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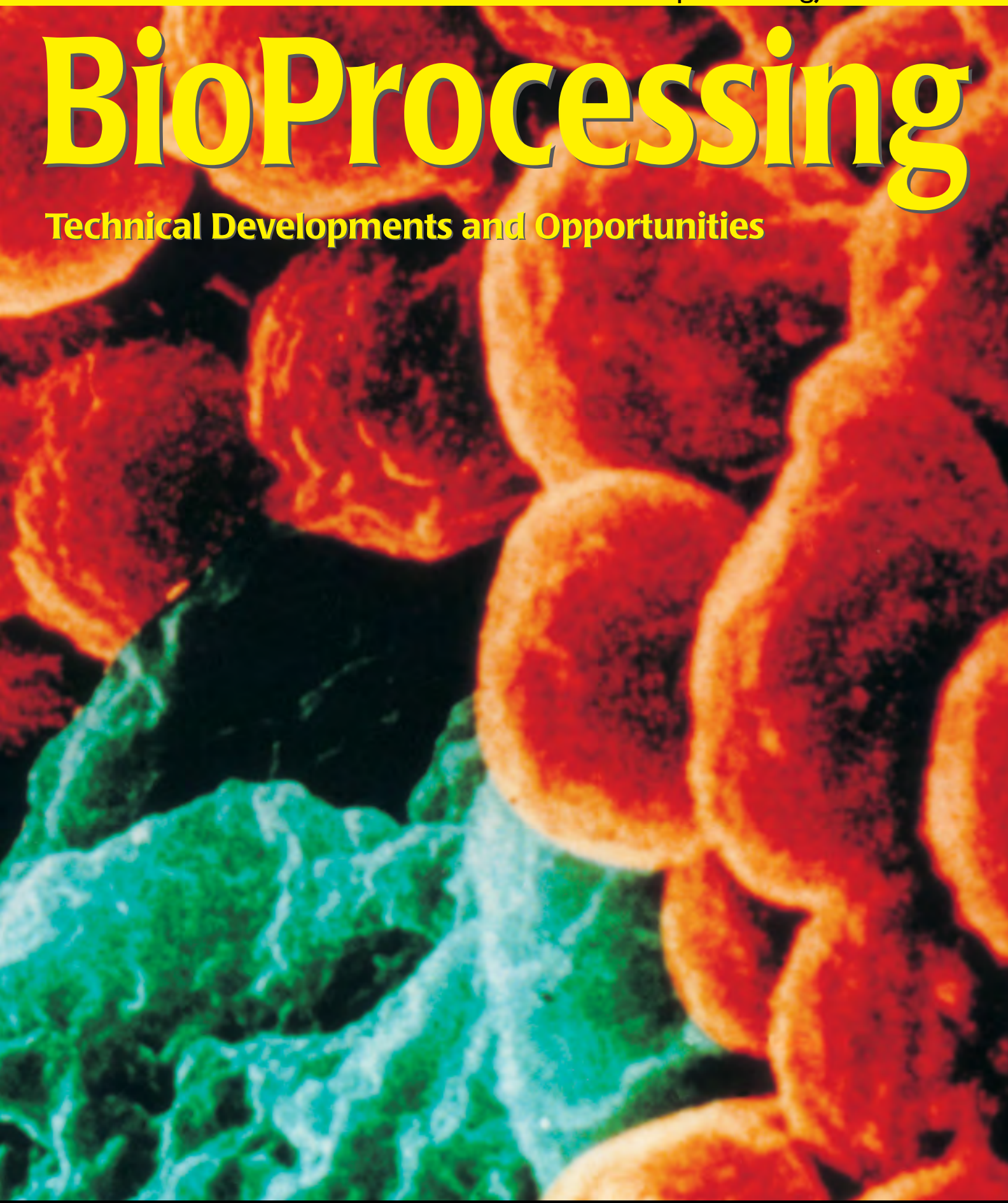

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Development of a Reference Material for Characterizing Adenovirus Vectors

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The development of reference testing reagents has been used successfully in the past to standardize measurements among laboratories, particularly for biological products such as recombinant cytokines. This approach was recommended by many parties with a stake in adenovirus vector delivery in order to address the fact that particle units and infectious units are not standardized in the field.[1] This has made interpretation of preclinical and clinical data, as it relates to the amount of adenovirus vector administered, difficult to compare across the field.

An Adenovirus Reference Material is being developed to define the particle unit and infectious unit for adenovirus gene vectors, and create a commonality for comparisons, especially for data related to vector safety.

