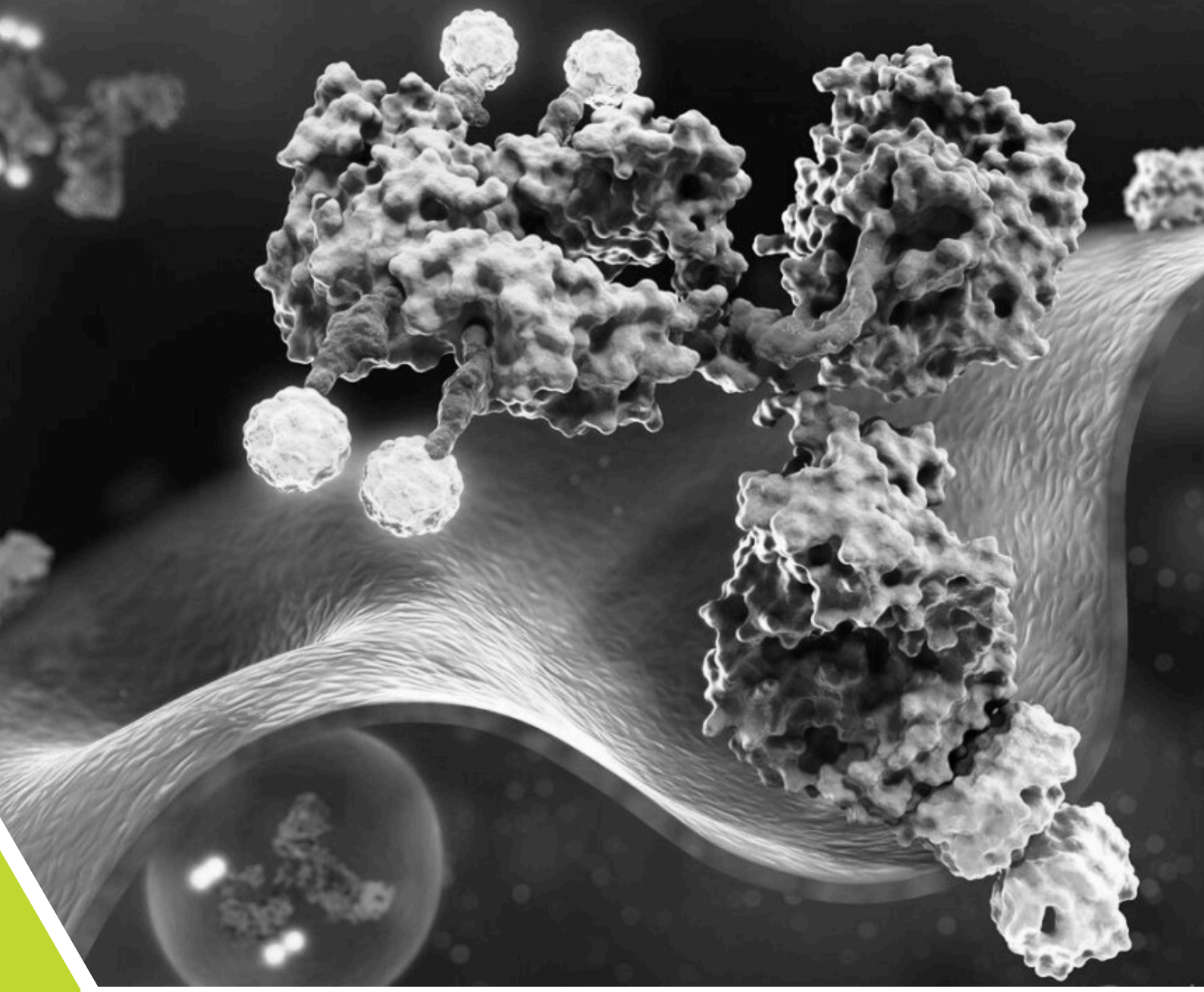


Antibody-Drug Conjugates

Overcoming Analytical Challenges in Next-Gen Cancer Therapies

White Paper

solvias

Executive Summary

Antibody-Drug Conjugates (ADCs) represent a revolutionary approach in cancer treatment by combining the selectivity of monoclonal antibodies with the potency of cytotoxic drugs. However, the unique complexity of ADCs — comprising an antibody, a linker, and a cytotoxic payload—introduces a range of analytical challenges.

