

Adventitious agent detection methods in bio-pharmaceutical applications with a focus on viruses, bacteria,
and mycoplasma

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Abstract

Adventitious agents present significant complications to biopharmaceutical manufacturing. Adventitious agents include numerous lifeforms such as bacteria, fungi, viruses, mycoplasma, and others that are inadvertently introduced into biological systems. They present significant problems to the stability of cell cultures and the sterility of manufacturing products. In this review, detection methods for bacteria, viruses, and mycoplasma are comprehensively addressed. Detection methods for viruses include traditional culture-based methods, electron microscopy studies, in vitro molecular and antibody assays, sequencing methods (massive parallel or next generation sequencing), and degenerate PCR (polymerase chain reaction). Bacteria, on the other hand, can be detected with culture-based approaches, PCR, and biosensor-based methods. Mycoplasma can be detected via PCR (including specific kits), microbiological culture methods, and enzyme-linked immunosorbent assays (ELISA). This review highlights the advantages and weaknesses of current detection methods while exploring potential avenues for further development and improvement of novel detection methods. Additionally, a brief evaluation of the transition of these methods into the gene therapy production realm with a focus on viral titer monitoring will be presented.

Keywords: Adventitious Agent Detection, Mycoplasma, Virus, Bacteria, Biological products, Viral vaccines, Product safety.

Introduction:

The World Health Organization (WHO) defines adventitious agents as microorganisms that have been unintentionally introduced into the manufacturing process of a biological medicine [1]. These microorganisms can be introduced throughout the manufacturing process including through starting materials and via human intervention [1]. Thus, regulatory agencies require testing for adventitious agents at various stages of the process. If an adventitious agent is detected, it is important to determine its species, origin, and evaluate its potential for human infection [1]. However, despite extensive efforts to detect adventitious agents, some have still been found in pharmaceutical products such as the presence of Porcine Circovirus 1 (PCV1) in a commercial rotavirus vaccine and novel rhabdovirus in the Sf9 cell line [2-4]. Thus, it is still challenging to efficiently detect adventitious agents using conventional methods.

It is important to note that none of the currently available methods represents a true, “ideal” detection method. The “ideal” detection method would be able to use minimal sample, detect all known adventitious agents and be able to identify potentially unknown agents, have a small limit of detection, be inexpensive, and be able to produce results in real-time or very rapidly. The required limit of detection will depend upon the agent. For example, well known human pathogens must be detected at smaller LODs to ensure patient safety and to meet regulatory requirements [1]. Thus, the development of novel, sensitive detection methods is of paramount importance to the pharmaceutical industry. The aim of this review is to assess traditional and novel detection methods for adventitious agents. These detection methods will range from simplistic to highly developed techniques. In addition, the adaptation of these methods from adventitious agent detection to use in other applications such as gene therapeutics production will be briefly noted.

Traditional Detection Methods:

Traditionally, bacteria and mycoplasma have been detected by culturing a sample of supernatant on agar medium [6]. Bacteria and mycoplasma may also be detected following the inoculation of embryonic chicken eggs via the yolk sac route [7]. Viruses, however, have been traditionally detected via

animal inoculation [5]. Additionally, cell culture may also reveal virus contamination if cytopathic effects are observed. Finally, a hemadsorption (HAD) test may be performed, but this test can only be performed on specific virus types [8, 9]. These traditional detection methods for viruses, mycoplasma, and bacteria are slow, laborious processes. Furthermore, these methods often do not identify the contaminating agent but simply confirm that an agent is present. Also, without the use of an established laboratory, labor and equipment costs to perform cell culture or animal inoculation tests can become expensive.

Microscopy Detection Methods:

Microscopy techniques are often used in parallel to traditional methods as a complementary or orthogonal detection method. Historically, bacteria have been directly observed using light microscopes whereas the effect of viruses on cell phenology has been observed as an indirect detection method [11, 12]. Finally, mycoplasma cannot be seen when the optics of a light microscope are focused on the cell monolayer but may be seen at the air/medium interface [12]. However, in recent years, more advanced microscopy detection methods have been developed for the detection of bacteria, mycoplasma, and viruses.

Electron Microscopy:

Electron microscopy has existed since the 1930s and has been used for the study of viruses since that time [13]. Transmission electron microscopy (TEM) offers higher resolution than traditional light microscopy and is the only imaging technique that allows for the direct visualization of viruses [14]. TEM can be used to document the presence of retroviruses and retrovirus-like particles and gauge the concentration of viral particles [14]. Thus, regulatory agencies have required the use of orthogonal methods to confirm the presence of contaminants including the use of TEM [12, 14]. Additionally, TEM has proven essential in identifying novel viruses and sub-types of viruses [12].

Images from TEM enable for exhaustive analysis for virus particles and present a “catch-all” method for identifying adventitious viruses [13]. However, this analysis is labor intensive. Thus, a novel

method presented by Ito et. al (2018) uses a fully convolutional neural network (FCN) approach to detect viral particles from TEM images [13]. Based on Ito et. al (2018), this FCN detection method outperformed similar methods used for the detection of viral particles within TEM images. As a complementary technique to TEM, immuno-electron microscopy (IEM) can be used for virus identification [15]. IEM can work directly with raw serum which minimizes sample preparation time. Thus, electron microscopy provides a method for detection and identification of adventitious virus particles.

