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Subunit vaccines: Analytical techniques for characterization, release and stability testing

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Introduction

Since the development of the first vaccines that comprised inactive or attenuated pathogens, there has been a drive to continue to produce safer vaccine formulations that are more effective, cause fewer side effects and are safer overall.

This has resulted in the development of a wide range of vaccine modalities, including viral vector-based component vaccines and DNA/RNA vaccines (Figure 1). One consequence of this has been the development of subunit vaccines, which are more chemically defined and have an enhanced safety profile. Subunit vaccines are vaccines that contain only the antigenic component(s) of a pathogen.

Essentially there are three types of subunit vaccines:

- Protein-based antigen vaccines
- Polysaccharide vaccines
- Conjugate vaccines

Protein-based subunit vaccines comprise an antigenic protein from a pathogen, usually an inactivated toxin (toxoid) or a virulence factor. Generally, these antigens are manufactured as recombinant proteins. With current protein engineering techniques, it is also possible to produce mosaic antigens, which are recombinant proteins containing two or more antigenic components. A modified version of the protein subunit vaccine uses synthetic peptides as the antigens; these peptides give rise to one or more protective epitopes.

As several pathogenic bacteria express a specific polysaccharide capsule as a virulence factor, polysaccharide vaccines use this feature and comprise the capsule polysaccharide alone. Since these capsule structures are complex, manufacturing such vaccines usually involves isolating the polysaccharide from cultures of the pathogenic bacterium. Increasingly, these polysaccharide vaccines often include several polysaccharides to ensure immune coverage over a range of serotypes.

Conjugate subunit vaccines comprise a carrier protein chemically linked to an antigen or antigens. The carrier protein is generally a protein such as CRM197, a nontoxic mutant of diphtheria toxin, or a toxoid, which will induce a strong immune response. Traditionally, the chemically linked antigens have been capsule polysaccharides; however, this approach is being used more in association with peptide antigens. Essentially, the carrier protein acts as an adjuvant to boost the immune response to the antigenic component(s).

The advantage of producing subunit vaccines is that they have no living component and hence are considered as safer than traditional vaccines. Subunit vaccines are also a response to the regulatory pressure to have more well-defined antigens. Additionally, from an analytical perspective, these vaccines are easier to characterize when compared to the highly complex virion and whole cell bacterial vaccines.

In the following, we discuss the analytical considerations for subunit vaccines. More specifically, we discuss the assays needed for subunit vaccine characterization, quality control (QC) release and stability testing.