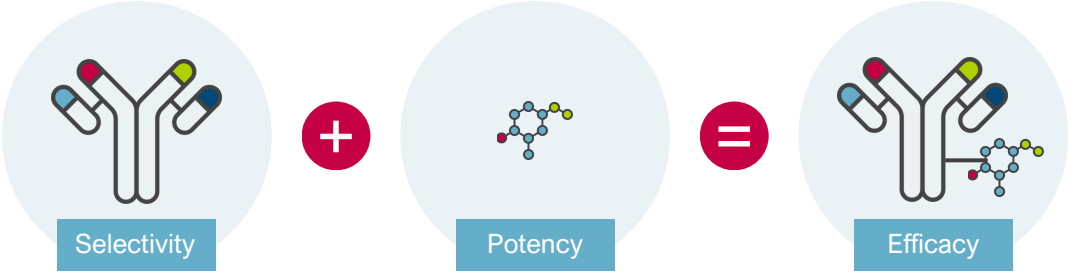
This white paper explores these critical challenges, focusing on the characterization, stability, and potency of each ADC component. It highlights the need for comprehensive and customized analytical strategies to ensure the safety, efficacy, and consistency of these therapeutics. From ensuring antibody integrity and linker stability to accurately assessing payload potency and drug-to-antibody ratios, each step demands precision to navigate the intricate pathways of ADC development.

The Search for a *Magic Bullet*

Cancer is a leading global cause of mortality, accounting for over 10 million deaths in 2020[1], with numbers rising post-pandemic.

Diagnosing cancer presents doctors with a choice between surgery, immunotherapy, radiotherapy, or chemotherapy[2], often used in combination. Chemotherapy, the most common approach[3], relies on small cytotoxic active ingredients. While historically successful, its low therapeutic index leads to severe side effects, primarily due to its impact on healthy, rapidly dividing cells[4].

The long search for targeted cancer therapies dates back to the early 20th century with Paul Ehrlich's concept of the "magic bullet"[5]. This vision seemed realized in 2000 when the FDA approved the first ADC, Mylotarg®, for acute myeloid leukemia (AML)[6]. ADCs consist of a monoclonal antibody scaffold, a linker, and a potent cytotoxic drug[7]. They align closely with Ehrlich's "magic bullet" idea, as the antibody targets specific cells, while the linker holds the cytotoxic drug until it encounters the right chemical or biochemical environment, then releases the therapeutic payload.



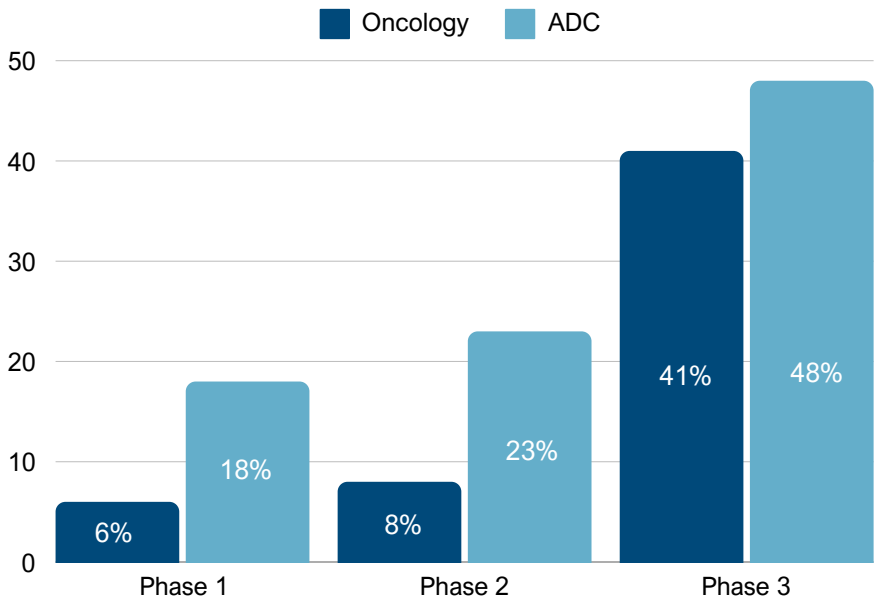
The ADC Market Surge

The market for ADCs has experienced significant growth over the years. As of now, 14 ADCs have been approved by the U.S. Food and Drug Administration (FDA), and over 100 more are in various stages of clinical development[8]. This rapid expansion is reflected in market projections, with sales for ADCs expected to surpass $30 billion by 2028, driven by a robust compound annual growth rate of 25%[9].