However, microscopy techniques tend to have higher limits of detection and are highly dependent on the homogeneity of the medium being sampled. Additionally, exceedingly small samples are used for detection, and, therefore, only a small fraction (or none) of a specific contaminant may be in the field of view if the concentration of the contaminant is exceptionally low. If, however, samples are preconcentrated with membrane filtration or similar methods, this problem may be minimized. This would increase the cost and time of this technique. Table 1 summarizes the historical methods and table 2 summarizes all the microscopy methods presented herein.

Fluorescence Microscopy and Other Optical Methods:

Technological developments in recent years have led to the development of portable microscopy [16]. For example, a smartphone-based fluorescence microscopy method has been developed to enable imaging of various fluorescently labeled objects such as viruses and bacteria [16]. Shrivastava et. al (2018) has presented a smartphone fluorescent microscopy-based detection and quantification method for bacteria from liquid samples [17]. Other smartphone-based detection methods for adventitious agents can be performed based on colorimetric, turbidity, pH, or luminescence-based endpoints [16]. A unique feature of these methods is that they can be used in real-time. Real-time detection can be implemented using fluorescent measurements or bioluminescence detection and periodical measurements. These smartphone detection methods have been applied in virus and bacteria detection and present a unique platform for adventitious agent detection. Table 3 summarizes these smartphone-based detection methods.

Immunoassays:

Immunoassays such as an enzyme-linked immunosorbent assays (ELISA) are a classical method for detecting and identifying adventitious agents [18, 19]. Recent publications highlight the development of novel immunoassays that present advantages over traditional ELISA. One such method, as presented by Pankratov et. al (2020), uses a cellulase-linked immunomagnetic assay for bacterial analysis [18]. This method, as described by the authors, could detect a single *E. coli* cell which shows the high sensitivity and specificity of the method [18].

Other immunoassays include cell-based activation immunoassays, lateral flow test strip immunoassays, immunochromatographic assays, and magnetophoretic immunoassays [15, 20]. These assays present advantages in detecting adventitious agents. The immunochromatographic assay presented by Li et. al (2018) was able to specifically detect *E. coli* cells in various sample types [21]. The cell-based activation immunoassay presented by Bar-Haim et. al (2018) can detect various bacteria and virus types faster than traditional ELISA [22]. Additionally, the lateral flow test strip immunoassays presented by Tominga et. al (2018) could detect and distinguish 72 distinct types of bacteria [19]. Finally, the magnetophoretic immunoassay presented by Kim et. al (2017) could detect the growth of a mycoplasma strain with lower false positives than other methods [20]. The major limitation of ELISA and other immunoassays is the need for prior knowledge of the adventitious agent. Table 4 summarizes the immunoassays presented herein.

PCR Methods:

Over the last 20 years, polymerase chain reaction (PCR) has been accepted as a gold standard for detecting various nucleic acid-based adventitious agents in pharmaceutical products [23]. Besides traditional PCR, real-time PCR, Droplet Digital PCR (ddPCR), multiplex PCR, and microfluidic PCR have been developed. Real-time PCR assays based on Primer-Probe Energy Transfer (PriProET) have more robust diagnostic capability as they require a shorter conserved region for hybridization making it less susceptible to single point mutations [24-26]. It is suitable for routine screening methods for raw

materials, cell banks, viral and vector seeds bank and animal materials. A virus-specific PCR test just prior to bioreactor harvest has successfully detected and identified a virus contaminant preventing further virus spread and reducing the financial burden of a complete shut down [27-29]. However, prior knowledge of the genome sequence of the virus, bacteria, or mycoplasma is essential to design specific primers [30].

The development of multiplex real-time PCR has enabled differentiation and quantification of viral or bacterial contaminants in a single assay. The risk of carrying-over contaminations is also reduced [31]. It provides increased throughputs and an increase in the number of targets tested in a single reaction by detecting co-infections [32, 33]. This PCR technique is often combined with other methods to detect adventitious agents. Proximity ligation allows for the detection of infectious agents by recognizing an antigen on the viral or bacterial surface with antibodies bound to DNA strands [29, 34]. Also, degenerate oligonucleotide primed (DOP) PCR can be used for non-specific amplification of a DNA sample and is often combined massive parallel sequencing [35, 36]. The digital droplet PCR (ddPCR) is a newly developed PCR technique which enables absolute quantification of target nucleic acids without the need of a standard curve [37, 38]. High throughput dd-PCR is available but needs better standardization and validation.

In addition to PCR, developments in biosensors have presented a unique technique for the detection of bacteria, mycoplasma, and viruses [39, 40]. The Ibis T5000 Universal Biosensor allows for sensitive and specific identification of microbial contaminants [41]. The technology is based on the coupling of broad-range PCR and electrospray ionization-mass spectrometry (ESI-MS) [40, 41]. DNA is amplified using family-specific PCR primers targeting organisms of interest. The various nucleic acids that exist in the sample are accurately measured by mass spectrometry and identified utilizing a database of sequence base composition of known microorganisms [40-42]. This enables broad adventitious agent investigation with PCR primers developed for a wide array of both known and unknown bacterial,

mycoplasma, and viral species [43]. However, the method does not determine whether the contaminant is viable or not and thus needs further validation [44, 45].