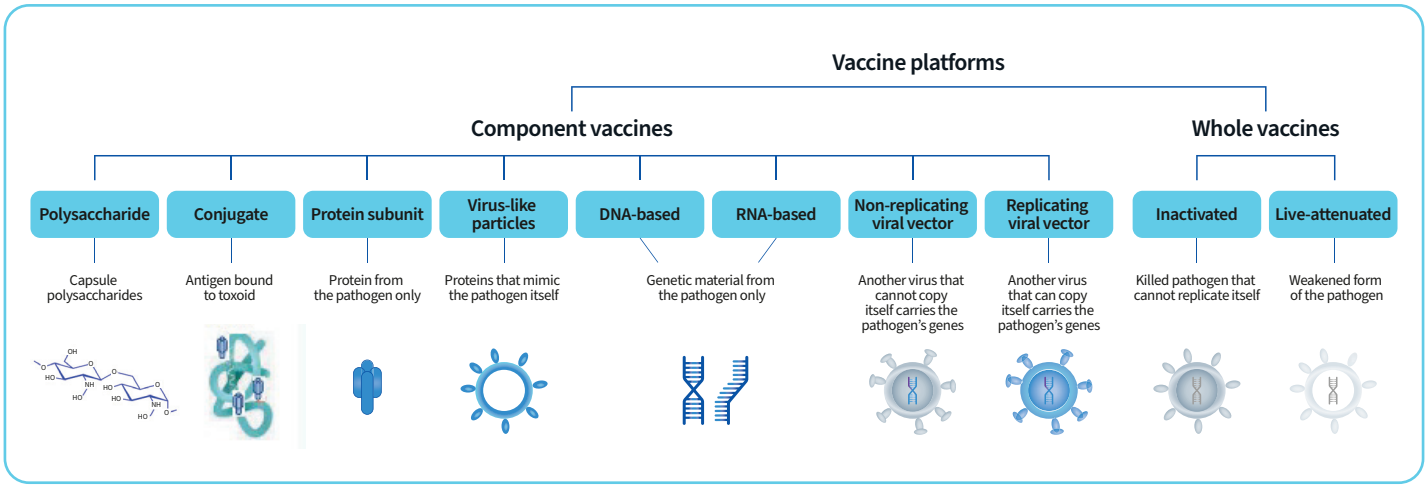


Figure 1: Schematic representation of vaccine modalities

Adjuvants

The major disadvantage of using subunit vaccines is that they are usually poor immunogens and generally need to be formulated with an adjuvant to improve efficacy and induce a potent immune response. Such adjuvants fall into two categories: (1) the particulate adjuvants and (2) the immunopotentiators.

With the former, traditionally, this has meant formulating the antigen with aluminum salts; and indeed, this "old technology" is still extensively used as it is low cost and effective and has an extensive safety record. The two most commonly used aluminum adjuvants are aluminum oxyhydroxide and aluminum phosphate, which are colloidal particles that readily bind to antigens by electrostatic interactions. In more recent years, other particulate antigens have been developed, including lipid emulsions and nanoparticles, which either bind or encapsulate the antigen(s). An increased understanding of the immune system has also led to the identification of immunopotentiators including proteins, oligonucleotides and lipid moieties that can be co-formulated with the antigen to enhance vaccine efficacy. Furthermore, increasingly, subunit vaccine formulations are combining adjuvants, including combinations of particulate and immunopotentiators.

Examples of adjuvants used in licensed and developmental vaccines are presented in Table 1.

Table 1: Adjuvants used in licensed and developmental subunit vaccines

Adjuvant	Comments
Aluminum salts	Commonly used adjuvant in many vaccines. Generally comprising aluminum oxyhydroxide and aluminum phosphate depending on antigen. Erroneously referred to as alum.
Calcium phosphate	Mineral adjuvant alternative to aluminum
Polyphosphazene	A polymer-based adjuvant
MF59®	Squalene-based oil-in-water emulsion
AddaVax™	Squalene-based oil-in-water emulsion based on MF59
Montanide™ ISA	Water-in-oil emulsion
Immune-stimulating complexes (ISCOMs)	Cage-like structures composed of saponins, cholesterol, and phospholipids
Matrix-M™	Quillaja saponin-based adjuvant
QS-21	7 Quillaja saponin-based adjuvant
Advax™	A polysaccharide adjuvant derived from delta inulin
Trehalose dibehenate (TDB)	A synthetic analog of mycobacterial cord factor
Pam3CSK4/Pam2CSK4	TLR2/6 agonist and potent activator of the pro-inflammatory transcription factor NF-κB
Macrophage-activating lipopeptide-2 (MALP-2)	A lipopeptide TLR-2/6 agonist
Polyriboinosinic: polyribocytidylic acid (Poly(I:C))	A synthetic double-stranded RNA and TLR-3 agonist
Monophosphoryl lipid A	Detoxified derivatives of bacterial lipopolysaccharide (LPS) and TLR-4 agonists
Glucopyranosyl lipid A	LPS derivative in oil-in-water emulsion and TLR-4 agonist
Flagellin	Recombinant bacterial flagellin from S. typhimurium; a TLR-5 agonist
Imidazoquinolines	Small molecule TLR-7/8 agonists, e.g., imiquimod and resiquimod

CpG oligodeoxynucleotides (CpG ODN)	TLR-9 agonist comprising synthetic DNA sequences that contain unmethylated CpG motifs
IC31®	A combination of oligodeoxynucleotide (ODN1a) and antibacterial peptide (KLKL(5)KLK) with TLR-9 activity
Dicyclic nucleotides	Stimulator of interferon genes (STING) agonist
Muramyl dipeptide (MDP)	N-acetyl muramic acid conjugated to L-Ala-D-isoGln; Nucleotide binding oligomerization domain containing 2 (NOD2) agonist
Virus-like particles (VLPs)	Self-assembling viral structural proteins
CRM197	Nontoxic mutant of diphtheria toxin generally used in conjugate vaccines
Cholera toxin subunit B	Nontoxic subunit of cholera toxin

