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Quality assessment strategy development and analytical method selection of GMP grade biological drugs for gene and cell therapy

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ABSTRACT

Biological drugs with gene and cell therapy potentials, including natural or rationally created biomacromolecules, recombinant proteins/enzymes, gene-carrying DNA/RNA fragments, oncolytic viruses, plasmid and viral vectors or other gene delivering vehicles with specific therapeutic genes and gene manipulation tools, and genetically modified and reprogrammed human cells comprise a large fraction of drug development candidates in modern precision and regeneration medicine. These drugs have displayed unique capabilities in treating patients with previously incurable diseases. However, most of the drug preparations have complex multimolecular structures and require specific biomanufacturing systems and many other additional biological active materials for drug synthesis, cell expansion, and production enhancement. Thus, the final products would have to be subjected to sequential extensive purification processes to exclude impurities and to concentrate the drug products after manufacturing. The quality evaluation for each drug product is an individualized process and must be specifically designed and performed according to the characteristics of the drug and its manufacturing and purification methods. Some of the Quality Control (QC) assays may be very costly and time-consuming, frequently with inconsistent test results from batch-to-batch. This review focuses on QC assessment strategy development for common gene and cell therapy drugs which use prokaryotic or eukaryotic cells for manufacturing or cell factories for *in vitro* expansions, especially for drug identification and concentration determination, impurity detection and quantification, drug potency, stability, and safety evaluations; and discusses some key issues for drug quality assessments in different categories and emphasizes the importance of individualized QC strategy design.

Introduction

With rapid progression in biomedical sciences, a number of discrete biological drug materials have been proportionally generated for diagnostic and therapeutic purposes yearly. Unlike synthesized or isolated small molecule drug products which have limited structure complexities and modification possibilities, and could directly interact with their target molecules, biologically active drugs are made by different biological materials and can cause multiple level changes in molecular and cellular levels after administration. Such drugs include, but are not limited to, countless peptides/proteins, synthesized functional RNA/DNA fragments, modified plasmids/cosmids/phages with inserted human genes, oncolytic viruses and gene delivering viral vectors, genetically manipulated human immune cells with target specificities, isolated or induced pluripotent stem cells (iPSCs) with committed differentiation capabilities, and *ex vivo* expanded tissues. These advancements help set up a new milestone in modern personalized precision medicine[1–12].

Except for some peptides, recombinant proteins, biomacromolecules, or short DNA/RNA molecules, which can either be synthesized *in vitro* or have a well-developed highly efficient purification/concentration process, such as affinity chromatography, vast majority of biological drugs used in gene and cell therapies need specially designed GMP grade manufacture systems for drug preparations. Depending on a drug's

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individual characteristics, a specific bioreactor (or "Cell Factory") is used with a precisely controlled biological microenvironment and numerous biological raw materials for cell growth, drug preparation, and yield enhancement. In the bioreactor, biological drug templates use specific prokaryotic or eukaryotic cells as miniature cell factories for drug synthesis and production. These living cells, supporting additives and their growth/metabolic products constitute undesired impurities and must be eliminated or reduced to an acceptable level prior to use. This does not include the enriched culture medium which provides a suitable environment for potential microbial co-growth and contaminations. Moreover, biological drug purification processes are challenging because the drug and different cellular contaminants share numerous similar physicochemical characteristics. Therefore, preparation of the final drug products sometimes requires multiple separation steps based on slight differences between the drug and impurities. Generally, the qualities of these drug products are evaluated for 1) identifiable impurities; 2) drug identification, concentration, and potency; 3) drug stability under different preservation and transportation conditions, including long-term stability; 4) biosimilars from different manufactured batches or manufacturers; and 5) safety of the drug[13,14]. These issues must be carefully assessed before releasing a drug. This review summarizes the common QC evaluation strategies for biological drugs manufactured in different bioreactor/cell factory systems and emphasizes the importance of individualized strategy development for each biological drug based on the Quality by Design (QbD) concept in different categories[15].

Critical aspects of quality assessment

Purity

The purity of the drug is the primary issue for drug quality evaluation when it is manufactured or made by bioactive cells. It can be divided into two categories: manufacture-related impurities and drug-self impurities. Most host cell impurities negatively impact drug efficacy and enhance or generate additional toxicities[16,17]. Since the manufacturing process for each biological drug is individually designed, a tailored QC assessment plan should be pre-planned based on drug characteristics, production environment and purification procedures. For prokaryotic cell-manufactured recombinant proteins and plasmid/cosmid/phage products, prokaryotic material contaminations, such as high level of endotoxins, residual bacterial proteins and DNA/RNA molecules, either released from dead cells or from lysate during drug extraction, are a serious issue and sometimes requires multiple and/or consecutive purification processes[18–20]. For eukaryotic cell-manufactured recombinant proteins, viruses and viral vehicles, and other biological macromolecules, the purification processes require more complex separation steps. These steps isolate the target drug from all the system contaminants including cell debris, liberated host cell proteins/DNA/RNA molecules and their degradation fragments, secreted components/micro-organelles, residual antibiotics, and potential pathogenic/oncogenic materials from human or non-human species. It is especially true for most intracellular recombinant proteins and some viral vectors which lack extracellular transport signals, and thus the drug products must be artificially released by breaking the manufacturing cells at the end of harvest[21–23]. A key fact that must be considered is that each purification process may potentially result in significant loss of drug products, and therefore the purification strategy needs to be carefully balanced for maximal yield of the drug. For cell-based drug products such as genetically modified Chimeric antigen receptor T (Car-T) cells and natural killer (NK or Car-NK) cells, embryonic stem cells and iPSCs, elimination of non-functional cells and aberrant differentiated cells during the expansion process is another issue that needs to be addressed and controlled[24–26].