High Throughput Sequencing (HTS):

High-throughput sequencing (HTS) allows for comprehensive detection for potential microbial contaminants including unknown viruses [46]. HTS detects the presence of any unexpected sequences that exist in a sample via non-specific massive sequencing and identifies the detected sequence by mapping the sequence to an existing database [47]. Multiple studies have proven that the sensitivity of HTS was comparable to that of qPCR assays [48, 49]. However, HTS is a complicated technology which involves various upstream sample handling process, different sequencing platforms, bioinformatic analysis tools and databases; therefore, development of control and method standardization is an essential requirement for future HTS to be considered as an ideal detection method [50].

The sensitivity of HTS is often influenced by genomic size, structure, and relative efficiencies in reverse transcription and cDNA synthesis in the case of RNA viruses [51, 52]. The difference in sensitivity of viral detection demonstrated in the multicenter study highlights the importance of enhancing sample preparation/processing strategies and the development of reference materials [50]. Furthermore, infectivity assays need to be combined as the hits identified by HTS analysis does not confirm whether the contaminant is viable or not. A comprehensive standardization in sample preparation, sequencing platform, reference materials, bioinformatics, and databases are critical for the future of adventitious agent detection [53-55].

Finally, a recent rise of Oxford Nanopore's MinION sequencing device has created a paradigm shift. The sequencing is based on the measurement of changes in electrical conductivity generated by different bases as the DNA strand is drawn to a nanopore. This affordable, pocket-sized MinION provides real-time long-read sequencing allowing for the detection of both known and unknown species [56-58]. With recent improvements in performance and further validation, it could be a viable option for

adventitious agent detection where immediate action is required. A summary of the PCR methods, HTS, and biosensors can be found in tables 4 and 5.

Discussion and Conclusions:

This review has presented both historical and more novel detection methods. From the presented tables and figure 1, methods with lower specificity and lower sensitivity tend to have lower costs associated with them whereas more robust, sensitive, and specific methods tend to have higher costs. Thus, it is the burden of the researcher to choose whether to incur the higher costs of specific methods at the price of reduced experimentation or to enjoy more experimentation with the risk of lower sensitivity. Additionally, each method presents wildly different limits of detection. Without clear guidance from regulatory agencies and given the fact that some adventitious agents may not be well understood, it is difficult to know whether a low limit of detection is necessary and worth the cost of some of the more sensitive methods. Thus, continuous efforts in improving detection methods including reducing costs, expanding sensitivity and specificity, and gaining better insight on the requirements by regulatory authorities is still of paramount importance in the pharmaceutical industry.

Currently, it appears that real-time PCR, high throughput sequencing, and some biosensors are the closest to “ideal” detection methods and further development of these methods may be the future of adventitious agent detection. The limited capability to detect a broad spectrum of both known and unknown agents by PCR-based method needs to be addressed to become more effective and versatile. High throughput platform development using the combination of family-specific primers and specific primer sets can be devised to detect a wide range of agents in a single assay. Moreover, further improvements in performance, speed, affordability, convenience, method standardization, and the establishment of reference materials would make gene sequencing a more attractive approach.

With such a broad variety of detection methods available, it is possible to consider the adaptation of these methods to other uses. As an example, some of the methods presented herein could be adapted for use in the production of gene therapeutics. This transition could lead to better manufacturing

techniques and yield significant opportunities for quality by design in gene therapeutic products. Specifically, as gene therapeutics use lentiviral or other virus-type delivery platforms, the use of real-time virus detection methods with quantification could lead to the ability to track the productivity of a gene therapy manufacturing platform. The methods presented previously that could fit this description (with some modification) include real time PCR, real-time immunoassays, and biosensors such as the Ibis T5000 Universal Biosensor. The ability to accurately monitor the production of viral vectors for gene therapeutics could enable for better process decisions and enhanced manufacturing techniques. Thus, the continued development of detection methods for adventitious agents is not only beneficial for safety and quality of pharmaceutical products, but it is also a potential avenue to enhance the manufacturing of future therapeutic products such as gene therapeutics.

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224 **Author Contributions**

225 Morris and Lee equally contributed to planning, manuscript writing, and review. Yoon was the
226 project PI and provided the main supervision and guidance to the research work. All the authors
227 reviewed and approved the manuscript. The authors declare that they have no competing
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References

1. Klug, B., et al., *Adventitious agents and live viral vectored vaccines: Considerations for archiving samples of biological materials for retrospective analysis*. Vaccine, 2016. **34**(51): p. 6617-6625.
2. Victoria, J.G., et al., *Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus*. J Virol, 2010. **84**(12): p. 6033-40.
3. Dubin, G., et al., *Investigation of a regulatory agency enquiry into potential porcine circovirus type 1 contamination of the human rotavirus vaccine, Rotarix: approach and outcome*. Human vaccines & immunotherapeutics, 2013. **9**(11): p. 2398-2408.
4. Ma, H., et al., *Identification of a novel rhabdovirus in Spodoptera frugiperda cell lines*. J Virol, 2014. **88**(12): p. 6576-85.
5. Kuzmin, I.V., *Virus Inoculation in Animals: The Mouse Inoculation Test*. Current Laboratories Techniques in Rabies Diagnosis, Research, and Prevention, 2015. **2**: p. 13-23.
6. Nikfarjam, L. and P. Farzaneh, *Prevention and detection of Mycoplasma contamination in cell culture*. Cell J, 2012. **13**(4): p. 203-12.
7. *Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications*, C.f.B.E.a. Research, Editor. 2010, Food and Drug Administration.
8. Leland, D.S., and C.C. Ginocchio, *Role of cell culture for virus detection in the age of technology*. Clin Microbiol Rev, 2007. **20**(1): p. 49-78.