Adjuvants have implications for subunit vaccine analysis. Because the adjuvant is a component of the vaccine product, additional analytical techniques are required to release and fully characterize the vaccine product; these additional assays are aimed at evaluating the adjuvant itself. Additionally, the adjuvant may interfere with the standard assays used to evaluate the antigen(s), which is particularly the case with the particulate adjuvants. Where interference does occur, interference-free assay variants need to be developed.

Regulatory analytical method expectations for subunit vaccines

Subunit vaccines are classed as biopharmaceuticals, and the principles of current good manufacturing practices (cGMP) apply to their manufacture. cGMP is designed to protect the integrity and quality of manufactured product intended for human use. With cGMP, it is expected that the manufactured subunit vaccine products will be demonstrated to have met predefined critical quality attributes (CQAs) in QC release tests, as well as will have a package of characterization data to demonstrate a comprehensive understanding of the vaccine product. The general principles for release testing and product characterization are described in ICH Q6B (Test Procedures and Acceptance Criteria for Biotechnological/Biological Products). As designated by ICH Q6B, the parameters that must be tested are:

- Appearance and description
- Identity
- Quantity
- Purity/impurities
- Potency
- General tests including microbiology

Additionally, there is a regulatory expectation that the stability of the subunit vaccine will be extensively tested during product development. The importance of stability is reflected in the extent of regulatory guidance that is available on the subject, exemplified by the following ICH guidelines:

- ICH Q1A(R2) Stability Testing Guidelines: Stability Testing of New Drug Substances and Products
- ICH Q1B Stability Testing: Photostability Testing of New Drug Substances and Products
- ICH Q1C Stability Testing for New Dosage Forms
- ICH Q1D Bracketing and Matrixing Designs for Stability Testing of Drug Substances and Drug Products
- ICH Q1E Evaluation of Stability Data
- ICH Q5C Stability Testing of Biotechnological/Biological Products

With the exception of pharmacopeial methods, analytics required for QC batch release and formal stability testing must be phase-appropriately validated, as outlined in ICH Q2(R1) (Validation of Analytical Procedures: Text and Methodology) (see below). ICH Q2(R1) is currently under revision (at the time of writing) and draft guidelines were released on March 24, 2022. Pharmacopeial methods just require verification to ensure product compatibility.

Increasingly, the regulatory expectation is that the vaccine CQAs will be identified using the Quality by Design (QbD) approach, incorporating risk assessment, as outlined in ICH Q8 (Pharmaceutical Development) and Q9 (Risk Management). More specific to analytical development is ICH Q14 (Analytical Procedure Development), which describes the analytical quality by design (AQbD) approach. With ICH Q14, regulatory authorities are aiming to harmonize the scientific application to analytical development and facilitate more efficient and sound scientific and risk-based approval that covers the life cycle of an analytical method.

One additional factor requiring consideration with respect to analytical methods is that of reference standards. Some of the analytical procedures will require reference standards for comparative purposes and especially so with potency assays. As with the biopharmaceutical products themselves, the reference standards need to be fully characterized. Indeed, reference standards are generally selected from a well-characterized batch—possibly an engineering batch for an early developmental process and from a cGMP batch for products in the clinical development phase.

Selection of QC release testing, characterization and stability assays

Regarding analysis of subunit vaccines, a large toolbox of analytical methods can be used to gain a comprehensive understanding of the product, in line with ICH Q6B guidelines. Overall, specific methods can be assigned to one of three categories: (1) QC batch release, (2) stability studies and (3) for information only (FIO) characterization assays. In reality, these functions can overlap, and selected analytical techniques can be used for more than one of the categories (Figure 2).