Drug identification/quantification assays and limitations

Accurately identifying the drug and quantifying its concentration is a key step for final drug release. For non-cell based biomacromolecules and therapeutic oncolytic viruses or gene delivering viral vehicles, the commonly used methods are specific antibody based immunological assays and quantification. Since most commercially available specific antibodies or assay kits may only recognize the primary and secondary structures of the protein immunogens, the immunological quantitation frequently includes molecules without intended functions, resulting in a common drug itself impurity issue. Functional biomolecules might vary from different manufacturing batches, even if the quantification results by the same assay indicate similar or comparable results. A typical example is Adeno-associated viral vector (AAV) quantitation using different antibodies against the same capsid proteins. The protein shell of AAV contains three distinct proteins, VP1, VP2 and VP3 in a ratio of 1:1:10. All three capsid proteins are encoded by the same *cap* gene as proprotein with different cleavage sites but share the same amino acid sequence fragment. Therefore, only antibodies which recognize conformational structure after capsid assembly have relative quantitation values for particle number determinations[27–31]. Carefully verifying the complementary-determining region (CDR) of a selected antibody would significantly increase the accuracy of the assay for active biomolecules.

For DNA/RNA-based gene materials, such as plasmid vectors, UV light absorbance at OD260 and OD280 provide a quick and accurate method for sample concentration and purity. For specific gene fragment identification in a gene delivering vector, either gene specific or viral vector specific quantitative Polymerase Chain Reaction (qPCR) can be used for quantitative purposes. Since most inserted genes have a length greater than 1000 base pairs (bp) and the entire viral or vector genome length is usually $>$ 3000 bp to 30,000 bp, and the designed lengths of most qPCR amplicons are often $<$500 bp for better amplification efficiency, a single qPCR result can only confirm the existence of a specific primer targeted portion in the product. As a common phenomenon of viral replications, a lot of viral particles are not properly transcribed/translated, assembled and packed, due to the rate differences between DNA/RNA replication and protein transcription/translation. Some viral vectors might not have any genetic materials and consist of a portion of the reagent with "empty" particles[32–34]. Since protein antigen based immunological assays cannot distinguish between "empty" and "full" particles, the assay may result in a large quantitation discrepancy between ELISA and qPCR results. Moreover, single qPCR cannot distinguish incomplete packed viral particles if the vectors were under-packed (one copy of ssDNA for AAV or one ssRNA for lentivirus instead of two identical copies per particle for these viruses), over-packed (multiple copies per vector) or partially packed with incomplete inserted gene [35–37]. These issues are a consistent challenge for correct concentration determination by routine immunological and molecular biological methods.

Genetically manipulated and *in vitro* expanded Car-T cells or NK cells, either autologous or allogeneic, have been proved as an effective treatment for cancers and some otherwise incurable diseases. Obtaining enough cells with therapeutic functions for single or multiple treatment is an essential step [38–40].. Quantifying the cells with their original characteristics and therapeutic function requires a combination of several assay methods, including cytology, immunology with flow cytometry and cell functional assays. The ratio of cells with therapeutic capability should be the dominant cell population in the final product, and the aberrantly differentiated cells or the cells without appropriate markers during the expansion process should be $<$2 – 5 % of the population. Besides that, expanded cells are frequently fractionally packaged according to dose requirement, and cryopreserved. The freeze/thaw cycle will bring an unfavorable environment to the cells, and result in decreased viability up to 24 h[41,42]. The final cell number determination requires the cells to reach a steady status after the

freeze-thaw cycle.

For stem cell-based therapeutics, determination of differentiation status of the cells and ratio of the cells with therapeutic potentials in the expanded population are more important than cell number count. *In vitro* expanded iPSCs are frequently under the differentiation pressure and consistent changes to spontaneous terminal differentiation[43–45]. Even a slight change in oxygen pressure or pH in the microenvironment would alter the intracellular transcription factor expression profile and force the cells into non-directed differentiation paths. The relative expression levels and expression orders of some key pluripotent and committed differentiation biomarkers and their fluctuation during cell proliferation and differentiation have provided substantial information concerning the position of the cells in their differentiation pathway during and after expansion and also indicates their homing trends after injection[46,47]. Determination of mRNA levels of such "stem cell" markers would provide valuable parameters to determine stemness and lineage committed status of the cells. The acceptance criteria for these stem cell markers and their cross-interactions must be predefined before the expansion, and the fluctuation and ratio changes during the expansion period must be within predefined acceptable ranges.

Drug potency

Genetically engineered biomacromolecules and intracellularly assembled viral particles generated either in prokaryotic or eukaryotic cells are often not perfect with various percentages of invalid drug ingredients[48–51]. Although these ineffective components can be detected and quantified by different immunological and molecular biological methods with the same intensity as active molecules, they do not have any significant therapeutic values for the patients and makes the dosing standardization difficult from batch-to-batch. Determining the drug bioactivity or potency is an important quality indicator. Most potency determination assays require confirmation of the therapeutic effect of the drugs in cell or tissue levels. Finding a model cell line, primary cells, or excised tissue with detectable and quantitative changes after contact with a biomacromolecule drug at a therapeutic concentration is crucial for the determination of drug potency. The detectable changes can be single or multiple related parameters. Examples include morphology, growth characteristics, gene activation, cell viability, metabolic changes, new or enhanced protein/peptide transcription/translation/secretions, etc. The quantitation results are often expressed as 50 % of the cells with appropriate reactions to the drug (EC_{50}, IC_{50}, or $TCID_{50}$) or by specific biomarker quantitation in minimal drug concentrations. The cell-based potency quantitation assays are suitable for all the non-cell biological drugs where the concentration has already been determined by immunological and/or molecular biological assays. The potency results can be used to compare with other quantitation assays to determine the drug quality and dosage, and to determine the biosimilars of the drugs from batch-to-batch or different manufactures.

When lacking a suitable cell-based potency assay, for instance, some drugs might not cause detectable changes in host cells or the changes are not significant, a more complex quantitation strategy with multiple approaches must be adapted. The results from each assay will be compared and cross-analyzed for estimated potency quantitation. For example, an AAV-based viral vector may require additional assays to determine its gene delivering efficiency, such as 1) analytical ultracentrifugation (AUC) and mass photometry for the determination of percentage of under-packed, over-packed and right-packed viral particles; 2) static and dynamic light scattering (SLS and DLS) for viral particle size and homogeneity; 3) electron microscopy (EM) for determination of percentage of "empty" and "full" viral particles; 4) multiple qPCR of different genome fragments for genome integrity in a particle; and 5) infectious titer determination by using receptor positive cell and gene expression analysis with flow cytometer or RT-qPCR based assays [52–61]. Combining different test results from all assays, or a select combination thereof, and other ex *vivo* experimental results, a tentative conclusion of effective titer can be derived for clinical uses. Similar strategies can also be developed for other biological drugs while a suitable potency assay cannot be as easily developed.