One of the key drivers of this growth is the comparatively lower risk associated with ADCs, as measured by the probability of technical and regulatory success (PTRS). ADCs have shown a notably higher PTRS than other oncology treatments, particularly in late-stage clinical trials. This higher success rate has spurred increased investment and deal activity, with several high-profile partnerships and acquisitions taking place in 2023 and 2024[10].

The strong performance of ADCs in clinical trials and their ability to deliver targeted therapies with fewer side effects make them an attractive option for pharmaceutical companies. As the pipeline continues to mature and expand, ADCs are expected to play an increasingly important role in the oncology landscape, offering both patients and investors promising opportunities in the fight against cancer.

Probability of Technical and Regulatory Success (PTRS)



* The FDA withdrew the US license to manufacture BLENREP in 2023.

The Analytical Challenges of each of ADCs' Building Blocks

ADCs are complex entities comprised of three distinct components, each presenting unique analytical challenges. Researchers must adopt both small- and large-molecule analytical approaches to navigate the intricacies of ADC characterization.

The Antibody

The antibody is designed to recognize and bind to a particular antigen that is highly expressed on the surface of the target cells. Since ADCs are primarily used in oncology, target antigens are often overexpressed in cancer cells[8], such as HER2, EGFR, CD19, and BCMA[11]. Currently, IgG1 is the most commonly used antibody in ADCs[12].

The evaluation of the antibody moiety starts with a congruent reference standard characterization, focusing on primary structure, higher-order structure, heterogeneity, and post-translational modifications (PTMs). Critical quality attributes, including integrity, size, charge heterogeneity, and bioactivity, are also part of this initial reference characterization and will guide CMC testing throughout the clinical development process.

Primary Structure (Identity)

The primary structure of an antibody is its amino acid sequence. Peptide mapping breaks the antibody into peptides and UV or MS analysis verifies that the sequence matches the expected structure. Amino Acid Analysis (AAA) confirms the overall amino acid composition and ensures the correct ratios. For antibodies like IgG1, N-terminal sequencing by Edman degradation checks specific amino acids at the N-terminus, such as lysines. These amino acids are crucial for stability and biological activity, which are essential for the ADC's therapeutic efficacy. The testing process for primary structure is the same for ADCs and regular monoclonal antibodies.

Higher Order Structure

Regulatory authorities typically require Circular Dichroism (CD) to check the 3D structure of antibodies. However, especially for ADCs with high conjugation indexes (large number of drug molecules attached to the antibody), Hydrogen/Deuterium Exchange Mass Spectrometry (HDX-MS) provides deeper insights. It compares the unconjugated antibody with the fully conjugated ADC to detect any structural changes caused by the conjugation.

Post-Translational Modifications (PTMs)

The main method used is peptide mapping mass spectrometry (Pep-Map MS). Some PTMs are particularly important for stability and function of the ADC and require specific tests:

- Cysteines: These amino acids form disulfide (S-S) linkages, which help maintain the antibody's structural integrity by covalently linking the separate amino acid chains. If these linkages are disrupted, the antibody may lose stability or function. MS is used to analyze these linkages. Similarly, unpaired cysteines (free thiols) can affect the structure and stability of the antibody if not properly controlled. These are tested by MS or by using the Ellman reagent, also known as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). When DTNB reacts with free thiol groups, it forms a yellow-colored product called 5-thio-2-nitrobenzoic acid (TNB). The intensity of the yellow color can be measured spectrophotometrically, which correlates with the concentration of free thiols present.
- N- and O-glycans: These are sugar molecules attached to the antibody, crucial for stability, solubility, and immune system interactions. Abnormal glycosylation can impact the antibody's function or increase the risk of immune reactions. To study them, a combination of LC-UV and LC-MS techniques is used to identify and analyze the glycan structures.

Critical Quality Attributes

Several techniques are used to assess critical quality attributes:

- Capillary electrophoresis, size exclusion chromatography (SEC with MALS), ion-exchange chromatography, capillary zone electrophoresis (CZE), and capillary isoelectric focusing (cIEF) are employed to assess the antibody's structural integrity and size distribution.
- Cell-based bioassays, binding ELISA, or surface plasmon resonance (SPR) are employed to test the bioactivity of the antibody, ensuring that it binds and functions as expected.