251 9. Ilchmann, A., A.A. Armstrong, and R.F. Clayton, *Schmallenberg virus, an emerging viral*
252 *pathogen of cattle and sheep and a potential contaminant of raw materials, is detectable by classical in-*
253 *vitro adventitious virus assays*. Biologicals, 2017. **49**: p. 28-32.

254 10. Nims, R.W. and P.J. Price, *Best practices for detecting and mitigating the risk of cell culture*
255 *contaminants*. In Vitro Cell Dev Biol Anim, 2017. **53**(10): p. 872-879.

256 11. Roingeard, P., *Viral detection by electron microscopy: past, present and future*. Biol Cell,
257 2008. **100**(8): p. 491-501.

258 12. Roingeard, P., et al., *Virus detection by transmission electron microscopy: Still useful for*
259 *diagnosis and a plus for biosafety*. Rev Med Virol, 2019. **29**(1): p. e2019.

260 This review of electron microscopy provides an in-depth evaluation of electron microscopy and highlights
261 its importance today.

262 13. Ito, E., et al., *Virus Particle Detection by Convolutional Neural Network in Transmission*
263 *Electron Microscopy Images*. Food Environ Virol, 2018. **10**(2): p. 201-208.

264 This article shows an interesting methodology for using statistical software to better understand images
265 captured by transmission electron microscopy.

266 14. Richert-Pöggeler, K.R., et al., *Electron Microscopy Methods for Virus Diagnosis and High-*
267 *Resolution Analysis of Viruses*. Frontiers in microbiology, 2019. **9**(3255).

268 This article reviews electron microscopy and details the resolution that can be obtained via this method. It
269 also details information regarding how electron microscopy can be used for viral diagnosis.

270 15. Verma, J., S. Saxena, and S. Gosipatala, *ELISA –Based Identification and Detection of*
271 *Microbes*. Analyzing Microbes- Manual of Molecular Biology Techniques - Springer Protocols, 2013.

16. Ding, X., et al., *Interfacing Pathogen Detection with Smartphones for Point-of-Care Applications*. Anal Chem, 2019. **91**(1): p. 655-672.

This review provides an example of the use of a handheld device for adventitious agent detection. It details the use of handheld devices to detect both viruses and bacteria.

17. Shrivastava, S., W.I. Lee, and N.E. Lee, *Culture-free, highly sensitive, quantitative detection of bacteria from minimally processed samples using fluorescence imaging by smartphone*. Biosens Bioelectron, 2018. **109**: p. 90-97.

This article details the use of a handheld device to detect bacteria from a sample that has not undergone purification.

18. Pankratov, D., et al., *Cellulase-Linked Immunomagnetic Microbial Assay on Electrodes: Specific and Sensitive Detection of a Single Bacterial Cell*. Analytical Chemistry, 2020. **92**(18): p. 12451-12459.

This article highlights a highly sensitive immunoassay technique that uses electrodes as a base for detection.

19. Tominaga, T., *Rapid detection of Klebsiella pneumoniae, Klebsiella oxytoca, Raoultella ornithinolytica and other related bacteria in food by lateral-flow test strip immunoassays*. J Microbiol Methods, 2018. **147**: p. 43-49.

This article highlights the use of a lateral-flow test strip immunoassay that has been used in the food industry for the detection of bacteria. It is hypothesized that this same method could be easily translated to the pharmaceutical industry.

20. Kim, J., et al., *Early detection of the growth of Mycobacterium tuberculosis using magnetophoretic immunoassay in liquid culture*. Biosens Bioelectron, 2017. **96**: p. 68-76.

21. Li, Q., et al., *Rapid detection of Escherichia coli O157:H7 by a fluorescent microsphere-based immunochromatographic assay and immunomagnetic separation*. Anal Biochem, 2019. **564-565**: p. 32-39.

This article highlights the rapid detection of a common contaminant (E. Coli) via immunochromatographic assays.

22. Bar-Haim, E., et al., *Early Diagnosis of Pathogen Infection by Cell-Based Activation Immunoassay*. Cells, 2019. **8**(9).

This article highlights the use of a cell-based activation assay which is used to diagnosis pathogen infection within cell culture.

23. Kumar, D., et al., *Use of PCR-based assays for the detection of the adventitious agent porcine circovirus type 1 (PCV1) in vaccines, and for confirming the identity of cell substrates and viruses used in vaccine production*. J Virol Methods, 2012. **179**(1): p. 201-11.

24. Zhang, X.J., et al., *Evaluation of a primer-probe energy transfer real-time PCR assay for detection of classical swine fever virus*. J Virol Methods, 2010. **168**(1-2): p. 259-61.

25. Hakhverdyan, M., et al., *Development of a real-time PCR assay based on primer-probe energy transfer for the detection of swine vesicular disease virus*. Arch Virol, 2006. **151**(12): p. 2365-76.