For cell-based drugs, such as Car-T and NK cells, the cell activation and proliferation index can be determined by providing a suitable *ex vivo* simulation environment. The drug potency is evaluated as 1) stable activation potentials of the cells (maintaining a stable expression of incorporated genetic materials, such as chimeric ScFv in expanded progenies); 2) proliferation potential after antigen stimulation; 3) cytokine release profile including cytokine release pattern, concentrations and their ratios; 4) non-specific activation by non-target cells and lymphocyte transformation factors for target specificity, and 5) target-bearing immunodeficiency murine models. The therapeutic dose determination and potency of the reagents for all the cell-based drugs ultimately rely on *in vivo* testing in humans by starting at a low dose and gradually increasing to the maximum tolerated dose[62–71].

Stability

As biological products, biomacromolecules, viruses and viral particles and *in vitro* manipulated cells, are extremely sensitive to harvest and purification methods, and storage and transportation conditions. Even a slight change in pH, temperature, and/or salt concentration will result in significant changes in drug potency[72–75]. Therefore, all biological drug products after purification and dose packaging should be subjected to instant and periodical stability evaluation which includes drug appearance, pH, osmolarity, effective drug concentration and potency. It is especially important for cell-based drug products, because dead cells will release numerous intracellular compounds into the storage medium and result in cell aggregation or precipitation. As a result, these biological drugs must be dissolved and stored in an "optimal" formulation buffer in appropriate storage conditions for direct use in humans. The medium or buffer of the drug must be equal to the physiological conditions of human blood, such as acceptable pH, physiological osmotic pressure, salt composition, and free of any kind of unacceptable organic and inorganic chemicals. Spontaneous aggregation and precipitation of the drugs during low temperature storage and transportation must be detected prior to drug administration and prevented by changing the reagent conditions. Some biological drugs may need a trace amount of human albumin or other proteins as a stabilizer or carrier. Their concentration, purity, interaction potentials with the drugs, and antigenicity must be predefined and limited within acceptable ranges.

Drug safety

A major concern for all biological drugs is the safety of humans. Unlike structure and target defined small molecules where safety and median lethal dose (LD_{50}) can be carefully studied in small animal models prior to use, proteins, viruses, gene delivery vectors and cell based biological drugs must be tested in humans for potential therapeutic effects and safety. The animal testing data is frequently not enough or incomparable for human safety evaluation, especially for cell based therapeutic reagents. The major safety concerns include: 1) contamination of microorganisms and their toxic products, such as endotoxin, during manufacturing. 2) Antigenicity of the drugs; individual biomacromolecules, viral vehicles, and cell-based drugs would present countless antigen determinants to human recipients, especially from allogeneic sources or other species[76–78]. All potential immune reactions, such as native immune reactions with preformed nature antibody and complement, humoral immune reaction with specific neutralization antibody induction and cellular immunity development with potential host cell/tissue/organ damages, Cytokine storm or Massive cytolytic syndrome, must be considered and predefined for insuring immediate and long-term drug effectivities and host safety. 3) Foreign gene incorporation or recombination resulting in unexpected

gene expression in the human body, especially for DNA/RNA/viral vector-based drugs. These reagents may carry the gene fragments from different viruses or manufacturing cells, such as transcription activators or antibiotics resistant genes to the host and generate unexpected non-therapeutic effects[79]. 4) Recombinant replication competent adventitious viruses, host cell oncogenes and aberrant signal transduction gene expressions. *In vitro* culture for cell-based therapeutic reagents may result in latent oncogene activation and tumorigenesis transformation of the cells and lead to tumor-formation after administration to the patients[80,81]. The tumorigenesis potential of the viral vectors and therapeutic cells must be identified and prevented by selected assay methods before clinical use, especially for stem cell reagents. The reagents with high possibilities for oncogene activation, expressions and tumorigenesis potentials should also be assessed in severe combined immunodeficiency murine models (*nude* or SCID mice) [82].

Quality control strategy development for biological drugs manufactured or made by living cells

General compendial tests for drug quality evaluation

All the biological drugs must meet the pharmacopeial standards, set by US Pharmacopeia (USP), European Pharmacopoeia (Ph. Eur.), and World Health Organization (WHO); and must satisfy anticipated regulatory concerns by using validated USP/FDA recommended analytic methods. The Table 1 summarizes generally used analytical assays for different biological drug categories. While some of the drug categories, such as *in vitro* synthesized DNA, RNA molecules and delivering lipid nanoparticles might require specific analytical methods, most drugs prepared by living cells or cellular reagents can be evaluated by some common assessment methods. Overall, the drug appearance should meet required color and clarity standards set for each individual biological drug, without any visible particles and precipitates. The pH of the drug materials must be within the physiological range (around 6 – 8) and the osmolarity of the reagents (salt + carrier protein + drug materials) should be within 280 to 330 mOms/L for cellular reagents. All the additives during manufacture and purification procedures, such as DNase, RNase, detergents, different inhibitors etc., must be removed completely or to a minimal acceptance range. All drug reagents manufactured under sterile conditions should be either free of bacterial endotoxins or within acceptance. For drug products prepared in *E coli*, special attention should be focused on endotoxin elimination. With repeatedly used bioreactor, *E coli* and other gram-negative bacterial contamination is also a frequent problem, because gram-negative bacteria tend to form a single cell layer, called biofilm, inside of the duct surface and liquid flow system and result in trace or significant amount of endotoxin contamination[83–85]. The minimum detection nadir of most currently used methods is around 0.01 to 0.05 EU/ml. Serial dilution of the sample will correctly identify the endotoxin concentration and determine the drug's acceptance or rejection.