The CMC testing panel, covering stability-indicating quality characteristics, is rounded out by description analyses, impurity testing, and microbiological safety assessments.

The Linker

The linker plays a critical role in defining the therapeutic index—the ratio between the therapeutic and toxic dose—of an ADC. An ideal linker prevents premature release of the cytotoxic payload and minimizes ADC aggregation[8], enhancing patient safety and reducing dosage requirements. Linkers are generally categorized as either cleavable or non-cleavable. **Cleavable linkers** release the drug payload in response to specific stimuli within target cells. Examples include hydrazone-like linkers that respond to pH changes within lysosomes and endosomes[13,14], disulfide-like linkers that respond to elevated glutathione (GSH) levels[15], and peptide-like linkers that respond to overexpressed cancerogenic proteases like cathepsin B[16]. **Non-cleavable linkers** remain intact until the ADC is fully degraded by enzymatic hydrolysis promoted by target cell inherent proteases.[17] Cleavable linkers offer precise, targeted drug release but are more prone to premature cleavage and off-target effects, whereas non-cleavable linkers provide greater stability in circulation but require a more complex design to ensure effective drug release.

Several analytical techniques are employed to assess the stability of linkers under various in vitro and in vivo conditions. Advanced methodologies like high performance liquid chromatography (HPLC), mass spectrometry (MS), gas chromatography (GC) and nuclear magnetic resonance (NMR) spectroscopy are complemented by molecule-specific supporting analyses, including Fourier-transform infrared spectroscopy (FT-IR) to elucidate the linkers identity, Karl Fischer titration for water content analysis, and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to analyze heavy metal content.

Typically, the linker is analyzed as part of a “drug-linker” construct, which combines the linker with the cytotoxic payload. Due to the high potency of these payloads, the toxicological profile of drug-linkers often fall below Occupational Exposure Limits (OELs) of 50 ng/m³, making standard safety measures insufficient.

Moreover, monitoring the rate of linker breakage during storage is critical, as excessive breakage can lead to higher levels of free warhead, increasing patient risk. To assess this, a reversed-phase high-performance liquid chromatography (RP-HPLC) test quantifies the amount of payload released. The unconjugated warhead is required as a reference to identify and measure this impurity and serves as an internal standard in the procedure. Due to their extreme toxicity, these warheads often have OELs in the single-digit nanogram range, demanding specialized containment systems and strict safety protocols. The main challenge lies in ensuring the safety of lab personnel while maintaining accurate testing procedures.

The Payload

The payload, or warhead, is the cytotoxic agent responsible for destroying cancer cells. ADCs payloads are multiple orders of magnitude more potent than conventional chemotherapeutics (IC50 in the nano – to picomolar range)[18,19]. This is necessary as only approximately 2% of the administered ADC molecules reach their target location[12]. Three main classes of payloads dominate ADCs:

- **Microtubule inhibitors:** Disrupt cell division by targeting microtubules, leading to cancer cell death. They are the most widely used payloads in FDA-approved ADCs due to their potency. Typical agents are monomethyl auristatin E (MMAE), monomethyl auristatin F (MMAF), and tubulysins.[20,21]

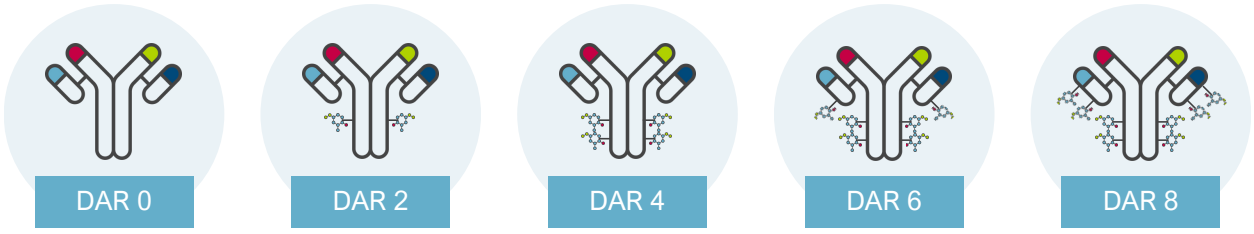
- **DNA-damaging agents:** DNA-damaging agents are generally more potent than microtubule inhibitors[22]. They work by inducing DNA double-strand breaks (e.g., calicheamicins)[23], cross-linking DNA (e.g., pyrrolobenzodiazepines (PBD)[24], preventing cancer cell replication through DNA intercalation (e.g., topoisomerase I inhibitors)[25], and triggering apoptosis via DNA alkylation (e.g., duocarmycins)[26]. DNA-damaging agents are the second most common payloads in ADCs.