26. Barfoed, A.M., et al., *Development of a primer-probe energy transfer based real-time PCR for detection of Marek's disease virus*. Journal of Virological Methods, 2010. **165**(1): p. 21-26.

27. Grillberger, L., et al., *Emerging trends in plasma-free manufacturing of recombinant protein therapeutics expressed in mammalian cells*. Biotechnol J, 2009. **4**(2): p. 186-201.

28. Kozlowski, S. and P. Swann, *Current and future issues in the manufacturing and development of monoclonal antibodies*. Adv Drug Deliv Rev, 2006. **58**(5-6): p. 707-22.

29. Barone, P.W., et al., *Viral contamination in biologic manufacture and implications for emerging therapies*. Nat Biotechnol, 2020. **38**(5): p. 563-572.

This article highlights the importance of detecting and understanding the different viruses that may contaminate biopharmaceutical products.

30. Farsang, A. and G. Kulcsár, *Extraneous agent detection in vaccines--a review of technical aspects*. Biologicals, 2012. **40**(4): p. 225-30.

31. Pallás, V., J.A. Sánchez-Navarro, and D. James, *Recent Advances on the Multiplex Molecular Detection of Plant Viruses and Viroids*. Front Microbiol, 2018. **9**: p. 2087.

32. Laamiri, N., et al., *A multiplex real-time RT-PCR for simultaneous detection of four most common avian respiratory viruses*. Virology, 2018. **515**: p. 29-37.

This article highlights the use of a real-time PCR for the detection of multiple viruses.

33. Thonur, L., et al., *One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3*. BMC Vet Res, 2012. **8**: p. 37.

34. Koos, B., et al., *Analysis of protein interactions in situ by proximity ligation assays*. Curr Top Microbiol Immunol, 2014. **377**: p. 111-26.

35. Blagodatskikh, K.A., et al., *Improved DOP-PCR (iDOP-PCR): A robust and simple WGA method for efficient amplification of low copy number genomic DNA*. PloS one, 2017. **12**(9): p. e0184507-e0184507.

This article highlights the use of Degenerate oligonucleotide primed PCR using a novel WGA method.

This method enables for amplification of low copy number genomic DNA.

36. McClenahan, S.D., C. Uhlenhaut, and P.R. Krause, *Evaluation of cells and biological reagents for adventitious agents using degenerate primer PCR and massively parallel sequencing*. Vaccine, 2014. **32**(52): p. 7115-21.

37. Americo, J.L., P.L. Earl, and B. Moss, *Droplet digital PCR for rapid enumeration of viral genomes and particles from cells and animals infected with orthopoxviruses*. Virology, 2017. **511**: p. 19-22.

This article highlights the use of droplet digital PCR (low volume required) for the detection of a specific virus in animals.

38. Quan, P.L., M. Sauzade, and E. Brouzes, *dPCR: A Technology Review*. Sensors (Basel), 2018. **18**(4).

This review highlights sensors that have been used for adventitious agent detection.

39. Bueno, J., *Biosensors in Antimicrobial Drug Discovery: Since Biology until Screening Platforms*. Journal of Microbial & Biochemical Technology, 2014. **S10**.

40. Jacob, D., et al., *Rapid, and high-throughput detection of highly pathogenic bacteria by Ibis PLEX-ID technology*. PLoS One, 2012. **7**(6): p. e39928.

41. Ecker, D., et al., *The Ibis T5000 Universal Biosensor: An Automated Platform for Pathogen Identification and Strain Typing*. Journal of the Association for Laboratory Automation, 2006. **11**: p. 341-351.

42. Lindsay, B., et al., *Survey of culture, goldengate assay, universal biosensor assay, and 16S rRNA Gene sequencing as alternative methods of bacterial pathogen detection*. J Clin Microbiol, 2013. **51**(10): p. 3263-9.

43. Ecker, D.J., et al., *Ibis T5000: a universal biosensor approach for microbiology*. Nature Reviews Microbiology, 2008. **6**(7): p. 553-558.

44. Sheets, R., et al., *Adventitious agents, new technology, and risk assessment, 19-20 May 2011*, Baltimore, MD. Biologicals, 2012. **40**(2): p. 162-7.

45. Bahadır, E.B. and M.K. Sezgintürk, *Applications of commercial biosensors in clinical, food, environmental, and biothreat/biowarfare analyses*. Analytical Biochemistry, 2015. **478**: p. 107-120.

46. Onions, D., and J. Kolman, *Massively parallel sequencing, a new method for detecting adventitious agents*. Biologicals, 2010. **38**(3): p. 377-80.

47. Shaman, J., et al., *Adventitious Virus Contamination Testing: Massively Parallel Sequencing in a Multimodal Solution for Biopharmaceutical Safety Testing*. BioProcessing Journal, 2013. **12**: p. 18-24.

48. Cheval, J., et al., *Evaluation of high-throughput sequencing for identifying known and unknown viruses in biological samples*. J Clin Microbiol, 2011. **49**(9): p. 3268-75.

49. Wylie, K.M., et al., *Sequence analysis of the human virome in febrile and afebrile children*. PLoS One, 2012. **7**(6): p. e27735.

50. Khan, A.S., et al., *A Multicenter Study To Evaluate the Performance of High-Throughput Sequencing for Virus Detection*. mSphere, 2017. **2**(5).

