Millipore®
Preparation, Separation,
Filtration & Monitoring Products

Application Note

Detection Of Anti-Drug Antibody (ADA) Using Single Molecule Counting (SMC™) Technology

Introduction to Immunogenicity

All biological therapeutics have the potential to induce an immune mediated response ranging from benign to severe adverse effects. These effects can encompass diminished clinical efficacy of the biotherapeutic being administered to hypersensitivity, allergic reaction or even cytokine storms.

Consequently, regulatory agencies are looking to understand the implications of immunogenicity and are directing the industry to integrate programs for immunogenicity risk management starting in early phase drug development in clinical and pre-clinical.

The Federal Drug Administration (FDA) and pharmaceutical experts in the area of immunogenicity testing have recently published guidelines for the design and optimization of immunoassays used in the detection of antibodies against biopharmaceutical drug products in patient samples in the absence of drug and more importantly, when drug is present. FDA recommends that screening and confirmatory IgG and IgM assays achieve a sensitivity of 100–500 ng/mL (FDA Guidance 2016).

The increased sensitivity recommended is based on the current state of the science observed in the FDA's filings as well as publicly available studies.

A more sensitive detection method may lead to earlier detection of a primary immune response or detection of IgG4 which the FDA can request on a case by case basis.

It has been seen that patients develop persistent ADA responses having levels lower than 100 ng/mL (AAPS Journal, Vol. 15, No. 1, January 2013).

Traditionally ELISAs or Electrochemiluminescence (ECL) have been used to identify the presence of anti-drug antibodies (ADA). Though effective for detection, ELISA

methods often fail to adequately measure specific antibody response in the presence of circulating protein therapeutic due to the limitation on sensitivity and problems presented on a plate-based format.

Multivalent IgM ADA binding to the antigen on a plate surface or in a microwell (spatial restriction) can prevent binding of the detecting reagent. This could lead to loss of early detection of the primary ADA response as IgM is the first isotype generated.

According to the FDA: 'ADA assays need to be sufficiently sensitive to detect low levels of ADA before the amount of ADA impact the PK, PD, safety, or efficacy.' The SMC™ technology offers a magnitude fold increase in sensitivity over current existing technologies.

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Immunogenicity and SMC™ Technology

SMC™ technology enables the development of ADA assays by the labelling of the drug with capture & detection reagents, and utilization of buffer reagents to develop and optimize the assays. The technology allows the ability to develop a homogenous species-independent assay format that is simple, easy to design and validate. The reduced number of wash steps aids in the detection of low affinity antibodies and decreases assay time. This assay format is often referred to as a "bridging assay" since the ADA acts as a bridge between the drug labelled capture & detection.

SMC™ technology, employing the SMCxPRO™ high sensitivity instrument, uses digital counting for low level protein detection and offers several advantages with a unique platform design, in addition to specialized chemistry for enhanced specificity.

Controlling the SMCxPRO™ system is an integrated software package that is 21 CFR part II compliant. The software has easy to use, flexible command and data interpretation functions and is also LIMS compatible.

By using a 642 nm laser focused 250 µm above the base of an Aurora plate, a rotating objective scans through the free-floating suspension exciting fluorochromes as they pass through the interrogation space.

A low noise Avalanche Photodiode (APD) counts individual photons as they are emitted. The focused interrogation space of acquisition reduces cross talk from well to well, flare from meniscus diffusion of light, as well as inherent interference from turbid solutions.

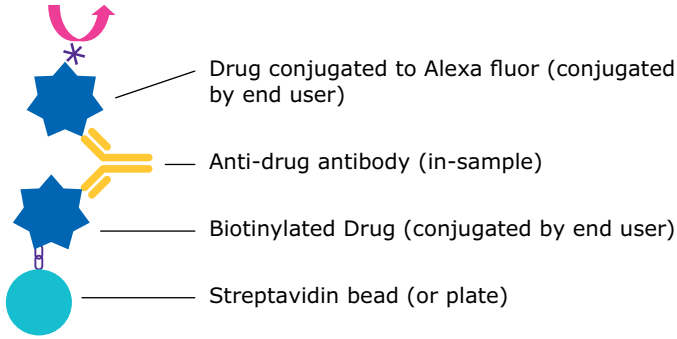


Figure 1. Bridging assay format for ADA detection in sample. immunocomplex Drug is Alexa and Biotin conjugated, and is captured on a magnetic streptavidin bead.

