparency can affect detection; therefore, the clearest bottles should be
216 selected for media fill operations.
217 Visual inspection of the bottles was performed by comparing each test container with its
218 corresponding negative control under adequate lighting conditions. To enhance the accuracy of
219 turbidity assessment, a reference marker (a thin rod or ruler) was placed behind each container
220 during examination. The visibility or disappearance of the reference line served as an indicator of
221 medium clarity and potential microbial growth. Loss of line sharpness or complete obscuration
222 was interpreted as evidence of contamination. This simple visual aid improves the reliability and
223 reproducibility of observations, particularly when evaluating semi-translucent containers.
224

225 **4. Discussion**

226 The results of this study support the feasibility and validity of using sub-nominal fill volumes—
227 specifically 25 mL and 50 mL—in 100 mL semi-translucent vaccine containers for media fill
228 simulations. All test containers inoculated with challenge microorganisms exhibited clear and
229 unambiguous growth, and no microbial contamination was observed in the negative controls.
230 These outcomes indicate that the reducing the media volume did not compromise the sensitivity
231 of contamination detection, nor did it interfere with visual inspection in semi-translucent plastic
232 containers.

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233 One of the primary concerns in using reduced media volumes is whether smaller liquid depths may
234 delay or inhibit microbial growth visibility, particularly in semi-closed or low-oxygen
235 environments. However, the results of this study demostrated that filling the culture medium with
236 25-50% of the nominal volume, if certain considerations are met, provides equivalent performance
237 to filling with full volume. The detection of Clostridium sporogenes under anaerobic conditions
238 further reinforces the suitability of the approach for both aerobic and anaerobic organisms.
239 Of course, it is recommended to adjust the filling speed proportionally to the reduced fill volume
240 to ensure that the exposure time of the containers under the filling heads accurately reflects the
241 real manufacturing process. This adjustment maintains the equivalence of process conditions
242 between the simulation and the actual aseptic operation. Furthermore, to ensure that the growth
243 medium adequately contacts all internal surfaces of the bottle and the inner part of the cap, each
244 container should be gently inverted or swirled several times after filling. This step facilitates
245 complete wetting of all potential contamination sites, thereby improving the representativeness of
246 the simulation and ensuring that any microbial contamination present during filling would be
247 detectable under incubation conditions (3).
248 Visual readability is another key factor in aseptic process simulation, especially when plastic
249 containers are not fully transparent. In this study, microbial growth—whether bacterial turbidity
250 or fungal mass—was consistently detectable through the container walls. Growth assessment was
251 intentionally based on visual inspection, in alignment with standard GMP media fill practices and
252 regulatory expectations, where turbidity detection remains the primary acceptance criterion. By
253 selecting containers with acceptable transparency and conducting observations over several days,
254 it was ensured that even subtle changes in the appearance of the culture medium could be reliably
255 detected.
256 The GPT (Growth Promotion Test) conducted with the same TSB formulation confirmed the
257 fertility of the media across all challenge organisms, further validating that the media fill
258 conditions were suitable for detecting contamination at the inoculated concentrations.
259 **5. Conclusion:**
260 The use of low inoculum levels (10–100 CFU) in this study provided indirect evidence of adequate
261 detection sensitivity under reduced-volume conditions. Although regulatory guidance provides
262 clear recommendations for media fill simulations, published studies specifically addressing
263 modifications to standard simulation conditions, particularly reductions in fill volume are limited.

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Most available reports focus on conventional full-volume fills [11] or discuss media fill design in general terms [12]. Some investigations have applied statistical approaches to evaluate aseptic processes [13] or sensitivity of media-fill challenge tests under various aseptic technique deviations [14].

The significance of this study lies in demonstrating that sub-nominal fill volumes can reliably support microbial detection while offering cost-effective and resource-efficient alternatives for high-volume aseptic manufacturing.

Importantly, the findings of this study are consistent with the regulatory flexibility in FDA and EU GMP guidelines, which allow for modified culture medium filling configurations, when properly justified and scientifically validated. The results demonstrate that, when properly designed, low-volume culture medium fillings can serve as effective and consistent alternatives to full-volume experiments, particularly in high-volume or cost-sensitive aseptic manufacturing scenarios.

**Author contributions**

Study concept and design: S. Z.

Acquisition of data: S. Z and R.L.

Analysis and interpretation of data: S. Z. and M.M.

Drafting of the manuscript: S. Z. and M.Z.

Critical revision of the manuscript for intellectual content: S. Z

Administrative, technical, and material support: R.L. and M.M. and M.Z.

Laboratory implementation Study supervision: R.L. and M.M. and M.Z.

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**Ethics**

Ethical approval was not applicable to this study as it did not involve human participants or animal experiments.

**Conflict of Interest**

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The authors declare that there is no conflict of interest. The study was conducted within the Razi Vaccine and Serum Research Institute as part of its scientific activities, without any external commercial influence.

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## Data Availability

All data generated or analyzed during this study are included in this published article.

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