Working Group Formation

The Adenovirus Reference Material Working Group (ARMWG) was formed to oversee the development of a well-characterized Adenovirus Reference Material (Adenovirus 5 Wild Type) and has been responsible for identifying the process to evaluate and select appropriate laboratories or institutions to manufacture, characterize, and distribute the material. The ARMWG consists of 38 volunteers representing the Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA), the American Type Culture Collection (ATCC), the U.S. Pharmacopeia (USP), the National Institute for Biological Standards and Controls (NIBSC), the Williamsburg BioProcessing Foundation (WilBio), five academic groups, 15 industry representatives, five contract manufacturers, four contract testing organizations, and two suppliers.

The Co-Chairs are myself and Estuardo Aguilar-Cordova (Harvard University). The Williamsburg BioProcessing Foundation has a Memorandum of Understanding with the FDA and is responsible for coordinating the activities overseen by the Working Group, and for facilitating the collection and distribution of information resulting from this project.

The ARMWG is an all-volunteer effort, and there is no funding to support this initiative. Although members of the ARMWG have held different points of view on key issues, working group discussions have lead to (near) consensus decisions.

This may seem an ideal situation, but the highly cooperative and collaborative spirit behind the ARMWG effort was driven by the death of a patient in 1999, the public perceptions about adenovirus gene therapy that followed this event, and a re-assessment of the work done in the adenovirus vector field.[2]

The ARMWG first established a list of the activities that were involved in manufacturing, characterization, storage, and distribution of a reference material. The ARMWG then established the criteria upon which selection of responsible groups would be made. This was done in conjunction with the establishment of a rough timeline for the activities that were grouped by phase: source materials, manufacturing, and characterization.

Groups were asked to submit proposals for performing each activity based on the established criteria. Proposals were circulated to all members of the ARMWG in advance of Working Group meetings. The FDA evaluated each proposal and CBER's representatives made recommendations to the ARMWG. Decisions on all proposals, and the selection of the group(s) to be assigned each project phase, were made by a vote of the Working Group. FDA representatives abstained from all votes.

All information regarding the development of the Adenovirus Reference Material is freely available. All ARMWG meeting minutes, including summaries of discussions related to decisions, proposals submitted, and groups selected for each activity, are posted on the WilBio website, www.wilbio.com. This information is also circulated to interested parties by email, such as organizations monitoring the development of the reference material (i.e., the NIH Office of Biotechnology Activities, USP, ASGT, NIBSC, and PhRMA).

Materials and Methods

The ARMWG has obtained donations of materials and services to produce more than 5000 x 0.5 ml vials of a reference material consisting of Adenovirus 5 WT. The ARMWG selected the formulation of 20 mM TRIS, pH 8.0,

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Table 1. Laboratories and Institutions Participating in Manufacturing Phase

Donation	Institution
293 Working Cell Bank, CGMP 100 vials	Vector & Vaccine Production Facility at the University of Alabama - Birmingham
Adenovirus 5 WT plaque-purified source material	Canji, Inc.
Adenovirus 5 WT Virus Bank, CGMP 250 x 10 ml vials	Canji, Inc.
Purified, formulated Bulk Ad5 WT Reference Material, >5L	Production & purification by Introgen, formulation by Selective Genetics, Inc.
Adenovirus Reference Material 5300 x 0.5-ml vials	Introgen Therapeutics, Inc.
Testing services to support production of virus bank and reference material	Althea Corp., MDS PharmaServices Q-One Biotech, Inc.
Supplies for Production	Amersham Pharmacia Biotech, Corning EM Processing, Hyclone, Invitrogen-Gibco Nunc, Osmonics, Inc., Stedim
Repository & Distribution	ATCC

25 mM Sodium Chloride, 2.5% (w/v) Glycerol for the reference material. The ARMWG also obtained manufacturing materials for generation of additional batches of the reference material, including a 293 cell production bank, an Adenovirus 5 WT production virus bank, a 293 cell testing bank, and repository services for storing and distributing the reference material and its manufacturing materials. The institutions involved in the manufacturing phase are shown in Table 1.

The Adenovirus Reference Material is being characterized by a consortium of more than twenty-four laboratories, with locations in the United States, Canada, the United Kingdom, and France. Characterization phase activities include a five-year long-term stability study, a short-term field use and shipping configuration stability study, particle concentration determination, infectious titer determination, sequence determination for the full length virus, and various analyses for impurities. Characterization is on track for public release of the Reference Material in spring, 2002. Participating laboratories and assigned characterization activities are detailed in Table 2.

Particle concentration. Fifteen laboratories are measuring particle concentration with a procedure developed by the ARMWG and based on OD260nm absorbance in the presence of SDS. The goal is to obtain 60 data points based on 30 independently derived sample dilutions. A small consortium of five laboratories is also determining particle concentration with a variety of orthogonal methods, including a Reverse Phase-HPLC assay,[3] an Anion Exchange HPLC assay,[4] quantitative real-time PCR, a PicoGreen assay,[5] and electron microscopy. Several other institutions are performing their institution-specific protocols for quantitative real-time PCR and Anion Exchange HPLC. They will provide the data and method details to the Working Group. The Working Group expects to be able to comment on the utility of the different orthogonal methods in comparison with the traditional absorbance method.