This article provides studies from various research centers that were used to evaluate the ability of high-throughput sequencing to detect viruses.

51. Paskey, A.C., et al., *Enrichment post-library preparation enhances the sensitivity of high-throughput sequencing-based detection and characterization of viruses from complex samples*. BMC Genomics, 2019. **20**(1): p. 155.

This article highlights the importance of post-library preparation to improve the sensitivity of high-throughput sequencing.

52. Charlebois, R.L., et al., *Sensitivity and breadth of detection of high-throughput sequencing for adventitious virus detection*. npj Vaccines, 2020. **5**(1): p. 61.

This article highlights the sensitivity and ability of high throughput sequencing to detect both known and unknown viruses.

53. Ng, S.H., et al., *Current Perspectives on High-Throughput Sequencing (HTS) for Adventitious Virus Detection: Upstream Sample Processing and Library Preparation*. Viruses, 2018. **10**(10).

This review highlights the importance of both upstream sample preparation and library preparation for high throughput sequencing to be successful.

54. Mee, E.T., et al., *Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing*. Vaccine, 2016. **34**(17): p. 2035-43.

55. Toohey-Kurth, K., S.D. Sibley, and T.L. Goldberg, *Metagenomic assessment of adventitious viruses in commercial bovine sera*. Biologicals, 2017. **47**: p. 64-68.

56. Lu, H., et al., *Oxford Nanopore MinION Sequencing and Genome Assembly*. Genomics Proteomics Bioinformatics, 2016. 14: p. 265–79

57. Tyler, A.D., et al., *Evaluation of Oxford Nanopore's MinION Sequencing Device for Microbial Whole Genome Sequencing Applications*. Scientific Reports, 2018. **8**(1).

58. Tyson J.R., et al., *MinION-based long-read sequencing and assembly extends the *Caenorhabditis elegans* reference genome*. Genome Res. 2018. 28(2): p. 266-74.

397 **Figure Legends**

398 **Figure 1:** Comparison of Detection Techniques: The above chart shows the advantages of each of the
399 detection techniques (red box) described previously along with the pre-processed materials (blue box).
400 The chart shows that no one method has all the characteristics of an “ideal” detection method. Thus,
401 further development in detection techniques may be able to provide a detection method that is closer to an
402 “ideal” method.

	Simple	Rapid	Inexpensive	Unknowns Detectable	Sensitive	Specific	Real Time
Growth on Agar Medium	✓						
Cell Culture	✓						
Light Microscopy	✓						
Handheld Devices	✓		✓				✓
Lateral Flow Strip Immunoassay	✓	✓	✓				
Cell Based Activation Immunoassay		✓				✓	
Magnetophoretic Immunoassay		✓				✓	
Real-time PCR		✓			✓	✓	✓
High Throughput Sequencing		✓		✓	✓	✓	
Transmission Electron Microscopy				✓	✓	✓	
Ibis T5000 Universal Biosensor				✓	✓	✓	
Cellulase-linked Immunomagnetic Assay					✓	✓	
Droplet Digital PCR					✓	✓	
Immunoelectron Microscopy					✓	✓	
Ideal Detection	✓	✓	✓	✓	✓	✓	✓

Figure 1: Comparison of Detection Techniques: The above chart shows the advantages of each of the detection techniques described previously. The chart shows that no one method has all the characteristics of an “ideal” detection method. Thus, further development in detection techniques may be able to provide a detection method that is closer to an “ideal” method.

Table 1: Adventitious Agent Detection Methods

Method:	Description	Advantages	Limitations	Limit of Detection	Cost ^a	Time	Reference(s)
Growth on Agar Medium	Supernatant is added to agar medium and allowed to incubate either aerobically or anaerobically for a period	Simple; Accepted by regulatory agencies; Well studied and documented.	Time consuming process; Does not identify the species of the adventitious agent	1 CFU (Sutton, 2011)	\$1.50-\$2.50 ^a per plate	12 hours – 14 days	6, 7
Animal Inoculation	Animals (such as mice, rats, rabbits, etc.) are inoculated with a small amount of virus containing material and observed for signs of illness	Effective; Accepted by FDA	Ethical concerns; Expensive; Time consuming	21 days; Limited based on observation by researcher (ERSA journal)	\$475-\$990 ^b per test (rabbit pyrogen test)	2-3 hours	5, 8, 9
Hemadsorption Test	Culture medium is replaced with a suspension of erythrocytes, and, if the cells are infected with virus, the erythrocytes will adhere to the cells	Well understood	Time consuming, no information on type of viruses/only can be used on certain viruses	Dependent on viral particle and presence of viral hemagglutinin	\$34 - \$75 per test (supplies and labor) (Newton, 2002)	1 hour	5
Cell Culture	Cells are inoculated with a sample that is suspected to be contaminated and observed for cytopathic effects and turbidity.	Simple	Time consuming	1 CFU; Must be estimated for specific experiments (Sutton)	\$100 - \$1,000,000 ^a (depends on available equipment and supplies)	2 – 14 days	5, 6, 8-10

^aPrice obtained from Thermo Fisher Scientific, Waltham, MA ^bPrice from Institute for In Vitro Sciences, Gaithersburg, MD