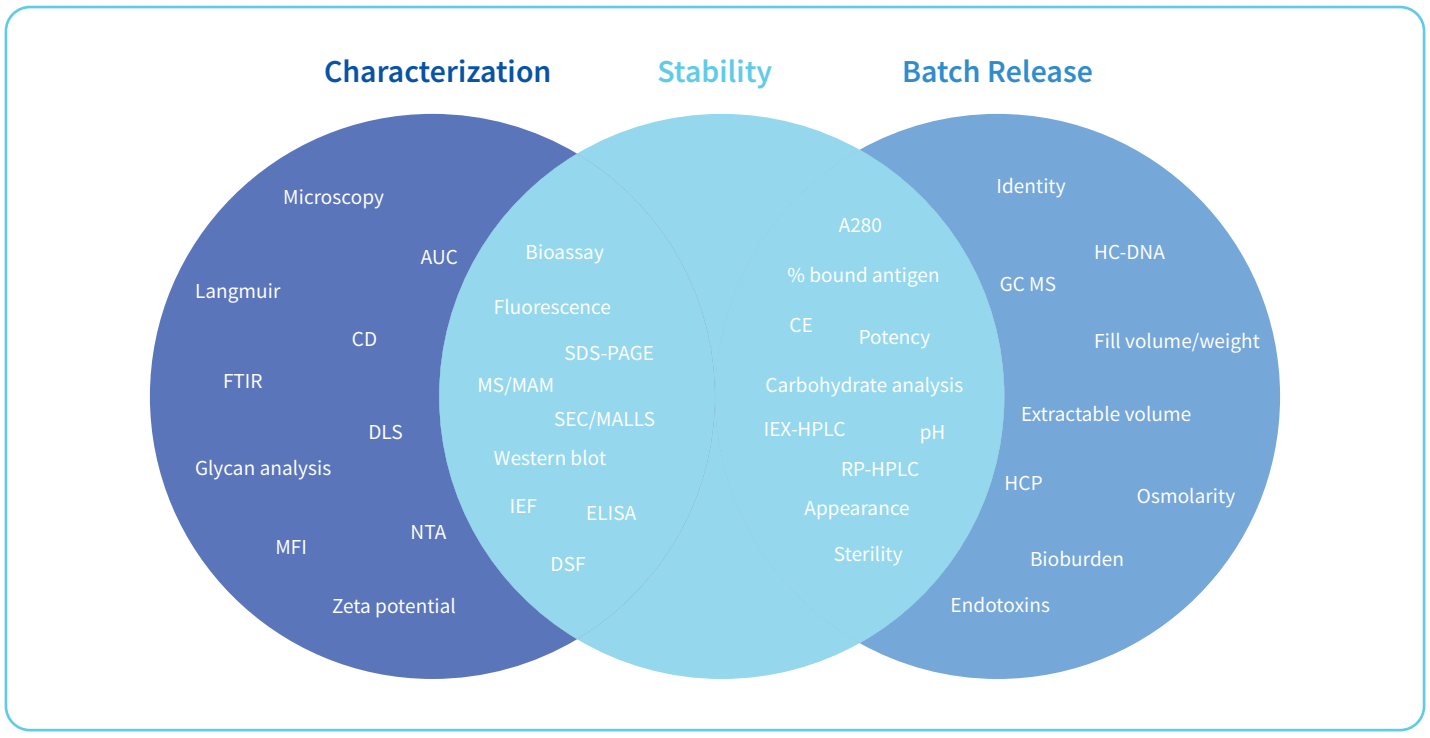


Figure 2: Schematic representation of vaccine modalities

Precisely which assays fit into which of the three categories is product specific and can change with increased product knowledge. With protein-based subunit vaccines, because the antigen must be formulated with an adjuvant, the characterization profiles of the drug substance and drug product are different. For the characterization of drug substance, a basic standard recombinant protein approach is usually adopted. With the drug product, the characterization requires both the antigen and adjuvant to be analyzed along with any potential interaction between the two components.

The analytical methods that can be applied to any QC release testing generally fall into one of two categories, namely, compendial and product-specific methods. Compendial methods are standardized techniques that apply to generic attributes such as pH, appearance and microbial purity/sterility (Table 2).

The product-specific assays have been specifically developed for the drug substance, drug product and possibly the adjuvant as well. These assays can be further subdivided into physico-chemical assays (Table 2) and biological/potency assays (see below). With the physico-chemical techniques, some of these can be considered "work horses" as they are commonly used with the majority of biopharmaceuticals, albeit being developed specifically for the subunit vaccine.