Infectious titer. Infectious titer is being determined in seventeen laboratories using a procedure developed by the ARMWG. The method is based on a read-out of the sample's cytopathic effect (CPE) after ten days on 293 cells in a 96-well plate format. The 293 cells for the infectious titer assays were supplied by the ARMWG so that every group would use the same system.

The Working Group selected a CPE-based assay because: 1) the group desired a method that could be performed by most laboratories, 2) the group desired a method that was not dependent on specific equipment (such as flow-cytometry based methods), and 3) the group desired a method that was easy to learn and perform by laboratory technical staff (as compared to plaque-formation assays which require more training to perform).

Each laboratory is performing the assay on two, independently created, square root of two-fold dilution series. This will result in a large number of data points from each laboratory. The Working Group also decided that the assay should incorporate a correction into the titer calculation to compensate for the slow diffusion of the adenovirus particle in solutions. The NAS titer calculation method was selected.[6]

Several individual laboratories are performing their in-house infectious titer assays, and will provide the data and method details to the Working Group as part of the characterization assessment. The methods include a plaque forming unit (pfu) assay, a flow cytometry-based infectious titer assay, and a different format CPE-based infectious titer assay.

Relatively few groups were interested in assessing the infectious titer of the reference material via in-house methods. The Working Group believes that most laboratories would prefer to assess the performance of their in-house infectious titer method without disclosing the data to the public. All information and data created under the Working Group's charter is available to the public, and no information is allowed to remain proprietary as a condition of participation in the development of the reference material.

After reviewing all of the available data and receiving the recommendations of the FDA representatives, the Working Group will determine and officially assign both the particle concentration as well as the infectious unit concentration (infectious titer) to the Adenovirus Reference Material. These assignments will allow the Adenovirus Reference Material to be used as a yardstick against which other methods can be compared for adenovirus vector particle unit and infectious unit measurements.

Stability testing. The Adenovirus Reference Material is also undergoing

Table 2. Laboratories and Institutions Participating in Characterization Phase

Donation	Institution
Testing phase 293 cell bank, 50 vials	Invitrogen-Gibco
Long-term stability study	Canji, Inc.
Short-term field use & shipping stability studies	Genetic Therapy Inc./Novartis, Croyle Laboratory at University of Texas (UT) - Austin Transgene (France)
DNA sequencing of complete vector	Canji, Inc., SeqWright
Particle concentration via ARMWG SOP and *orthogonal methods or **institutional method	AFSSAPS (France), *Berlex Biosciences, **Biotechnology Research Institute (Canada) *Canji, Inc., **Cell Genesys, Cobra Therapeutics (UK), Covance Laboratories (UK) *Croyle Laboratory at UT - Austin, *Harvard University Gene Medicine Initiative, Genetic Therapy, Inc./Novartis, **Onyx, Q-Biogene, Inc. (Canada), *Schering Plough Research Inst., **Transgene, Vector & Vaccine Production Facilityn at University of Alabama - Birmingham
Infectious titer via ARMWG SOP and *institutional method	AFSSAPS, AppTec Laboratories, ATCC, *Berlex Biosciences, Biotechnology Research Institute (Canada), Canji, Inc., *Cell Genesys, *Cobra Therapeutics, Covance Laboratories, Croyle Laboratory at UT - Austin, Harvard University Gene Medicine Initiative, Genetic Therapy, Inc./Novartis, Onyx, Q-Biogene, Inc., Q-One Biotech (U.K. & U.S.) Schering Plough Research Institute, Transgene, Vector & Vaccine Production Facility at University of Alabama - Birmingham
Residual host cell DNA	Althea
Residual host cell proteins	Canji, Inc.
Free hexon	Schering Plough Research Institute
31K MW precursor protein form	Schering Plough Research Institute
Particle size distribution	Croyle Laboratory at UT - Austin
Photon correlation spectroscopy	Transgene

stability testing and purity analysis. A five-year stability study was set up to monitor the reference material when stored frozen at -80°C, which represents the ARMWG-recommended ultra-low storage condition of below -55°C. A more limited study was designed to assess stability when the reference material is frozen at -80°C, but then stored long-term at -20°C.

Stability is being monitored via particle concentration by OD260nm in SDS, particle quality and concentration via an Anion Exchange HPLC Assay,[4] activity by Infectious Titer Assay (both the ARMWG procedure and a flow cytometry-based institution-specific method also incorporating the NAS calculation), particle size by photon correlation spectroscopy, particle aggregation by absorbance ratio (OD320nm to OD 260nm), and, for selected time points, a qualitative assessment of micro-aggregation via electron microscopy and assessment of sterility as a correlative for container integrity.