Another *sine qua non* test for all the biological drugs is the sterile test. Due to special features of biological manufacturing, microbial contamination is a frequently occurring problem, especially with extended culture period and multiple intervention of the culture. On the other hand, some biological drugs are also manufactured in bacterial cell factories, which can result in a large amount of bacterial genetic materials in drug products. Even with stringent or multiple purification processes, these microbial residuals are still detectable by sensitive molecular techniques[86–88]. The purpose of sterile testing is to confirm the reagent free of live microbial contamination and to ensure the sterility of the drugs, even trace amounts of genetic materials of microbes can be detected in the reagents. The classical sterile test is to culture the drug products in selected liquid medium at 30–35 °C and 18 – 24 °C incubators, respectively for at least 14 days to eliminate possibility of any residual live bacteria and fungi in the products. In some special situations, such as to eliminate potential inhibitory effect of bactericides/fungicides in the products or for microbial identifications, a membrane filtration method or directly streaking the samples onto different identification agar plates can be adapted accordingly. For contamination source tracking purposes, the bacterium and fungus in positive samples must be further identified by different microbial classification techniques. Some recently developed sterility tests, such as early detection of microbial proliferation, PCR, and immunological methods, are not acceptable for defining "sterile", because the detection limit of each assay cannot meet sterile standards[89,90]. In addition, mycoplasma contamination must be excluded for drugs generated in mammalian cells and cell-based reagents before and after drug preparations.

Optical characteristic of the samples is another important parameter for protein, DNA/RNA based drug products. Optical absorption of UV light at wavelengths of A260 and A280 and their ratio represents protein, DNA, RNA concentration respectively and drug purity[91]. Using SLS and DLS technology, the size and homogeneity of different nanoparticles[92] can be determined, and the drug purity can also be evaluated according to size peak distributions and relevant optical parameters. It is especially useful for protein wrapped viruses and viral vectors, such as AAV and AV vectors.

Plasmid based drug products manufactured in prokaryotic cells

Plasmid vectors with genetically manipulated sequences and inserted materials have broad uses for human medicine. The vectors can be used *in vitro* either by transforming/transfecting target cells to generate useful medical materials such as antibodies, cytokines, biomaterials, and oncolytic viruses/viral vectors, or by modifying the target cells for gene editing purposes[93–96]. The vector can also be directly used *in vivo* for disease treatment and vaccination. Since most plasmid vectors were expanded in *E coli*, host cell material contamination is a major issue. In addition, the integrity of plasmid vectors and sequence correctness should be addressed appropriately as important quality indexes.

Due to the fact that a significant amount of residual host cell (*E coli* or other prokaryotic cells) proteins in a plasmid reagent would interrupt the transfection efficiency of the drugs and cause unexpected side effects *in vivo*, elimination of residual host cell proteins is a necessary step for plasmid drug purification. Bacterial proteins can be digested with powerful serine proteinase or other specific proteinase to ensure the products are free of any residual antigenic and functional proteins. Routinely used quantitation methods for residual *E coli* proteins and exogenous proteinase are ELISA based assay systems with different polyclonal and monoclonal antibodies against highly natural conserved bacterial structure proteins or total lysate proteins/specific proteinase. An effective digestion and purification process will result in a great reduction of residual host protein to minimal acceptable levels. The characteristics of residual host cell (*E coli*) proteins can be further determined by resolving the sample in SDS-PAGE, followed by silver staining or W. Blot and/or analytical mass spectrometry[97–98].

Co-transfection of eukaryotic cells with residual host cell (*E coli*) DNA and RNA would result in unexpected transcription and translation of *E coli* proteins, which will greatly interrupt the cell function and transfection efficiency, even causing the death of the transfected cells. Eliminating residual host cell DNA from a plasmid reagent is hard because they share similarities with plasmid DNA and may require multiple purification steps. A commonly used residual host DNA quantitation method is qPCR-based host cell DNA amplification. The method utilizes a highly conserved *E coli* sequence for 23S and 16S RNA ribosome[99,100]. Some primer pairs are also non-specific for *E coli* only and can be used for other microbial contamination identification. The quantitation results with different primer pairs which target the same gene in different fragment regions might vary slightly depending on multiple factors that determine the PCR sensitivity and specificity.

While residual host cell (*E coli*) RNA can be easily eliminated by

Table 1
Quality Assessment of GMP Grade Biological Drugs for Gene and Cell Therapies

