
Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products

Guidance for Industry

DRAFT GUIDANCE

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For questions regarding this draft document, contact (CDER) Susan Kirshner at 301-827-1731; (CBER) Office of Communication, Outreach and Development, 800-835-4709 or 240-402-8010; or (CDRH) Office of Communication and Education, 800-638-2041 or 301-796-7100.

**U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)
Center for Devices and Radiological Health (CDRH)**

**April 2016
Pharmaceutical Quality/CMC
Revision 1**

Assay Development and Validation for Immunogenicity Testing of Therapeutic Protein Products

Guidance for Industry

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TABLE OF CONTENTS

I.	INTRODUCTION.....	1
II.	BACKGROUND	2
III.	GENERAL PRINCIPLES.....	2
IV.	ASSAY DESIGN ELEMENTS.....	4
A.	Testing Strategy	4
1.	<i>Multi-Tiered Testing Approach.....</i>	<i>4</i>
2.	<i>Immunoglobulin Isotypes.....</i>	<i>5</i>
3.	<i>Epitope Specificity</i>	<i>5</i>
B.	Assay Cut Point.....	6
C.	Sensitivity.....	7
1.	<i>Assay Sensitivity.....</i>	<i>7</i>
2.	<i>Drug Tolerance.....</i>	<i>8</i>
D.	Specificity and Selectivity.....	8
1.	<i>Matrix Interference</i>	<i>9</i>
2.	<i>Minimal Required Dilution.....</i>	<i>10</i>
E.	Precision.....	10
F.	Reproducibility.....	11
G.	Robustness and Sample Stability.....	11
H.	Selection of Format.....	11
I.	Selection of Reagents	12
1.	<i>Development of Positive Control Antibodies.....</i>	<i>12</i>
2.	<i>Development of Negative Controls.....</i>	<i>13</i>
3.	<i>Detection Reagent Consideration.....</i>	<i>13</i>
4.	<i>Controlling Non-Specific Binding.....</i>	<i>14</i>
J.	Reporting Results for Qualitative and Semi-Quantitative Assays	14
K.	Other Considerations for Assay Development	15
1.	<i>Pre-Existing Antibodies</i>	<i>15</i>
2.	<i>Rheumatoid Factor</i>	<i>15</i>
3.	<i>Monoclonal Antibodies</i>	<i>15</i>
4.	<i>Conjugated Proteins</i>	<i>16</i>
5.	<i>Products With Multiple Functional Domains.....</i>	<i>16</i>
V.	ASSAY DEVELOPMENT	16
A.	Development of Screening Assay.....	16
B.	Development of Confirmatory Assay	17
1.	<i>Selection of Format for Confirmatory Assay.....</i>	<i>17</i>
2.	<i>Cut Point of Confirmatory Assay.....</i>	<i>17</i>
C.	Development of Titering Assay.....	17

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1. Titer Determination	17
2. Cut Point of Titering Assay.....	18
D. Development of Neutralization Assay	18
1. Selection of Format for Neutralization Assay.....	18
2. Activity Curve of Neutralization Assay.....	19
3. Considerations for Matrix Interference for Neutralization Assay.....	20
4. Cut Point of Neutralization Assay.....	21
5. Additional Considerations for Neutralization Assay.....	21
VI. ASSAY VALIDATION	22
A. General Considerations for Assay Validation	22
B. Validation of Screening Assay	24
1. Sensitivity of Screening Assay.....	24
2. Cut Point of Screening Assay.....	24
C. Validation of Confirmatory Assay	24
D. Validation of Titering Assay	25
E. Validation of Neutralization Assay.....	25
VII. IMPLEMENTATION OF ASSAY TESTING.....	26
A. Obtaining Patient Samples.....	26
B. Concurrent Positive and Negative Quality Controls	27
C. Confirmation of Cut Point in the Target Population.....	27
VIII. DOCUMENTATION.....	28
REFERENCES.....	29

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1 **Assay Development and Validation for Immunogenicity Testing of**
2 **Therapeutic Protein Products**
3 **Guidance for Industry¹**
4

5
6 This draft guidance, when finalized, will represent the current thinking of the Food and Drug
7 Administration (FDA or Agency) on this topic. It does not establish any rights for any person and is not
8 binding on FDA or the public. You can use an alternative approach if it satisfies the requirements of the
9 applicable statutes and regulations. To discuss an alternative approach, contact the FDA office
10 responsible for this guidance as listed on the title page.
11