Table 2: Some typical QC release and characterization analytical methods for protein and conjugate subunit vaccines

Parameter	Assay	Method
General tests	pH	Pharmacopeial – pH meter
	Osmolality	Pharmacopeial – osmometer
	Moisture content	Pharmacopeial – Karl Fischer (lyo only)
Appearance and description	Appearance	Pharmacopeial – appearance, color and clarity methods
Identity	Primary sequence	Peptide mapping
		MAM
		Western blotting
Quantity	Protein quantitation	A280
		BCA
	Immuno-quantitation	ELISA

Purity and impurities	PTMs	MAM
		Peptide mapping
	Hydrolysis/fragments	CE SDS/SDS PAGE
	Charged isotypes	cIEF
		Ion exchange HPLC
	Hydropathy – hydrophilic/ hydrophobic variants	HIC HPLC
		Reverse-phase HPLC
	Glycan profile	LC/MS, HILIC HPLC
	Aggregation	SE HPLC/MALLS
		DLS
	Subvisible particles	HIAC
		Microflow imagery
Higher order structure	Antigen structure	Intrinsic and extrinsic fluorescence
		FTIR
		Circular dichroism
		Differential scanning calorimetry
Adjuvant	% bound antigen	Protein analysis
	Particle size and size distribution	Microscopy
		Microflow imagery
		Dynamic light scattering
	Particle charge	Zeta potential
	Immunopotentiating adjuvant	Adjuvant specific
Safety	Sterility	Pharmacopeial – microbiology
	Endotoxin	Pharmacopeial – LAL

With polysaccharide-based vaccines and polysaccharide conjugated vaccines, additional saccharide-specific assays will be required, such as described in Table 3.

Table 3: Release and characterization analytical methods for polysaccharide-containing vaccines

Parameter	Assay	Method
Quantity	Saccharide quantitation	Anthrone assay
		Sulfuric acid/phenol assay
Identity	Polysaccharide identity	GC MS
		ELISA
Higher order structure	Polysaccharide structure/conformation	NMR
		FTIR
		LC MS

Analytics for particulate vaccines

Most of the recombinant protein antigen subunit vaccines involve a particulate adjuvant. The most common types of particulate adjuvant are the aluminum salt (generally referred to as alum); however, increasingly, liposomal and other nanoparticle adjuvants are being developed. Analysis of subunit vaccines that have a particulate adjuvant can be problematic as most analytical methods are solution based. Issues such as sedimentation, light scattering and instrument clogging can make particulate vaccines incompatible with many standard analytical techniques. This is particularly an issue with the aluminum salt adjuvants, which need special consideration when it comes to analytical development. Assays may need to be modified to address the particulate nature of the vaccine.

Direct and indirect analysis of particulate vaccines

Where the particulate nature of the subunit vaccine presents a problem for the standard analytics, a two-approach strategy can be employed involving (1) indirect methods, which comprise desorbing or solubilizing the antigen for subsequent analysis, and (2) direct methods, which analyze the intact vaccine formulation.

With the indirect method approach, desorption/solubilization can involve pH adjustment, solubilizing salts, chaotropic agents and even surfactants, depending on the antigen's interaction with the adjuvant. As the desorption/solubilization procedure has the potential for modifications to the antigen and/or adjuvant, adequate controls are needed to demonstrate that the extraction procedure is not introducing artifacts. Once desorbed or solubilized, the antigen can be buffer exchanged and analyzed using the standard techniques (Table 2), assuming that the desorption procedure is compatible with the specific technique.

With the particulate subunit vaccines, the pharmacopeial methods can be considered as direct methods, as they are applied to the formulated drug product, and generally, the particulates do not cause interference; however, verification is required to ensure the particular nature of the vaccine does not cause interference.

Direct quantification of the antigen while bound to a particulate adjuvant can also be problematic, so actual dosages are inferred rather than measured directly. Light absorption methods usually are not applicable due to light scattering effects. In contrast, some fluorescence-based quantitation methods can be used. Alternatively, with appropriate antibodies raised against the antigen, immunological methods, optimized for reduced nonspecific binding, can be applied to particulate vaccines. With such enzyme-linked immunosorbent assays (ELISAs), a reference standard vaccine is required to enable quantitation against a standard curve.