QC Assessment Categories		Therapeutic RNA & DNA molecules & non-viral delivering vehicles	mRNA based drug products	Plasmid vectors with DNA inserts, produced in prokaryotic cells	Oncolytic viruses	DNA/RNA viral vector-based gene delivering & editing reagents	Therapeutic cell reagents, including *in vitro* edited cell (Car-T & NK cells)	Stem cells from different sources & iPSCs
Identity	**Special Analytical Assays**	qPCR, RT-PCR, ddPCR for specific gene fragments & fragment analysis	RT-PCR for specific gene fragments, & fragment analysis	qPCR & ddPCR	Virus identification by immunological & molecular biological assays, EM	Immunological assay for specific vector proteins. Molecular biological assay for specific gene fragments, EM	Cell type & homogeneity, specific markers & marker quantification	Stemness & differentiation markers, cell morphology
	Structure & Composition Accuracy	Sequence accuracy, including vector integrity	Sequence accuracy[166], size & integrity confirmation, LNP homogeneity	Sequence accuracy for both vector & insert, fragment analysis by restriction enzymes	Whole genome sequence or signature gene sequences, viral particle integrity analysis	Sequence accuracy for entire vector/ insert(s)	% cells with specific markers (Flow Cytometer analysis)	Intracellular mRNA profiles (gene expression profile)
	Specific characteristics of the drug, including biosimilar	Functional gene integrity	5′ capping efficiency. 3′poly(A) tail length	% of supercoiled plasmid DNA	Receptor specificity & target cell specificity	Specific receptor affinity & tropism for specific cell populations	% cells without designed gene expression	whole genome sequencing & genotyping (STR & Karyotype stability)
Concentration & Drug Preparation	**Quantitation Methods**	UV absorbance-based quantitation, qPCR, RT-PCR & HPLC	UV absorbance, A260/A280 ratio, RT-PCR, CE & HPLC	UV absorbance, A260/A280 ratio, vector & gene specific qPCR, ddPCR	qPCR, ddPCR, ELISA, DLS & HPLC determined copies/particles	qPCR, ddPCR, ELISA, DLS & HPLC determined particle numbers	Viable cell count, cell surface markers for homogeneity & cell stages	Cell count & homogeneity, cell surface & intracellular markers for homogeneity
	Standardized Concentration Units	mg or μg/mL	mg or μg/mL	gene copies/mL or mg/mL (for pure DNA samples)	viral particles/ mL	particles #/mL or gene copies/ mL	cells/mL (plus % therapeutic effective cells)	cells/mL (plus % of stem cells with specific markers)
	Drug Preparations	General reagent tests①	General reagent tests①	*General reagent tests⑦*	General reagent tests①	General reagent tests①	General reagent tests①	General reagent tests①
Potency or Bioactivity	**Drug Activities**	Gene expression efficiency by *in vitro* transcription/ translation & proper function assays	*in vitro* protein translation, expression efficiency & integrity, intracellular protein expression	*in vitro* inserted gene expression & efficiency	Cytotoxicity for different cancer cell lines & specificities	*In vitro* infection of specific target cells or infectivity spectrum of human/ mammalian cells/tissues	Cell viability, % effective cells, *in vitro* & *in vivo* cell functions	Cell viability & stemness
	Analytical Methods	*in vitro* & intracellular protein expression efficiency & quantitation	*ex vivo* transfection & protein expression by immunological assays	*in vitro* transcription & translation, intracellular expression & function assays	Plaque assay with specific target cells. Tumor-bearing murine models (nude & SCID mice)	*in vitro* infectivity assays with susceptible cells	*in vitro* proliferation & activation assay with or without stimulations	*in vitro* proliferation/ differentiation capability, stem stability, growth characteristics in 3D culture
	Target Reactions	Dose determined gene expression in susceptible cells, target-based functional assays	Functional assays after expression in selected cells/ tissues	Transformation efficiency. Gene product quantitation by ELISA or immunochemistry staining	Cell viability④. Define the IC_{50}, $TCID_{50}$	Gene expression quantitation by flow cytometer & RT-qPCR, transfection efficiency (MOI)	Cell activation & cytokine release profile, target killing & elimination	Stem cell related transcription factor expression & mRNA profile, ratio analysis of stem cell factors
Impurity	**Manufacture/ Processing Impurity②**	Impurity from *in vitro* synthesis, assembling, gene carrying vector, & manufactures	Residual RNA template, RNA aggregation & fragmentation, residual RNA polymerase	All the manufacture impurities②	All the manufacture impurities②	All the manufacture impurities②	Dead cells & cell debris, terminal differentiated & aberrant cells	Dead cells & cell debris
	Residual Additives	Additives during synthesis, purification & product enrichment processes	Residual solvent, free nucleotides	All the additives during drug preparations③	All the additives during drug preparations③	All the additives during drug preparations③	Residual Car-T constructs, transfection reagents, residual cytokines or other additives	Residual reprograming reagents. Residual cytokines or cell

(*continued on next page*)

Table 1 (*continued*)

QC Assessment Categories		Therapeutic RNA & DNA molecules & non-viral delivering vehicles	mRNA based drug products	Plasmid vectors with DNA inserts, produced in prokaryotic cells	Oncolytic viruses	DNA/RNA viral vector-based gene delivering & editing reagents	Therapeutic cell reagents, including *in vitro* edited cell (Car-T & NK cells)	Stem cells from different sources & iPSCs
								proliferation enhancers
	Drug Impurity (defect or non-functional drugs)	Ratio of defect molecule, unexpected loops, spontaneous aggregation & precipitation	RNA fragments, loops, RNA with mismatched nucleotides or without a cap or short poly (A) tail	Linearized or nicked plasmid, gene mutations & absence, fragmentation	% of non-infectious viruses (by comparing the infectious titer with protein & DNA/RNA quantitation)	% of defective viral particles, unassembled viral protein, DNA/RNA fragments	% of cells without appropriate functions & cell under spontaneous apoptosis	% of terminal differentiated cells & aberrant cells
Unexpected microbial contaminations and Drug Safety	**Human Hazards**	Microbial contamination, manufacturing impurities, & harmful gene products	Microbial contamination, manufacture impurities. Unexpected immune reactions	Sterility, incomplete endotoxin elimination, harmful gene recombination	Non-specific cytotoxicity, massive tumor lysis, persistency of viremia & viral recombination	Non-specific infectivity, viremia, virus recombination, abnormal immune reactions	Microbial & endotoxin contamination, cytokine over release, rejection, tumorigenesis	High % of cell without functions, cell aggregation, tumorigenesis, & microbial contamination
	***in vitro* Test**	Bioburden, endotoxin, & toxicology study of gene products	Bioburden, endotoxin. *in vitro* cell proliferation & cytotoxicity study, pre-existed immune reactions	Bioburden & endotoxin, manufacture impurities②, gene recombination	Bioburden & endotoxin, manufacture impurities②, viremia, immune reactions	Bioburden & endotoxin, manufacture impurities②, viremia, abnormal immune reactions	Sterility, mycoplasma & endotoxin detection, cytokine caused damages, HLA mismatch	Sterility, mycoplasma & endotoxin, oncogene activation, cell aggregation, HLA mismatch
	***In vivo* Test**	Acute & chronic toxicity tests in mammalian animals	LD_{50} & protein expression & host immune reactions in murine models	Maximal tolerance doses in laboratory mice.	Safety study in tumor bearing SCID mice, dose incremental study in clinical trial patients	Safety study in laboratory animals.	Safety study with target cell bearing SCID mice, dose incremental study in clinical trial patients	Tumorigenesis in SCID mice, animal model for stem cell homing & host immune reactions
Stability	**Short Term (0 - 72 hrs.)**	Drug concentration, integrity, potency & degradation	mRNA stability, spontaneous RNA aggregation & precipitation	Drug concentration, integrity, potency & degradation	Potency consistency, particle stability, spontaneous aggregation & precipitations	Potency consistency, particle stability, spontaneous aggregation & precipitations	Cell viability & potency consistency, cell aggregation, pH & osmolality changes	Cell viability & stemness consistency, cell aggregation, pH & osmolality changes
	Long Term (minimal 6 mo. to 1 yr.)	Drug concentration, integrity, potency & degradation. Reagent pH, osmolality & aggregates	RNA stability, stability for appearance, pH, osmolality, visible or micro aggregation	Concentration, integrity, potency & degradation, appearance, pH, osmolality & aggregations	Potency, stability, aggregation & precipitations, pH & osmolality consistency	Potency, stability, aggregation & precipitations, pH & osmolality consistency	Cell viability & potency, cells aggregation, pH & osmolality consistency	Cell viability & stemness, cells aggregation, pH & osmolality consistency

① General tests for biological reagents, including appearance (visible particles, aggregates & precipitations), specific salt concentrations, conductivity, pH, osmolality, reagent stabilizers

② Manufacturing impurities include impurities from accessory gene fragments of gene-carrying vectors, residual host cell proteins, DNA, RNA fragments, cell debris from dead or artificially lysed cells

③ All the additives during drug preparations, such as animal proteins, antibiotics, enzymes, anti-foaming reagents, eluted components from purification process, non-physiological inorganic & organic components.