12
13
14
15 **I. INTRODUCTION**
16

17 This guidance provides recommendations to facilitate industry’s development and validation of
18 immune assays for assessment of the immunogenicity of therapeutic protein products during
19 clinical trials. Specifically, this document includes guidance regarding the development and
20 validation of screening assays, confirmatory assays, titering assays, and neutralization assays.^{2,3}
21 For the purposes of this guidance, immunogenicity is defined as the propensity of the therapeutic
22 protein product to generate immune responses to itself and to related proteins or to induce
23 immunologically related adverse clinical events. The recommendations for assay development
24 and validation provided in this document apply to assays for detection of anti-drug antibody(ies)
25 (ADA).⁴ This guidance may also apply to some combination products on a case-by-case basis.⁵

¹ This guidance has been prepared by the Office of Medical Policy in the Center for Drug Evaluation and Research in cooperation with the Center for Biologics Evaluation and Research and the Center for Devices and Radiological Health at the Food and Drug Administration.

² This document specifically does not discuss the development or validation of anti-drug antibody(ies) (ADA) assays for animal studies; however, some concepts discussed are relevant to the design of ADA studies for nonclinical testing. Refer to the International Conference on Harmonisation (ICH) guidance for industry *S6(R1) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals* for more information regarding immunogenicity assessments in animal toxicology studies. Also see the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*, where the topic “Utility of Animal Studies” is covered in more detail. We update guidances periodically. To make sure you have the most recent version of a guidance, check the FDA guidance Web page at <http://www.fda.gov/RegulatoryInformation/Guidances/default.htm>.

³ For information on clinical immunogenicity assessment of proposed biosimilar biological products, see the guidance for industry *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product*.

⁴ This guidance does not pertain to immunogenicity assays for assessment of immune response to preventative and therapeutic vaccines for infectious disease indications.

⁵ General information on combination products is available at <http://www.fda.gov/CombinationProducts/default.htm>.

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26 This document does not discuss the product and patient risk factors that may contribute to
27 immunogenicity.⁶ This guidance, including any discussions of terminology used in this
28 guidance, does not apply to in vitro diagnostic products.⁷ This guidance revises the draft
29 guidance for industry *Assay Development for Immunogenicity Testing of Therapeutic Proteins*
30 issued in December 2009. The information in this guidance has been reorganized for clarity and
31 includes new information on titering and confirmatory assays.

32
33 In general, FDA’s guidance documents do not establish legally enforceable responsibilities.
34 Instead, guidances describe the Agency’s current thinking on a topic and should be viewed only
35 as recommendations, unless specific regulatory or statutory requirements are cited. The use of
36 the word *should* in Agency guidances means that something is suggested or recommended, but
37 not required.

II. BACKGROUND

41
42 Patient immune responses to therapeutic protein products have the potential to affect product
43 safety and efficacy.⁸ The clinical effects of patient immune responses are highly variable,
44 ranging from no effect at all to extremely harmful effects to patient health. Detection and
45 analysis of ADA formation is a helpful tool in understanding potential patient immune responses.
46 Information on immune responses observed during clinical trials, particularly the incidence of
47 ADA induction and the implications of ADA responses for therapeutic protein product safety
48 and efficacy, is crucial for any therapeutic protein product development program. Accordingly,
49 such information, if applicable, should be included in the prescribing information as a subsection
50 of the ADVERSE REACTIONS section entitled “Immunogenicity.” Therefore, the development
51 of valid, sensitive, specific, and selective assays to measure ADA responses is a key aspect of
52 therapeutic protein product development.

III. GENERAL PRINCIPLES

53
54
55
56
57 The risk to patients of mounting an immune response to a therapeutic protein product will vary
58 with the product. FDA recommends adoption of a risk-based approach to evaluating and
59 mitigating immune responses to or immunologically related adverse clinical events associated

⁶ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*, where these topics are covered in more detail.

⁷ Per 21 CFR 809.3(a), “in vitro diagnostic products are those reagents, instruments, and systems intended for use in the diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body. These products are devices as defined in section 201(h) of the Federal Food, Drug, and Cosmetic Act (the act), and may also be biological products subject to section 351 of the Public Health Service Act.”

⁸ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*.