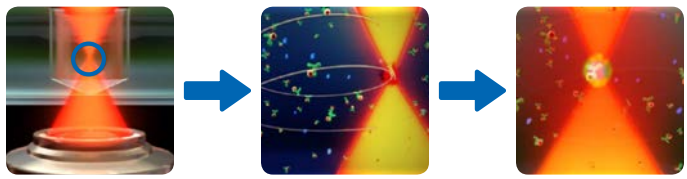
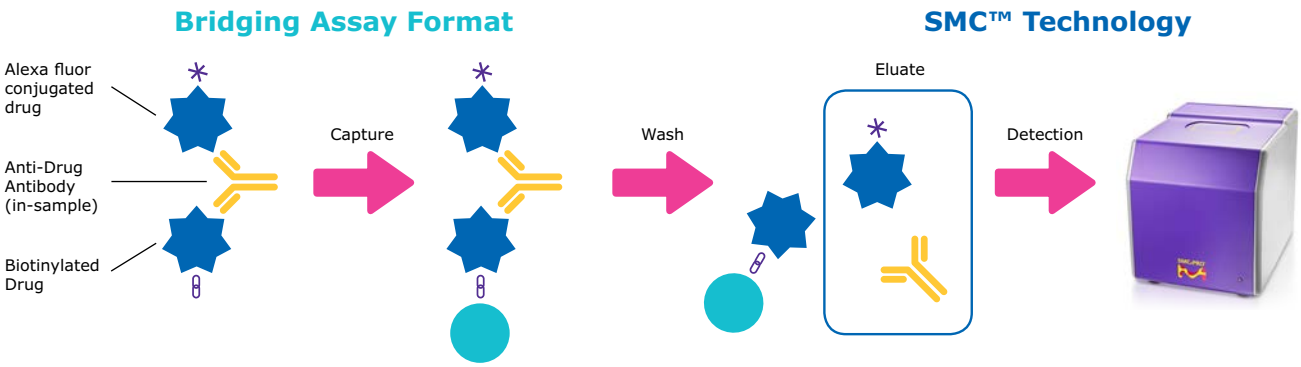


Figure 2. Counting of Alexa conjugated drug as it traverses through interrogation window.

Simple Workflow

Upon completion of the derivatization of the drug for use as capture and detection, the workflow for assay development is as follows:



Offline Sample Incubation

- ADA in sample is incubated for 2hr or overnight

Complex Capture

- Complex is captured onto blocked beads
- Wash to remove unbound antibodies

Elution

- Complex is dissociated, beads are magnetically separated and eluate transferred to read plate.

Single Molecule Counting

- Rotating laser scans sample
- Alexa-conjugated drug is excited and photons generated is counted by an APD

In developing an immunogenicity assay, optimization is required to fully validate for the immunological system being studied. Considerations such as those listed below can be easily studied with the SMC technology platform:

- Drug tolerance
- Cut point/Matrix Tolerance
- Sensitivity/Dynamic range of the assay
- Reproducibility

Further Optimization

Further optimization of different variables can take place to produce the most sensitive assay. These include:

- Drug concentration (capture and detection reagent)
- Assay Diluents (to mitigate HAMA or other interfering factors)
- Sample volume
- Number of wash steps
- Incubation time
- Standard / sample diluent
- Determination of minimum required dilution (MRD)
- Evaluation of Drug interference / tolerance

Drug Tolerance

In bridging assays of this type, it is important to minimize the amount of free (unlabelled capture or detection reagent) drug to quantify and drive the equilibrium in favour of quantifying ADA's in samples.

Drug tolerance is an important consideration in immunogenicity and is a challenge faced where the ability to quantify ADA in matrix is reduced in the presence of high drug concentration as result of competition. Several methods have been used to overcome this challenge, which include acid dissociation.

By using a platform such as SMC™, with better sensitivity, may help overcome this by simple dilution, thereby eliminating the need for acid dissociation.

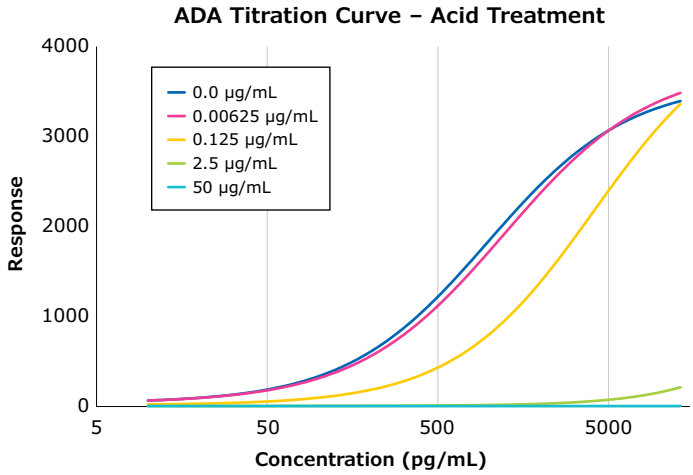


Figure 4. Example of drug tolerance on SMC Technology.