④ Cell viability assays include morphology observation, dye exclusion, metabolic disruption, intracellular component release & death-related signals.

specific RNase digestion, the absence of host cell RNA needs to be confirmed for drug release. Both residual host cell DNA and RNA can be visualized by agarose gel electrophoresis analysis, SDS-PAGE, or capillary electrophoresis (CE)[101]. Identified extra host DNA and RNA bands can be further semi-quantified according to their relative densities to the dominant plasmid. Ideally, residual DNA should be <10 ng/ml, and the RNA concentration should be <5 % of total plasmid DNA concentration for *in vitro* uses and <1 % for *in vivo* injection. The minimal acceptable concentration of non-plasmid DNA and RNA is, however, still controversial, especially for *in vivo* uses. It must be determined for each individual drug according to its characteristics and usefulness. To eliminate any unexpected side effects of the reagent, the residual host cell DNA and RNA must be fragmented, and the length of the fragments must be <200 bp.

Plasmid quality plays important roles in its *in vitro* transformation and transfection efficiency, and *in vivo* efficacy. During manufacturing, some plasmid vectors suffer from bacterial endonuclease digestion and being linearized or nicked. Usually, only the supercoiled plasmid vectors can be successfully transfected into the cells and further transcribed and translated[102–104]. The linearized or nicked plasmid DNA will be rapidly destroyed before reaching the cell transcription machinery. The ratio of open linearized and nicked plasmid DNA to supercoiled plasmid DNA determines the plasmid quality and effective doses. Commonly used methods include analytical High Performance Liquid

Chromatography (HPLC), CE, agarose gel or SDS-PAGE analysis according to the differences of each isotype in surface charge, molecular sizes, and shapes. The gel analysis results can further be semi-quantified by densitometry analysis. These analytical methods require pre-optimization with high quality calibration samples to ensure the accuracy of the results. As general acceptance criteria, supercoiled plasmid DNA should be greater than 95 % of the total sample DNA for *in vitro* transfection and > 99 % for *in vivo* use. For long-term stored samples, the spontaneous aggregation and degradation of plasmid vectors should be monitored periodically and right before use, especially for the samples stored at an out of optimal condition with low temperature, low salt, or non-physiological pH.

The concentration and quality of a plasmid-based drug product is generally determined by 1) UV absorption for specific wavelengths at A230, A260, A280 and their ratios to each other. 2) ratio of supercoiled plasmid to total DNA concentration, and 3) its transformation and transfection efficiencies with different target cells (EC_{50} titer or dilution) [105,106]. The DNA sequence of the insertions in plasmid vectors should be analyzed prior to release to ensure the insertion has shown correct sequence and location. Entire plasmid DNA sequence should also be checked to ensure the plasmid backbone and appropriate transcription machinery were well preserved during manufacture.

Viral vector-based gene therapeutic drug products

Many pathogenic and non-pathogenic human viruses can be reconstructed to oncolytic viruses or gene delivery vectors. Through changing viral surface ligand affinity or protein composition for specific cell surface receptor, the tropism of a viral reagent can be unique or general and can target on either dividing or nondividing cells or both for transient or permanent gene expression and editing, according to the therapeutic purposes[107–109]. Viral vectors have been proved to be irreplaceable gene delivering vehicles for some specific human diseases. Typically, the viral vectors can be grouped into two categories for QC assay selections. One is viral protein enwrapped DNA/RNA viruses, such as AAV and AV. Another is lipid bilayer membrane wrapped, viral protein capsid DNA/RNA virus, such as lentiviral/retroviral vectors, RSV vectors, and herpes simplex viral (HSV) vectors. All the viral vectors are manufactured in different genetically defined eukaryotic cell lines, such as insect cells, mammalian cells and non-human primate or human cells, through co-transfections of multiple plasmid vectors[110,111].

The percentage of defective viruses in final viral products varies frequently from different manufacturing batches, depending on cell viability, microenvironment, nutrition exhaustion rate, waste accumulations, and many other uncontrollable factors. Even if the ratio of viral genetic materials and viral proteins is proportional by multiple quantitation methods (qPCR, ELISA, UV absorption in specific wavelengths), it does not mean the therapeutic dose decision can be made based on each individual assay or their combination[112–114]. It is especially true for lentiviral/retroviral and other RNA based viral vectors, because of the high replication errors in a reversed genetic information flow[115,116]. Either a reliable potency evaluation or more complex combination assays which reveal different therapeutic efficiency characteristics (*e.g.* mRNA synthesis of the inserts and/or protein expression quantification in susceptible cells) are needed for the determination of therapeutic doses.

The viral vector genome quantitation method by qPCR or RT-qPCR requires a highly purified sample as standard to generate a reliable calibration curve to quantify the unknowns. Since all PCR primer pairs have only amplified a fragment of the target, the quantitation results for recombinant viruses and viral vectors based on single qPCR and RT-qPCR technology is often overestimated and inconsistent with the potency assay results[117,118]. Digital PCR or droplet digital PCR allocates the samples to less than one copy per reaction chamber or oil droplet and counts the numbers of chambers/droplets with PCR amplicons. With optimal serial dilutions, the percentage of positive droplets can be proportionally determined from a massive count (> 20,000 counts/sample), and the copy numbers of a target fragment in an unknown sample can be directly obtained without the need of a calibrator[119–121]. With further instrument improvement, like multicolor flow cytometry technology, multiple fluoresceins with different wavelengths of emission light can be detected at the same time in a single chamber/droplet. It becomes practicable to use multiple primer pairs labeled with different fluorescein dyes, simultaneously detect different portions of a single gene. Using this gene walking strategy, the integrity of recombinant vectors can be ascertained. It will further improve the accuracy of vector quantitation.