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60 with therapeutic protein products that affect their safety and efficacy.⁹ Immune responses may
61 have multiple effects, including neutralizing activity and the ability to induce hypersensitivity
62 responses. Immunogenicity tests should be designed to detect ADA that could mediate
63 unwanted biological or physiological consequences.

64
65 Screening assays, also known as binding antibody (BAb) assays, are used to detect all antibodies
66 that bind to the therapeutic protein product. The specificity of BAb for the therapeutic protein
67 product is established using confirmatory assays. ADA are further characterized using titering
68 and neutralization assays. Titering assays are used to characterize the magnitude of the ADA
69 response. It is important to characterize this magnitude with titering assays because the impact
70 of ADA on safety and efficacy may correlate with ADA titer and persistence rather than
71 incidence (Cohen and Rivera 2010). Neutralization assays assess the ability of ADA to interfere
72 with the therapeutic protein product-target interactions. Therefore, neutralizing antibodies
73 (NAb) are a subset of BAb. It is important to characterize neutralizing activity of ADA with
74 neutralization assays because the impact of ADA on safety and efficacy may correlate with NAb
75 activity rather than ADA incidence (Calabresi, Giovannoni, et al. 2007; Goodin, Frohman, et al.
76 2007; Cohen and Rivera 2010). Similarly, it may be important in some cases to establish NAb
77 titers. Additional characterization assays, such as isotyping, epitope mapping, and assessing
78 cross-reactivity, e.g., to endogenous counterparts or to other products, may be useful.

79
80 The optimal time to design, develop, and validate ADA assays during therapeutic protein product
81 development depends on the risk assessment of the product (Mire-Sluis, Barrett, et al. 2004;
82 Gupta, Indelicato, et al. 2007; Shankar, Devanarayan, et al. 2008; Gupta, Devanarayan, et al.
83 2011). The sponsor should provide a rationale for the immunogenicity testing paradigm,
84 preferably at the investigational new drug application (IND) stage, during phase 1. Because
85 ADA assays are critical when immunogenicity poses a high clinical risk (e.g., assessment of a
86 therapeutic protein product with a non-redundant endogenous counterpart) and real-time data
87 concerning patient responses are needed, the sponsor should implement preliminary validated
88 assays early, before and during phase 1, and obtain data in real time. Real-time assessments
89 entail analyses of the samples as soon as possible after sampling, before banking of the samples,
90 and prior to additional dosing when the dosing regimen allows. In lower risk situations, the
91 sponsor may bank patient samples so they can be tested when suitable assays are available. FDA
92 encourages sponsors to test samples during phase 1 and phase 2 studies using suitable assays.
93 Samples derived from pivotal studies should be tested with fully validated assays. At the time of
94 license application, the sponsor should provide data supporting full validation of the assays.
95 Recommendations regarding the timing of ADA sample collection can be found in section
96 VII.A.¹⁰

97

⁹ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*.

¹⁰ See the guidance for industry *Immunogenicity Assessment for Therapeutic Protein Products*, where immunogenicity risk assessment and mitigation considerations are covered in more detail. Guidance on appropriate assay development and validation for immunogenicity testing is also available in the ICH guidances for industry *Q2A Text on Validation of Analytical Procedures* and *Q2B Validation of Analytical Procedures: Methodology*.

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98 Assays for detection of ADA facilitate understanding of the immunogenicity, safety, and efficacy
99 of therapeutic protein products. However, the detection of ADA is dependent on key operating
100 parameters of the assays (e.g., sensitivity, specificity), which vary between assays.¹¹ Although
101 information on ADA incidence is typically included in the prescribing information under an
102 “Immunogenicity” subsection of the ADVERSE REACTIONS section, FDA cautions that
103 comparison of ADA incidence among products, even for products that share sequence or
104 structural homology, can be misleading. This is because detection of ADA formation is highly
105 dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of
106 ADA (including NAb) positivity in an assay may be influenced by factors such as method,
107 sample handling, timing of sample collection, concomitant medications, and disease condition.
108 Therefore, comparing immunogenicity rates among therapeutic protein products with structural
109 homology for the same indication is unsound, even though fully validated assays are employed.
110 When a true comparison of immunogenicity across different therapeutic protein products that
111 have homology is needed, it should be obtained by conducting a head-to-head clinical study
112 using a standardized assay under the same conditions that has equivalent sensitivity and
113 specificity for both therapeutic protein products.¹²