When an antigen binds to a particulate adjuvant, the interaction between the two components can result in effects upon the antigen structure. One disadvantage of indirect analysis is that any characterization of such effects due to antigen-adjuvant interaction is lost. As any antigen-adjuvant interaction is intrinsic to the formulation, the appropriate analytics need to be performed with the antigen *in situ*, tox the adjuvant. Such direct methods are mainly aimed at understanding the effect of the antigen-adjuvant interaction on the higher order structure (HOS), which can be crucial for inducing a neutralizing immune response. As HOS methods are largely biophysical (Table 4), modifications are usually required due to particulate interference.

Table 4: Some typical direct analysis methods for particulate subunit vaccines

Parameter	Assay	Method
Quantity	Protein quantitation	o-phthalaldehyde assay
	Immuno-quantitation	ELISA
Higher order structure	Antigen structure	Intrinsic and extrinsic fluorescence
		FTIR
		Circular dichroism
		Differential scanning calorimetry
		Differential scanning fluorimetry
		In situ proteolysis with LC MS

Determining the antigen-adjuvant binding parameters

With particulate subunit vaccines, one aspect of the antigen-adjuvant interaction that might be crucial to the efficacy and stability of the vaccine is the strength of binding between the two components. With some antigens, binding strongly to the adjuvant reduces stability and decreases potency. Hence, some understanding of the binding parameters can be important for formulation development and characterization purposes.

One method for evaluating the binding parameters of the antigen and adjuvant is linear Langmuir analysis. This is a simple binding assay that can determine the maximum binding capacity B_{max} and the adsorption coefficient K (strength of binding parameter). While this method does not directly measure the binding parameters on the formulated vaccine, it does evaluate the interaction between the drug substance and the adjuvant to instruct formulation development. Nevertheless, the method is useful in characterizing the antigen-adjuvant interaction. A method that directly assesses the antigen-adjuvant interaction is to use partial desorption, selecting desorption conditions (see above) that result in approximately 50% desorption of the antigen from the adjuvant. While this method is a less quantitative method than linear Langmuir, the advantage is that it can be used to evaluate the antigen-adjuvant interaction during storage; a parameter that can change with time and storage conditions.

Potency assays

In determining the potency of biopharmaceuticals, it is expected that attempts are made to develop a cell-based bioassay that reflects the intended mode of action of the product (Figure 3). With vaccines, due to the complexity of the immune system, such an option is not always possible *in vitro*. The standard historical approach has required the implementation of an animal-based *ex vivo* assay to show adequate immune response. Following immunization of an appropriate animal species, usually mouse, blood sampling is performed at a time point optimal for the immune response. Sample measurement endpoint(s) to be determined depend on the purpose and intended immune response of the vaccine, but the most common is to measure B-cell responses through quantifying anti-antigen IgG levels in the samples using an immunoassay such as ELISA. Alternative, or supplementary, approaches include evaluating T-cell responses with enzyme-linked immunosorbent spot (ELISpot) assay, or flow cytometry may also be used where scientific justification is provided. Further characterization of the immune response is possible by, for example, determining the T-helper subtypes Th1/Th2 ratio and/or cytokine levels.