Since most viral vectors and some other biological reagents are globular-shaped nanoparticles with a homogenous diameter around 20 – 200 nanometers, they would display a unique SLS and DLS pattern when light beam wavelength is much greater than the nanoparticle diameters. Based on SLS and DLS intensity and associated optical signature parameters of the particles, the relative concentration of the particles can be calculated using synthetic nanoparticles with the same diameter as a calibration[122,123]. A newly developed instrument, Stunner, combines UV and visible optical spectrum scanning and SLS/DLS measurement together, and has been used for detections and quantification of different protein enwrapped DNA or RNA viral vectors. By cross analysis of all the optical information from a viral vector product, the reagent purity, particle numbers, and other unique characteristics including empty and full ratio, particle aggregations, and small macromolecule contaminations, are correctly determined. By comparing with other methods, such as AUC, HPLC elution, and electron microscopy (EM) for Empty and Full determination, the Stunner provides a rapid, more accurate, and repeatable quantitation method for purified AAV and AV drug products. For membrane wrapped viral vectors, such as lentiviral and retroviral vector, SLS and DLS has also provided useful information for reagent purity, particle size, homogeneity and concentrations, although UV and visible light scanning has little value for particle number estimation due to unproportional light absorption by the lipid bilayer. The Stunner analysis of the samples also provides useful information for residual chemicals (iodixanol) or non-drug particles (extra light scattering peaks with particle diameters less or greater than vector sizes)[94,124,125].

Residual host cell protein/DNA/RNA contamination is another major issue that needs to be addressed before drug release. When viral vectors are manufactured with mammalian or other eukaryotic cells, a number of different contaminants could be found in final drug products, which include 1) secreted components/exosomes and intracellular organelles from the host cells; 2) released or exfoliated host proteins by rapid rotation or from dead cells; 3) host cell DNA and RNA molecules with functional gene fragments; 4) infectious viral recombination during co-transfection or adventitious mammalian viruses; 5) residual accessory gene fragment of plasmid vectors with transcription efficiency, and 6) additional colloidal proteins, stimulation factors, antibiotics, chemicals and cell debris. Identification and quantitation of these unwanted contamination materials based on manufacturing processes is an important step for drug quality assessment – Identification and quantification of Critical Quality Attributes (CQAs)[126–130].

An essential quality assurance test is to accurately identify and quantify the residual host and the plasmid DNA contamination with potential transcriptable gene fragments, especially for potential oncogene activities. Using commercial qPCR kits, both SV40 and E1A from plasmid vectors can be well detected and quantified. The residual host cell DNA contamination can be identified by qPCR which targets naturally conserved abundant SINEs (Alu elements), LINE-1, or gene for ribosomal RNA sequences of host genome. Because most functional genes have a sequence length greater than 200 bp, a qPCR positive result with an amplicon greater than 200 to 500 bp indicates the contamination of potential functional host genes. Therefore, the primer designs for specific DNA sequence fragments should include different lengths of the amplicons, from < 200 bp to >500 bp; and perform the assay separately

or simultaneously. When all the qPCR results for amplicons greater than 200 bp have shown negative results, the reagent can be determined as free of harmful host DNA fragments, even if the result for the same sequence with a fragment length of <200 bp is positive[131–133].

The infectivity (potency or efficacy) of oncolytic viruses and viral vectors can be determined and quantified by infection of susceptible cells *in vitro* with a serial dilution strategy. Target gene expression can be quantified either by virus induced cytopathic effects (CPE) or by comparing target gene expression (mRNA level) with a selected housekeeping gene, ie. relative RT-qPCR. The latter identifies the gene expression efficiency after vector transduction and eliminates the observer's bias[134–137].

Cell based therapeutic reagents

With a breakthrough success in bone marrow transplantation for different hematological diseases, cell therapy has become an advanced treatment for many human diseases lately. The newly developed Car-T and NK cell therapy brings new hope for cancer eradications and therapeutic benefits for patients with severe autoimmune diseases. With rapid progress in stem cell biology, different human terminally differentiated cells can be reversed or induced to pluripotent stem cells. The iPSCs have the capabilities to repair tissue damage and age-related tissue and organ degenerations through committed differentiations. All the cell-based reagents require intensive *in vitro* expansion to obtain enough cells for single or multiple injections. During expansion, numerous nutritional factors, cytokines, chemicals, and human proteins are added to enhance cell proliferation and to maintain a fixed undifferentiated or committed early differentiated status[138–143]. With exhaustion of the nutrition and waste accumulation in the culture medium and mitosis drifting, the cells might aberrantly differentiate and lose their original designed functions. Thus, the *in vitro* cultured cell products require frequent and intensive quality evaluations to confirm the drug maintains desired status and functions during the expansion.

1. Cell concentration, sterility, and stability

A basic requirement for cell therapy is providing accurate cell numbers with specific markers for dosage determination. Since most treatments require cell numbers greater than 10^7 cells per dose, and in some cases, especially for allografts, the cells are cryopreserved in small aliquots with relatively low cell numbers/mL, the cell samples often need to be pooled or *in vitro* expanded shortly for injection. Increased manipulation processes would raise the possibility for unexpected cell death and potential contamination by different microbes. The tests to confirm the accurate cell concentrations with appropriate cell characteristics plus rapid sterility/mycoplasma and endotoxin tests must be performed prior to the drug administration to the patients. The viable cells with specific anticipated markers in the cell suspension must be greater than 95 – 98 % to avoid potential side effects caused by dead cells. The cell stability also needs to be assessed as delayed injection can occur in some uncontrollable situations. The difference between initial cell counts after collection and the 24th hour count during the 2–8 °C storage should be <2–5 %[144,145].