114
115 The recommendations on assay development and validation provided in this guidance are based
116 on common issues encountered by the Agency upon review of immunogenicity submissions.
117 Sponsors should contact FDA for any product-specific guidance. Isotyping and cross-reactivity
118 assay designs should be discussed with FDA. Other publications may also be consulted for
119 additional insight (see Mire-Sluis, Barrett, et al. 2004; Gupta, Indelicato, et al. 2007; Shankar,
120 Devanarayan, et al. 2008; Gupta, Devanarayan, et al. 2011). In general, FDA recommends that
121 sponsors develop assays that are optimized for sensitivity, specificity, selectivity, precision,
122 reproducibility, and robustness (see sections IV.C through G).

123

124

IV. ASSAY DESIGN ELEMENTS

126

127 This section applies to all types of assays for detection of ADA, unless specified otherwise.

128

A. Testing Strategy

129

130

1. Multi-Tiered Testing Approach

132

133 FDA recommends a multi-tiered ADA testing approach because of the size of some clinical trials
134 and the necessity of testing patient samples at several time points. In this paradigm, a rapid,
135 sensitive screening assay is initially used to assess clinical samples. The initial screening assay
136 should be sensitive to low levels of low- and high-affinity ADA (see section V.A). Samples
137 testing positive in the screening assay are then subjected to a confirmatory assay to demonstrate

¹¹ See the United States Pharmacopeia (USP) General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* for a broader discussion of various assay types.

¹² For information on proposed biosimilar products, see the guidance for industry *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product*.

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138 that ADA are specific for the therapeutic protein product. For example, a competition assay
139 could confirm that antibody is specifically binding to the therapeutic protein product and that the
140 positive finding in the screening assay is not a result of non-specific interactions of the test serum
141 or detection reagent with other materials in the assay milieu such as plastic or other proteins.
142

143 Samples identified as positive in the confirmatory assay should be further characterized in other
144 assays, such as titering and neutralization assays. In some cases, assays to detect cross-reactivity
145 to other proteins with homology, such as the corresponding endogenous protein, may be needed.
146 Further, tests to assess the isotype of the antibodies and their epitope specificity may also be
147 recommended once samples containing antibodies are confirmed as positive.
148

2. Immunoglobulin Isotypes

151 The initial screening assay should be able to detect all relevant immunoglobulin (Ig) isotypes.
152 For non-mucosal routes of administration, and in the absence of anaphylaxis, the expected ADA
153 isotypes are IgM and IgG. For mucosal routes of administration, IgA isotype ADA are also
154 expected. Although FDA expects that all relevant isotypes be detected in screening assays, it is
155 not necessary that the screening assay establish which isotypes are being detected. For example,
156 assays using the bridging format may provide no information on which isotypes are being
157 detected. Bridging assay format can theoretically detect antibodies of most isotypes, but may not
158 detect IgG4 isotypes. In some circumstances the sponsor should develop assays that discriminate
159 between antibody isotypes. For example, for therapeutic protein products where the risk for
160 anaphylaxis is a concern, antigen-specific IgE assays should be developed. In addition, the
161 generation of IgG4 antibodies has been associated with immune responses generated under
162 conditions of chronic antigen exposure, such as with factor VIII treatment, and in erythropoietin-
163 treated patients with pure red cell aplasia (Matsumoto, Shima, et al. 2001; Aalberse and
164 Schuurman 2002). Consequently, depending on the clinical concern, assessing for specific
165 isotypes may be needed.
166

3. Epitope Specificity

169 FDA recommends that the sponsor direct initial screening tests against the whole therapeutic
170 protein product and, when relevant, its endogenous counterpart. For some therapeutic protein
171 products, the sponsor may need to investigate the ADA to specific epitopes to which immune
172 responses are specifically generated. For example, determination of epitope specificity is
173 recommended for some fusion molecules because the region where the two molecules join may
174 form a neoantigen, and immune responses to this region may arise. Because of epitope
175 spreading, immune responses to other parts of the molecule may ensue, leading to the generation
176 of antibodies to the therapeutic protein product or its endogenous counterpart (Prummer 1997;
177 Miller, Korn, et al. 1999; Disis, Goodell, et al. 2004; Thrasyvoulides, Liakata, et al. 2007; van
178 der Woude, Rantapaa-Dahlqvist, et al. 2010; Hintermann, Holdener, et al. 2011). For these
179 therapeutic protein products, FDA encourages sponsors to investigate the initiating event in the
180 immune cascade. This knowledge may allow for modification to the protein to reduce its
181 potential immunogenicity. Similarly, for therapeutic protein products with modifications, such
182 as PEGylation, sponsors should develop assays to determine the specificity of ADA for the

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183 protein component as well as the modification to the therapeutic protein product. Also see
184 sections IV.K.4 and 5.