- **Small molecule immunomodulators:** These compounds are designed to interact with specific molecular targets in cancer cells, disrupting key survival pathways. While small molecule immunomodulators show promise, they are still in development and have not yet been FDA-approved for use in ADCs. One significant group within this category is the immune-stimulating antibody conjugates (ISACs)[27]. The analytical characterization of these warheads is primarily an organic chemistry task and is beyond the scope of this paper.

Evaluating the potency of the cytotoxic payload is essential to ensure the ADC's therapeutic efficacy. Techniques such as cell viability assays, enzyme-linked immunosorbent assays (ELISA), and flow cytometry are employed to determine the payload's ability to induce apoptosis in cancer cells. Detailed insights into these assays can be found in the cell-based bioassay section later in this white paper.

Drug-to-Antibody Ratio (DAR)

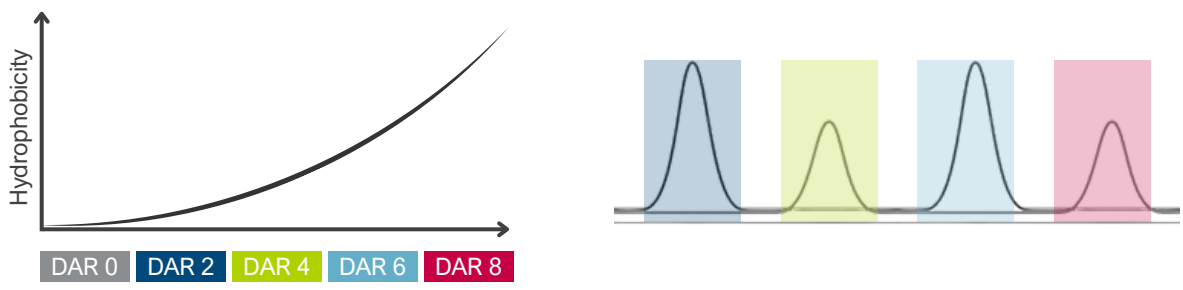
In addition to the individual quality attributes of each ADC component, several ADC-specific critical quality attributes (CQAs) must be defined. One of the most crucial of these is drug-to-antibody ratio (DAR), the number of drug molecules conjugated to each antibody. For instance, a DAR of two means two drugs molecules are conjugated to a single antibody. Different ADCs can have varying DARs, influenced by the properties of the antibody, the type of linker used, and the conjugation method.



A typical conjugation reaction yields a mixture of antibodies with different DARs, ranging from unconjugated antibodies to those with up to eight attached drug molecules. The challenge lies in accurately characterizing and documenting this distribution. DAR data is often reported as the average DAR, representing the mean DAR across all conjugated species within a batch. Maintaining a consistent average DAR across production batches is essential, as it has significant implications for both potency and safety. A lower DAR can reduce the treatment's efficacy per dose, while a higher DAR can increase toxicity risks, making precise control of DAR critical for successful ADC development and manufacturing.

The total number of drug molecules conjugated to an antibody is only the first layer of heterogeneity in ADCs. The second layer involves the specific positions where the drugs are attached. While DAR 0 and DAR 8 each have a single isomer, DARs between 2 and 6 can have multiple isomers due to the various possible conjugation sites on the antibody. Monitoring both the total drug load and its distribution across different conjugation sites is crucial during ADC development and is typically achieved using HPLC-based techniques.

Most drugs used in ADCs are hydrophobic. This change in hydrophobicity can be leveraged to determine DAR and drug load distribution using hydrophobic interaction chromatography (HIC). In this process, antibodies with low hydrophobicity (lower drug conjugation) elute first as the salt concentration decreases, followed by those with higher hydrophobicity (higher drug conjugation). This produces a chromatogram with distinct peaks corresponding to the different conjugated species. The size of each peak reflects the proportion of that species in the sample, allowing the determination of how much of each species is present.