2. Materials and reagents for cell drug formulation and storage

All the materials, medium, buffer, and anti-frozen reagents used for cell cryopreservation and expansion for final drug products should be pre-evaluated for proper salt and colloid composition, pH and osmolarity to make certain they meet normal human physiological conditions and are free of any microbial genetic materials and endotoxin. A rapid sterility test would be suitable to replace a time-consuming culture based sterile test for immediate injection, but standard culture based sterile test must be performed prior to long term drug storage. It is especially important that the Car-T cell reagents must be free of microbial material and endotoxin, because endotoxin caused toxic reactions are difficult to distinguish from cytokine storms caused by cytotoxic T cells[146–148].

3. Cell characterization

Cell characterization is required for all expanded, artificially manipulated cells and stem cells before clinical use, including their genotyping and phenotyping. For Car-T cells, the positive cells with specific surface ScFv expression and its intracellular stem should be dominant in the cell preparations by flow cytometer. The cells must present desired cytotoxic T cell characteristics, activate by specific antigen challenging, and release appropriate cytokines[149–151].

For stem cell-based reagents, the stemness and committed differentiation stage of the cells can be defined through different stem cell factor expressions[152–155]. More than a hundred different transcription factors and cell surface molecules have been linked to stem cell self-renewing and committed differentiation. Different combinations of transcription factor expressions indicate the stemness status of the cells and potential lineage differentiation. The relative expression of selected transcription factors compared to uniformed housekeeping gene expression predict cell positions in their differentiation pathway. For example, a cell that expresses SOX2, OCT4, and Nanog at same time indicates the cells still maintain their pluripotent stem status, but the ratio changes of these transcription factors along with other transcription factor expressions would indicate the cells starting a committed lineage differentiation. Carefully selecting specific transcription factor combinations and relevant intracellular/surface protein expressions will help to predict the destination of the cells and their therapeutic effects [156,157].

4. Tumorigenesis

Ex vivo expansion of iPSCs and other tissue-specific blast cells might have enlightened cellular oncogene activation during *in vitro* manipulation and extended culture and obtained tumorigenesis potentials after introduction into the human body[158]. It is especially true for patients with potent immunodepletion therapies. Evaluating the cell reagents for their tumorigenesis competency is an indispensable task, especially for stem cell based cellular drugs. Karyotypic stability, whole genomic sequencing and gene expression profiles of the cells (by mRNA expression profile) is the first step to define potential oncogenesis. With an *in vitro* tumor facilitating environment, such as 3D culture for tumor formation, the tumorigenesis of the cellular products can be evaluated [159]. The final exclusion of tumorigenesis potential of the cells depends on direct subcutaneous inoculation of the reagents with matrix gel onto *nude* or *SCID* mice[82,160].

5. Other human pathogens and histocompatibility

As a basic safety issue, some well-known pathogens inside human cells should be prescreened and excluded for allogeneic cell applications, such as HIV, HBV, HCV, HPV, retrotransposons with transcription potential and other adventitious Viruses. These human pathogens can either insert their genomic material into the host genome or serve as circular extranuclear DNA fragments and pass their genetic information to the progeny cells. It is especially important for iPSCs related implantation therapies[161,162]. For allogeneic cell therapy, pre-existing immune reactive antibodies and cytotoxic cells of the host must be prescreened prior to administration[163,164]. For this purpose, HLA typing of the cell products should be predefined, including potential development of graft-versus-host disease (GVHD) by a reconstituted immune system[165].

Conclusions

Due to the structure and composition complexities and specific manufacture and purification processes, the quality assessment plan and analytical methods for different biological drugs should be individually designed and rationally selected. The key factors for a drug's quality assessment include: 1) molecular structure, composition, and intracellular modification, if the drug is manufactured by the bioreactor with different cells. This includes all the peptides/proteins, DNA and RNA gene fragments, and other non-cell products. 2) The intended biological activities and their interaction targets in the levels of molecular, cellular and specific tissues, and anticipated therapeutic outcomes in humans plus potential side effects and toxicities. 3) The percentages of defective or non-perfect drug products in each manufacturing process; 4) the stability of the products during purification and long-term storage, and 5) available analytical methods for each quality concerned categories and assay limitations in exploring factual quality values of the products. For therapeutic plasmid and viral vectors, the correct backbone sequences with inserts must be defined and confirmed by sequencing analysis. Oncolytic viruses and different viral vectors need to display well assembled specific viral proteins with correct ratio, and the viral ligand for specific receptor molecules must be present on the surface of the Viruses. For genetically modified specific human cells and stem cells with committed differentiation, cell type, homogeneity, morphology with specific genotyping and phenotyping, and specific cell markers should be well defined by specific tests.

Biological drug manufacturing and purification processes play important roles in drug quality. The cell factories and all the additives during the processes must be predefined for the selection of final impurity identification/quantification methods. The targeted impurities need to be effectively eliminated to an acceptable level in final drug products, according to international standards. Therefore, all selected QC assays for individual drug products must be qualified and validated periodically to ensure the accuracy and consistency of the test results. The safety of the drugs, such as endotoxin and microbial contamination, relevant side effects and toxicities of the drugs, and pre-existing or induced host immune reactions after administration, should be identified by *in vitro* and *in vivo* tests. A set of quality evaluation tests for drug identity, concentration and potency should be organized for the evaluation of batch-to-batch or lot-to-lot consistency and biosimilars.

While some biological drug products might require several different analytical methods for a single quality aspect evaluation, the test results sometimes present conflicting data from each other. The final quality assessment depends on carefully weighing the *pros and cons* of each test method and cross analyzing all the data together to draw a correct conclusion. Reagent quality and instrument precision also contribute to obtaining reliable data. They must be regularly validated and calibrated with standard samples. With rapid progress in biological sciences, all the established QC analytical methods are subjected to periodical review for further improvement. Additionally, new test methods and instruments are constantly developed for precision analysis of the samples and elimination of potential assay limitations. These new assay methods and instruments should be evaluated and adapted to QC analytical test lists once they are confirmed and validated.

CRediT authorship contribution statement

Quan-en Yang: Writing – review & editing, Writing – original draft. **Nicole Lee:** Writing – review & editing, Writing – original draft. **Nicole Johnson:** Writing – review & editing, Writing – original draft. **Jennifer Hong:** Writing – review & editing, Writing – original draft. **Jenny (Qinghua) Zhao:** Writing – review & editing, Writing – original draft. **Xiulian Sun:** Writing – review & editing, Writing – original draft. **Jian Zhang:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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