185

B. Assay Cut Point

187

188 The cut point of the assay is the level of response of the assay that defines the sample response as
189 positive or negative. Information specific to establishing the cut point for the respective assay
190 types is provided in sections V and VI. Establishing the appropriate cut point is critical to
191 ensuring acceptable assay sensitivity.

192

193 The cut point of the assay can be influenced by a myriad of interfering factors, such as pre-
194 existing antibodies, rheumatoid factor (RF), human anti-mouse antibodies, and the levels of
195 product-related material or homologous proteins in the matrix. These factors should be
196 considered early on in assay development when defining the cut point. Because samples from
197 different target populations and disease states may have components that can cause the
198 background signal from the assay to vary, different cut points may be needed for discrete
199 populations being studied.

200

201 The cut point should be statistically determined using samples from treatment-naïve subjects.¹³
202 By performing replicate assay runs with these samples, the variability of the assay can be
203 estimated. During assay development, a small number of samples may be used to estimate the
204 cut point. This may be done with as few as 5–10 samples from treatment-naïve subjects.

205

206 The specific approach employed to determine the cut point will depend on various factors.
207 Specifically, because the cut point should identify any samples that produce a signal beyond that
208 of the variability of the assay, the sponsor should consider the impact of statistically determined
209 outlier values as well as true-positive samples when establishing the cut point. The sponsor
210 should provide justification for the removal of any data points, along with the respective method
211 used to determine their status as outliers. Positive values and samples may derive from non-
212 specific serum factors or the presence of pre-existing antibodies in patient samples (Ross,
213 Hansen, et al. 1990; Turano, Balsari, et al. 1992; Coutinho, Kazatchkine, et al. 1995; Caruso and
214 Turano 1997; van der Meide and Schellekens 1997; Boes 2000). Although pre-existing
215 antibodies to a variety of endogenous proteins are present in healthy individuals, these can be
216 much higher in some disease states. The sponsor should identify those samples with pre-existing
217 antibodies, for example, through immunodepletion approaches, and remove them from the cut
218 point analysis. If the presence of pre-existing antibodies is a confounding factor, it may be
219 necessary to assign positive responses or a cut point based on the difference between individual
220 patient results before and after exposure. It is possible to arrive at a reasonable value to define
221 assay cut point through careful design consideration, such as utilizing the minimal required
222 dilution (MRD) of the sample, removing statistical outliers from analyses, minimizing the impact

¹³ Treatment-naïve subjects could be healthy individuals or a patient population not exposed to therapeutic protein product, depending on the stage of assay development or validation and on the availability of samples. Sponsors should provide justification for the appropriateness of the samples used.

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223 of interfering factors, improving assay drug tolerance, and using an approach to account for pre-
224 existing antibodies.

225

226 C. Sensitivity

227

228 1. Assay Sensitivity

229

230 The sponsor should determine the sensitivity of the assay to have confidence when reporting
231 immunogenicity rates. Assay sensitivity represents the lowest concentration at which the
232 antibody preparation consistently produces either a positive result or readout equal to the cut
233 point determined for that particular assay.¹⁴ FDA recommends that screening and confirmatory
234 ADA assays achieve a sensitivity of at least 100 nanograms per milliliter (ng/mL). Although
235 traditionally FDA has recommended sensitivity of at least 250–500 ng/mL, recent data suggest
236 that concentrations as low as 100 ng/mL may be associated with clinical events (Plotkin 2010;
237 Zhou, Hoofring, et al. 2013). However, it is understood that neutralization assays may not
238 always achieve that level of sensitivity.