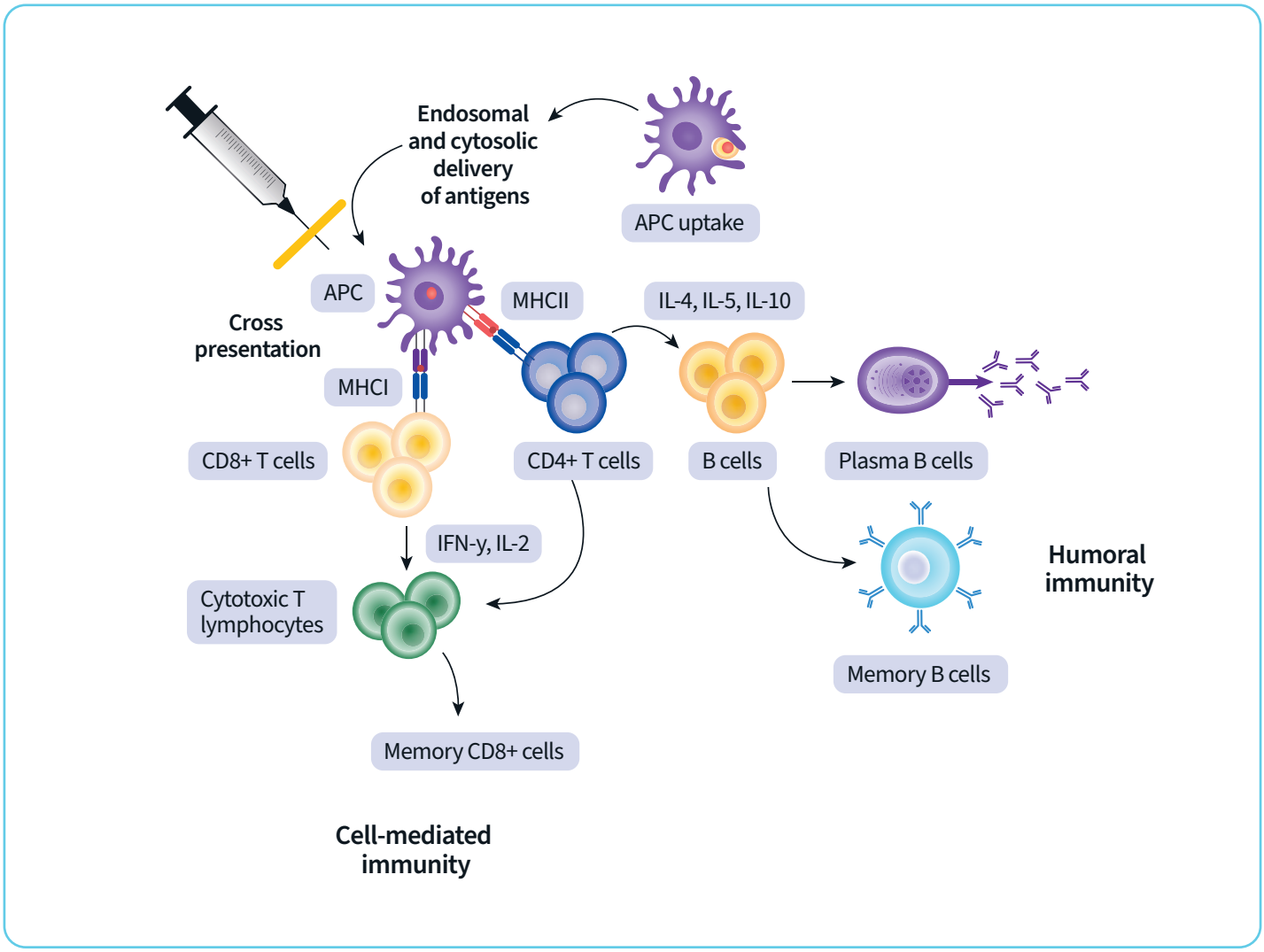
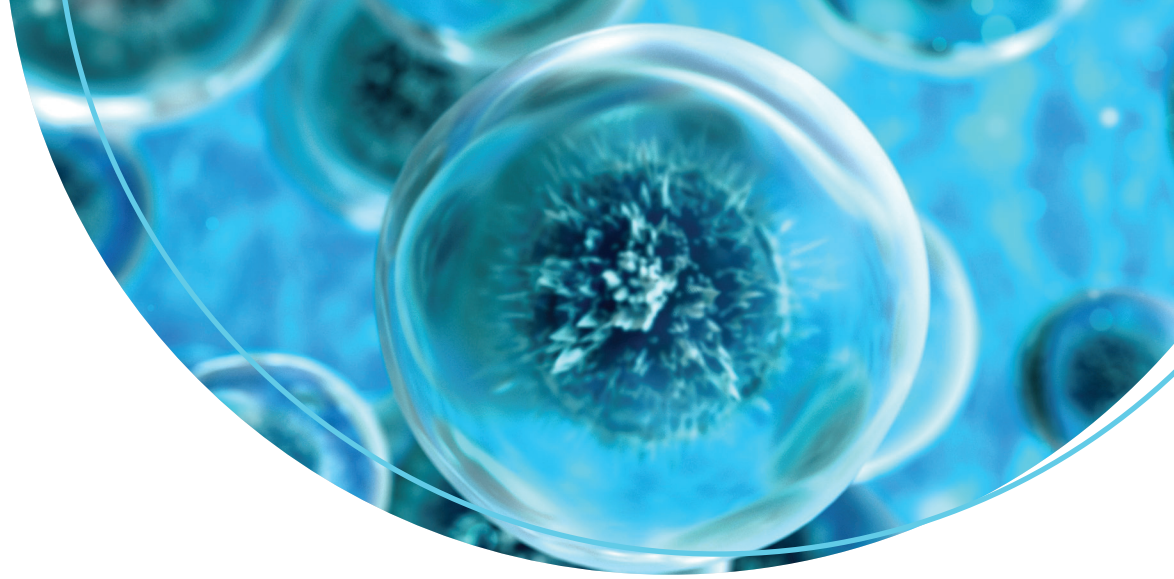