Cell-Based Bioassays in ADCs

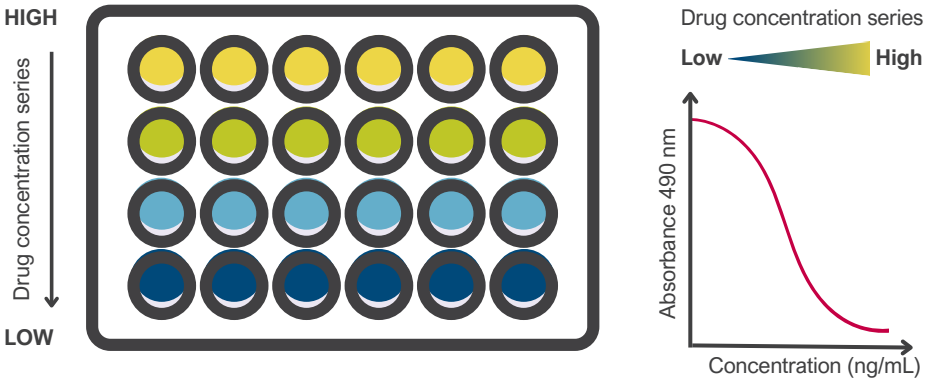
Cell-based bioassays (CBBAs) play a critical role in ADC development. These assays can evaluate how well an ADC induces a biological response, particularly given its complex mechanism of action that includes both the antibody's targeting ability and the cytotoxic payload's efficacy. CBBAs are highly custom because they need to be tailored to the specific characteristics of the ADC and its target cells. Factors such as the ADC's unique mode of action, the biology of the target cell line, and the cytotoxic payload all influence assay design, making a one-size-fits-all approach impractical.

A key challenge to address when developing a CBBA is biological variation. Sources of variation between cell lines include factors such as cell doubling time, antigen density on the cell surface, and toxin sensitivity. Therefore, optimizing key variables like incubation time (the period during which the cells are exposed to the drug), cell density, and the concentration series of the drug is crucial. The more precisely these variables are defined during assay development, the higher the quality and reproducibility of the potency curve, which leads to more accurate and robust assays.

Before using cells in an assay, they are cultured for 3-4 weeks. During this period, a master cell bank (MCB) and working cell banks are prepared. The MCB is cryopreserved after being characterized and tested for purity, identity, and stability, serving as the primary source for future use. A small portion is thawed to create working cell banks.

The CBBA is typically performed on a 96-well plate, with cells evenly distributed. The drug is applied in a concentration series, with the highest concentration at the top of the plate and the lowest at the bottom. After an incubation period, a detection reagent is applied, leading to a color change based on cell viability. This color change needs to be converted to a readout we can analyze.

A common reagent used is MTS, which starts off yellow. Living cells reduce MTS to blue formazan in the presence of NADPH or NADH, both of which are produced by metabolically active cells. Wells with live cells will turn blue, while those with predominantly dead cells remain yellow. The intensity of the blue color is directly proportional to the number of live cells, as formazan absorbs light at 490 nanometers. Higher absorbance at this wavelength indicates more live cells, while lower absorbance reflects more cell death.