Table 2: Microscopy Methods

Method	Description	Advantages	Limitations	Limit of Detection	Cost ^{a,b}	Time	Reference(s)
Light Microscopy	Specimens are observed under light microscope with or without oil immersion	Simple, well understood	No information on species of contaminating agent	200 nm size (Sutton 2011) or 200,000 particles per square millimeter (Forouhi 2020)	\$200 - \$5,000 ^a (for microscope)	10-30 min. (depending on preparation)	10-12
Transmission Electron Microscopy	Specimens are observed via a transmission electron microscope and a high voltage electron beam is used to create an image of the specimen.	Allows for the direct visualization of viruses, can be used with difficult to detect or unknown viruses	Expensive equipment, complex, Time consuming sample preparation	< 10 nm (Sutton 2011) (specimens must be prepared) 10E7 pfu/mL (Rayjonec, 2019)	\$2,000,000 ^b or higher for the microscope, \$200 ^c per specimen for fixation (off-site), \$100-\$200 ^c for use of microscope (off-site)	3 hours -14 days for fixation, embedding, sectioning, staining, and imaging (on-site or off-site preparation)	12-14
Immunoelectron Microscopy	A sample is suspended in a suitable medium (such as phosphate buffered saline) and antiserum is added. The mixture is warmed, centrifuged, and the pellet is examined by negative stain electron microscopy.	Used extensively for the diagnosis of viral infections (est. 1940s)	Complex sample preparation, previous knowledge of the appropriate antibodies to be used required	10 ⁵ - 10 ⁷ particles per mL (Li 2013) or 10E4 PFU/mL (Baca 2015)	\$50,000 - \$1,000,000 ^b for microscope \$100 for specimen preparation (off-site), \$50-\$65 ^c for specimen mounting and coating (off-site), \$100-\$200 ^c for use of microscope (off-site)	3 hours -14 days for fixation, embedding, sectioning, staining, and imaging (on-site or off-site preparation)	14

^aPrice obtained from Olympus Life Sciences, Waltham, MA ^bPrice obtained from TSS Microscopy, Hillsboro, OR, ^cPrice obtained from Indiana University, Bloomington, IN

Table 3: Smart-phone based and Biosensor-based Methods

Method	Description	Advantages	Limitations	Limit of Detection	Cost ^a	Time	Reference(s)
Smartphone-based Fluorescence Microscopy	Samples are fixed and stained on glass slides (using a fluorescent-in-situ-hybridization approach with pre-designed rRNA-targeting PNA probes) and then are analyzed directly on the smartphone screen. The smartphone is integrated with a 3D printed optomechanical attachment.	Compact, lightweight, cost-effective, simple	Detection limitations, requires PNA probes	10 CFU/mL (Shrivastava, 2018)	\$2,000 or greater ^a	5min – 1 hour (Young-Ho Shin et al 2021)	17
Smartphone-based Optical Methods	Smartphone-based Optical Methods rely on certain endpoints such as pH, turbidity, color, etc. for a measurement to be determined. With each of these methods, something like a smartphone microplate reader or other connected/accessory device may be used to quantify the desired endpoint based on a picture.	Compact, lightweight, portable	Low-throughput, low sensitivity, additional components sometimes required	20 CFU/mL (Nelis, 2020)	\$2,000 or greater ^a	5min – 1 hour (Young-Ho Shin et al 2021)	16

^aPrice obtained from ioLight, Hicksville, NY

Table 4: Immunoassays

Method:	Description	Advantages	Limitations	Limit of Detection	Cost ^{a,b,c,d}	Time	Reference(s)
Cellulase-linked immunomagnetic assay	This assay utilizes a sandwich antibody (Ab/aptamer-bacterium-Ab/aptamer) labelled with cellulase assembled on a micrometer sized magnetic bead applied to a nitrocellulose-modified-film. The cellulase then digests the nitrocellulose film which changes the electrical properties of the electrodes. This change can be measured.	High sensitivity, high specificity	Needs to be assessed for more species	Detection of a Single E. Coli cell [25]	\$515/100 assays ^a	15 min - 3 hours (RB Jamal 2020)	18
Cell-Based Activation Immunoassay	B- or T- lymphocytes or monocytes and granulocytes are used to detect and quantify specimens in samples.	Quick, high specificity	Prior information required, complex	10 ⁵ CFU/mL	\$300 - \$700 ^d	24 hours	22
Lateral Flow Test Strip Immunoassay/ Immuno-chromatographic Assay	A liquid moves via capillary action through polymeric strips on which molecules that can interact with the analyte are attached.	Low cost, simple, rapid	Low sensitivity	10 ⁴ -10 ⁹ CFU/mL (Ziegler 2020)	\$110/10 assays ^a \$1,660/100 tests ^b	15 min (RB Jamal 2020)	19
Magnetophoretic Immunoassay	An immunoassay (similar to ELISA) that uses magnetic beads, radioisotopes, or fluorescent labels to detect a specific analyte. A magnetic label is conjugated to either the antibody or antigen and a magnetic reader is used to record the magnetic change induced by the beads.	High specificity, can be fully automated, rapid	Required conjugation of magnetic beads	0.3 pM or 5-50 CFU/mL	\$1,400 or greater ^c	< 3 hours (RB Jamal 2020)	20