A short-term field use and shipping configuration study is being performed. The field use study focuses on the impact of 1,2, or 3 three freeze-thaw cycles, the impact of thaw and subsequent storage at 2-8°C over 7 days, and the impact of thaw and subsequent storage at room temperature over 7 days. Eight methods will be used to determine stability: the ARMWG procedure for OD260nm in SDS, the ARMWG procedure for infectious titer, a flow cytometry-based infectious titer assay, an Anion-Exchange HPLC assay, assessment of appearance, pH, and assessment of aggregation status via photon correlation spectroscopy and dynamic light scattering. The shipping condition stability study examines the impact on the Reference Material when packaged on dry ice using the ATCC shipping configuration with the package held for two days at 40°C plus an additional day at 50°C. Together these studies will provide users the information they need to handle the Reference Material.

Purity. A variety of purity analyses will also be performed by participating laboratories. This includes analysis of free hexon levels, analysis of 31K MW protein content,[7] analysis of residual 293 host cell protein level,[8] analysis of residual 293 host cell DNA level via quantitative real-time PCR utilizing primers and probes against the human 18S gene,[9] and analysis of viral particle aggregation via photon correlation spectroscopy and dynamic light scattering methods.

Repository. The ATCC was selected by the Working Group to be the repository for the Adenovirus Reference Material, plus related materials such as the two cell banks and the virus bank. The ATCC will distribute the Adenovirus Reference Material to the public for a nominal administrative fee, once the Working Group gives the go-ahead. The Working Group plans to provide a document summarizing the manufacture and characterization of the Adenovirus Reference Material for distribution with the material itself. The Working Group also plans to publish and present the characterization package for the Adenovirus Reference Material, so that scientists in the field are aware of this resource.

Goals for Working Group

FDA representatives have indicated that the FDA plans to require sponsors to qualify or validate their analytical methods against the Adenovirus Reference Material, so that reported

particle units and infectious units are comparable to those reported for the Reference Material. The timeframe for incorporation of the Adenovirus Reference Material-defined units into sponsor methods has not yet been announced. However, once sponsors and scientists begin using the units defined by the Adenovirus Reference Material, then the analysis of the safety and efficacy of adenoviral vectors, across the product class, will become more meaningful.

The main goal of the Working Group was to create an Adenovirus Reference Material that would allow units of measure to be standardized in the shortest possible timeframe. This effort began with public discussion of the expectations for a Reference Material in October 2000, and formal creation of the Working Group in early 2001. The Working Group expects the Reference Material to be available to the public in the spring of 2002.

Secondary goals for the Working Group were for the Adenovirus Reference Material to be something that could be used in all kinds of laboratories, and could be made in multiple lots, based on the same starting materials. The last goal increases the chances that there will be closer comparability between different batches of the Reference Material, should these need to be produced in the future.

The Working Group's goals did not, and do not, include standardization of analytical methods, or the endorsement of specific production processes. Although the Working Group determined that it was desirable to use a single method to establish unit assignments for the Reference Material, the methods were selected for their scientific merit, as well as their practical application in laboratories with limited instrumentation. The methods selected are not "special," in and of themselves, but are representative of the field.

Additionally, the definition of Reference Material units and the choice of analytical methods was aimed at achieving the practical goals stated earlier, and not the more abstract goal of determining the absolute number of intact viral particles or the absolute number of infectious units.

A final goal of the Working Group was to make the process used to produce and characterize the Adenovirus Reference Material transparent. For that reason, all information, all submitted proposals, all available data, recommendations by the FDA, plus the discussions and decisions of the Working Group are freely available to anyone via the internet, as well as via conferences notes and publications.

The Working Group believes it has established a model for the development of reference materials for viral vectors and other biological materials. The success the Working Group has had in achieving its mission has been due to a clear understanding of goals, the transparency of the process, and the highly collaborative effort that had input from all arenas in the field. This input came from regulatory authorities, standardization organizations such as USP and NIBSC, academic laboratories, industry/sponsor representatives from Quality Assurance, Quality Control, and Manufacturing, contract manufacturing and testing organizations, and suppliers, including representation from outside the U.S.

Another factor in completing the mission quickly, was the fact that nearly every company actively developing adenoviruses as therapeutic products was involved in the Working Group, or in submitting proposals to participate. These groups put aside their proprietary stances and cooperated to achieve something of benefit to the entire field. Compromise was required by participants, and was achieved because the Working Group continually asked how proposals and decisions would fit with the goals.

Questions about the Adenovirus Reference Material or the Working Group can be addressed to Stephanie Simek or Steven Bauer at CBER/FDA, to the Working Group co-chairs, or to Keith Carson at the Williamsburg BioProcessing Foundation.

Acknowledgments:

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