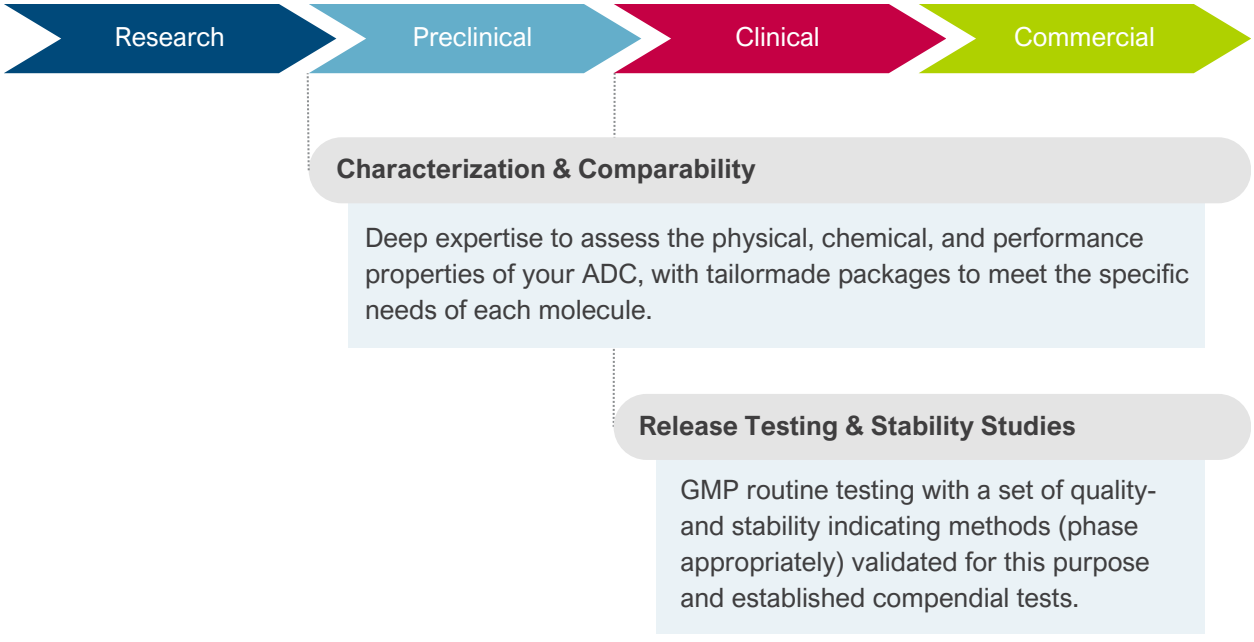
Your Trusted Partner

Antibody-drug conjugates (ADCs) are transforming oncology with their potential for targeted, less toxic cancer treatments. However, their complex structure — combining biological and chemical components — poses significant challenges in analytical testing and regulatory compliance. Solvias addresses these challenges with comprehensive analytical capabilities, leveraging our deep expertise, experience, and state-of-the-art technology to keep your development on schedule and within budget.

We understand that ADCs are more than the sum of their parts and no two ADCs are the same. Our team of experts is well-versed in handling every component — antibodies, linkers, and payloads — while collaborating closely with your team to design customized analytical strategies that align with your project's specific goals. Our flexible and client-centric approach means you get tailored solutions that keep your ADC development on track.

In our cutting-edge toxicology facilities, we handle products with OELs as low as 5 ng/m³, enabling us to support free drug analysis, a critical component of every ADC release testing panel. We offer comprehensive DAR analysis using LC-UV and LC-MS, combined with strong expertise in bioassays, to help accelerate the development of ADCs and bring these life-saving therapies to market faster.

From comprehensive characterization and stability assessments to custom assay development and regulatory support, Solvias offers an integrated suite of solutions that covers all aspects of ADC development:



For further information and details about these analytical testing services, or to get in touch with an expert, contact us at **info@solvias.com**.

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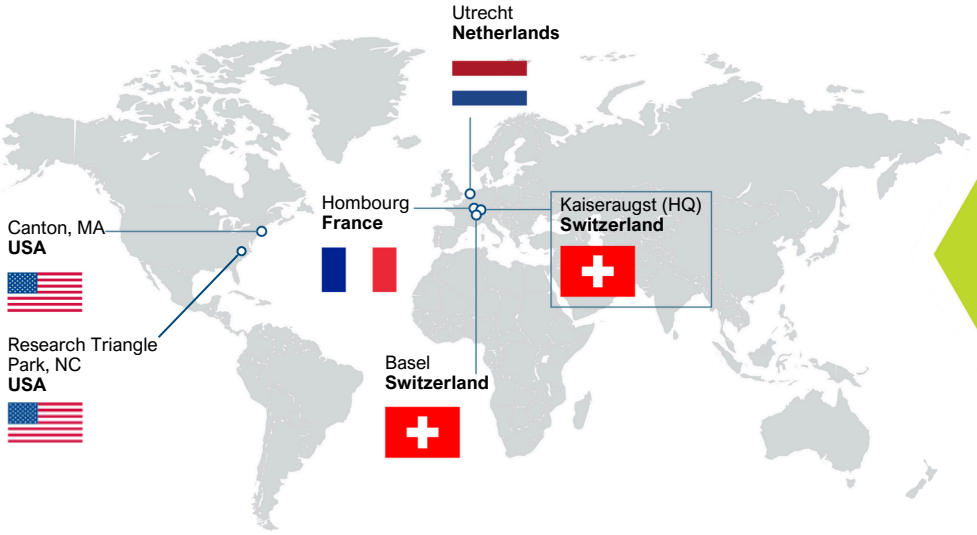

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