^aPrice obtained from Thermo Fisher Scientific, Waltham, MA, ^bPrice obtained from Abcam plc., Cambridge, UK, ^cPrice obtained from Tiger Medical, Inc, Irvington, NJ,

^dPrice obtained from RayBiotech, Inc, Peachtree Corners, GA

Table 5: PCR and HTS

Method	Description	Advantages	Limitations	Limit of Detection	Cost	Time	Reference(s)
Real time-PCR	Processed sample is amplified with specific primer set and a probe, and Ct value is assessed for identification and compared with standards for semi-quantification in real-time.	Sensitive, specific, quick	No unknown detection, no information on infectivity, semi-quantitative	1 - 10 copies/μl	\$15,000 ^a - \$80,000 ^b \$1-5/reaction	30min – 2 hours	23-25
Multiplex PCR	With multiple non-interfering primer sets, PCR reactions targeting for multiple sequence are performed within the same well, which are detected by different probes.	Increased throughput, Reduced carrying over contamination	Primer interference, Difficult optimization and validation, semi-quantitative	10 - 30 copies/μl	\$15,000 ^a - \$80,000 ^b Reaction cost varies depending on the number of targets	1-3 hours	32, 33
Droplet Digital PCR	A nucleic acid sample is partitioned in water-in-oil droplets in which PCR reactions occur. After PCR amplification, the individual fluorescent positive and negative droplets are quantified via Poisson distribution to determine the number of DNA copies in the starting sample.	Sensitive, reproducible, Absolute quantitation, no standard curve required, insensitive to PCR inhibitors	More expensive, more hands-on time, restricted dynamic range	1 copy/μl	Quote requested ^c \$3-5/reaction	3 hours	37, 38
High Throughput Sequencing	Samples are screened for presence of any unexpected sequence via non-specific massive sequencing and the detected sequence is identified by mapping the sequence to the existing database.	large breadth of detection, unknown detection, high throughput	Time consuming, complicated, standardization and reference material establishment, No information on contaminant viability	~ 10 copies/reaction	\$200,000~400,000 ^d \$20 -1,500/GB	10-48 hours	46-48, 50-54
MinION Sequencer	Target DNA strand is drawn into nanopore, and each DNA base is identified by measuring different electrical conductivities.	Low cost, small size, direct sequencing, real time data collection and analysis, fast	High quality and high copy number required	10 copies/reaction *LOD is inversely proportional to turnaround time. With high-copy, quick detection is possible	\$1,000 - \$285,455 ^e \$90 - 1,600/sample	Full: 6-10 hours Quick: 10 min – 1 hour	56, 57, 58

^aPrice obtained from Qiagen, Hilden, Germany, ^bPrice obtained from Thermo Fisher Scientific, Waltham, MA, ^cPrice obtained from Bio-Rad Laboratories, Hercules, CA

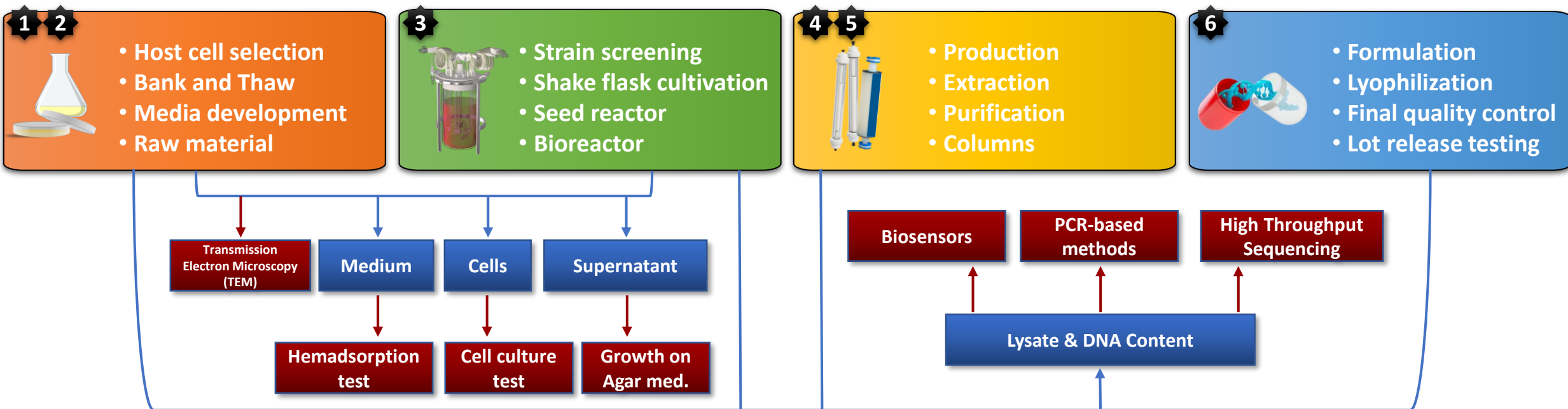
^dPrice obtained from Illumina, San Diego, CA, ^ePrice obtained from Oxford Nanopore Technologies, Oxford, UK

Cell line Development

Upstream

Downstream

Product Release



Testing at different stages of production

- 1 Cell bank
- 2 Pre-production cells
- 3 Pre-filtered harvest or post-production cells
- 4 Control cells
- 5 Post-filtered cells
- 6 Final filled product