Figure 3: Schematic representation of the vaccine-induced immune response

APC, antigen-presenting cell; IL-4, interleukin-4; IL-5, interleukin-5; IL-10, interleukin-10; MHCI, major histocompatibility complex class I; MHCII, major histocompatibility complex class II



With prophylactic subunit vaccines against microbial pathogens, in some cases, a microbial challenge assay is required. With this type of assay, following immunization and subsequent induced immune response, animals are challenged with the infectious agent at appropriate doses and the endpoint is disease progression or "time to death" (animals being euthanized at a predetermined point in disease progression). With challenge assays, additional assays might be required to evaluate the antibody response in neutralizing the virulence factor to which the vaccine is targeted. Such challenge assays are the exception, as apart from the obvious ethical issues, they require special facilities and animal handling due to the use of virulent pathogens.

While *ex vivo* potency assays are the historically accepted method, there is a strong drive to move away from animal testing where at all possible under the 3Rs principle of reduction, refinement, and replacement. Such an approach promotes the use of alternative methods whenever possible, reducing the number of animals used, refining the experimental techniques to minimize harm, and replacing animals with non-animal models when feasible. For QC release testing, there is a trend toward developing immunological-based pseudo-potency assays. Although not directly demonstrating an immune response, such assays do require quantitative assessment of the CQAs of the vaccine product that assures potency and hence efficacy of the vaccine *in vivo*. Typically, these *in vitro* assays use monoclonal antibodies (mAbs) that can bind directly to conformational epitopes on the virulence factor and neutralize the biological activity. Such neutralizing mAbs effectively demonstrate that the corresponding antigen epitope is structurally intact and therefore capable of eliciting a neutralizing antibody response by B-cells. The caveats regarding the pseudo-potency approach are (1) having sufficient and appropriate mAbs to address the main neutralizing epitopes and (2) demonstrating that the immunological endpoints correlate with the *ex vivo* assay. This latter point requires adequate bridging study data to be submitted before the *in vitro* assay is accepted as a QC release method. The *in vitro* approach, provided it demonstrates good correlation and is stability indicating, has many benefits, including increased precision, accuracy, reduced cost and ease of performing.

Due to the inherent variability of all biological potency assays, a relative potency format is usually required that quantitates the assay response of the test sample relative to a reference standard. The underlying assumption in the relative potency assay is that the sample behaves biologically as a dilution of the refence standard. For this reason, the reference standard should be a very well-characterized batch of the material, relevant to the stage of product development. At later stages of development, this should be a GMP batch manufactured using the same production process as the intended release batches. The test sample response in the potency assay is assessed, using predefined acceptance criteria, against the reference standard to ensure it is equivalent (sym. similar) in biological activity. Only once this is shown to be true can the potency be calculated. Typically, the potency assay measurement is plotted as response vs. dose across a range of concentrations used. The number of dose points used and the data-fitting algorithms applied in fitting the dose-response model can vary based on the assay system and responses achieved. For more information on developing potency assays, see the e-book Potency Assays 101.

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