Further Implications of Immunogenicity — Adaptive Immune Response Assessment

Screening assays do not necessarily need to identify isotypes but need to be capable of binding multiple relevant classes or sub-classes. A number of isotypes play a major role in the immunogenic response. For instance:

- IgE-specific assays may be informative for products with a history of high risk of anaphylaxis.
- IgG4-specific assays may be informative for products that are chronically administered, or on erythropoietin-treated patients with pure-red cell aplasia.
- IgE and IgG4-specific assays may be requested on a case-by-case basis by the FDA due to hypersensitivity. The Compliment cascade can also be mediated by IgM and IgG.

These responses ultimately leads to generation of an inflammatory response through the formation of anaphylatoxins, such as C1q, C4a, C3a, and C5a.

Engagement of FcR or CR (Complement receptor) on cells, through immune complex cross-linking, results in the production of chemokines and growth factors that have a cascade effect on trafficking and growth of T and B cells.

This leads to release of cytokines and chemokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IL-21, IFN-g) which ultimately leads to tissue damage.

We have several products that can assist in the assessment of these responses and are available for the Luminex® platform using MILLIPLEX® kits.

Human High Sensitivity T Cell

◉ (Cat. No. HSTCMAG-28SK)
(Cat. No. HSTCMAG28SPMX13)♦
(Bulk Cat. No. HSTCMAG28PMX13BK)♦
(Cat. No. HSTCMAG28SPMX21)
(Bulk Cat. No. HSTCMAG28PMX21BK)

Fractalkine/CX3CL1	IL-12 (p70)♦
GM-CSF♦	IL-13♦
IFNγ♦	IL-17A/CTLA8
IL-1β♦	IL-21
IL-2♦	IL-23
IL-4♦	I-TAC/CXCL11
IL-5♦	MIP-1α/CCL3
IL-6♦	MIP-1β/CCL4
IL-7♦	MIP-3α/CCL20
IL-8/CXCL8♦	TNFα♦
IL-10♦	

Human Immunoglobulin Isotyping

◉ (Cat. No. HGAMMAG-301K)

IgA	IgG3
IgG1	IgG4
IgG2	IgM

Human IgE – Singleplex

(Cat. No. HGAMMAG-303E)

IgE

Human Complement Panel 1

(Cat. No. HCMP1MAG-19K)

Adipsin/Factor D	C5a
C2	C9
C4b	Factor I
C5	Mannose-binding Lectin (MBL)

Human Complement Panel 2

(Cat. No. HCMP2MAG-19K)

C1q	Factor B
C3	Factor H
C3b/iC3b	Factor P/Properdin
C4	

Legend key for MILLIPLEX® MAP kits

♦ Available in Cat. No. listed
◉ Available for custom premix

Mouse High Sensitivity T Cell

(Cat. No. MHSTCMAG-70K)
(Cat. No. MHSTCMAG-70KPMX)
(Bulk Cat. No. MHSTCMAG-70KPXBK)

GM-CSF	IL-10
IFNγ	IL-12 (p70)
IL-1α	IL-13
IL-1β	IL17A/CTLA8
IL-2	KC/GROα/CXCL1
IL-4	LIX
IL-5	MCP-1/CCL2
IL-6	MIP-2/CXCL2
IL-7	TNFα

Mouse Immunoglobulin Isotyping

◉ (Cat. No. MGAMMAG-300K)

IgA	IgG2b
IgG1	IgG3
IgG2a	IgM

Mouse IgE – Singleplex

(Cat. No. MGAMMAG-300E)

IgE

SMC™ Immunogenicity Assay Development Kit (Cat. No. 03-0175-00)

Combining our Immunoassay portfolio to study the impact on the immunogenicity of a therapeutic can provide great insight into the mechanism of the response. The SMC™ technology can offer increased sensitivity which may assist in the detection of low affinity antibodies and lead to earlier detection of primary ADA response, overcome matrix effects and may reduce drug tolerance.

The MILLIPLEX® kits can offer insight into the mechanism of the response and also help understand the immune complex mediated responses to the ADA.

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To place an order or receive technical assistance

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For other countries across Europe and the world, please visit: **EMDMillipore.com/offices**
For Technical Service, please visit: **EMDMillipore.com/techservice**

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