239

240 The assays should have sufficient sensitivity to enable detection of low levels of ADA before the
241 amount of ADA reaches levels that can be associated with altered pharmacokinetic,
242 pharmacodynamic, safety, or efficacy profiles. Because assessment of patient antibody levels
243 will occur in the presence of biological matrix, testing of assay sensitivity should be performed
244 with the relevant dilution of the same biological matrix (e.g., serum or plasma, with the same
245 anticoagulant as the diluent, from the target population). The final sensitivity should be
246 expressed as mass of antibody detectable/mL of undiluted matrix. Therefore, assay sensitivity
247 should be reported after factoring in the MRD. Assay sensitivity should not be reported as titer.
248 During development, sensitivity should be assessed using both individual as well as pooled
249 samples from treatment-naïve subjects so that the suitability of the negative control can be
250 established.

251

252 Assay sensitivity should be determined by testing serial dilutions of a positive control antibody
253 of known concentration in pooled negative control matrix. The dilution series should be no
254 greater than two- or threefold, and a minimum of five dilutions should be tested. Alternatively,
255 sensitivity can be calculated by interpolating the linear portion of the dilution curve to the assay
256 cut point. As noted previously, assay sensitivity should be reported in mass units per volume of
257 undiluted matrix.

258

259 A purified preparation of antibodies specific to the therapeutic protein product should be used to
260 determine the sensitivity of the assay so that assay sensitivity can be reported in mass units/mL
261 of matrix. Antibodies used to assess sensitivity can take the form of affinity purified polyclonal
262 preparations or monoclonal antibodies (mAb).

263

264 A low positive system suitability control containing a concentration of ADA slightly above the
265 sensitivity of the assay should be used to ensure that the sensitivity of the assay is consistent

¹⁴ See the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies* for a discussion on *Relative Sensitivity*.

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266 across assay runs. The low positive system suitability control should be designed to fail in 1% of
267 the runs (see section IV.I.1).

268

269 **2. *Drug Tolerance***

270

271 Therapeutic protein product or the endogenous counterpart present in the serum may interfere
272 with the sensitivity of the assay. Specifically, complexes formed between ADA and the
273 therapeutic protein product, also called ADA-drug complexes, that prevent detection of ADA in
274 the test format can form if product-related materials are present in the test sample. This is
275 because ADA assays are generally designed to detect uncomplexed ADA. The assessment of
276 assay sensitivity in the presence of the expected levels of interfering therapeutic protein product,
277 also known as the assay's drug tolerance, is critical to understanding the suitability of the method
278 for detecting ADA in dosed patients.¹⁵ FDA recommends that the sponsor examine assay drug
279 tolerance early in assay development. The sponsor may examine drug tolerance by deliberately
280 adding different known amounts of purified ADA into individual ADA-negative control samples
281 in the absence or presence of different quantities of the therapeutic protein product under
282 consideration and determining quantitatively whether the therapeutic protein product interferes
283 with ADA detection. Results obtained in the absence and presence of different quantities of the
284 therapeutic protein product under consideration should be compared. There should be a
285 relationship between the quantity of antibody and the amount of therapeutic protein product
286 required for a specified degree of inhibition. Data from pharmacokinetic studies may be useful
287 in establishing optimal sample collection times. Acid dissociation pretreatment or other
288 approaches may be used to disrupt circulating ADA-drug complexes, which may lead to
289 increased assay drug tolerance. Interference from the therapeutic protein product can be
290 minimized if the sponsor collects patient samples at a time when the therapeutic protein product
291 has decayed to a level where it does not interfere with assay results.

292

293 **D. *Specificity and Selectivity***

294

295 Demonstrating assay specificity and selectivity is critical to the interpretation of immunogenicity
296 assay results. Specificity refers to the ability of a method to detect ADA that bind the therapeutic
297 protein product but not assay components such as surfaces or reagents. The assays should
298 exclusively detect the target analyte, in this case the ADA.¹⁶ The selectivity of an ADA assay is
299 its ability to identify therapeutic protein product-specific ADA in a matrix such as serum or
300 plasma that may contain potential interfering substances. Assay results may be affected by
301 interference from the matrix or from on-board therapeutic protein product.¹⁷ Lack of assay
302 specificity or selectivity can lead to false-positive results, which could obscure relationships
303 between ADA response and clinical safety and efficacy measures. Demonstrating the specificity

¹⁵ See the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

¹⁶ See the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

¹⁷ See the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

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304 and selectivity of antibody responses to mAb, Fc-fusion protein, and Ig-fusion proteins poses
305 particular challenges because of the high concentration of Ig in human serum. The sponsor
306 should clearly demonstrate that the assay method specifically detects anti-mAb and not the mAb
307 product itself, non-specific endogenous antibodies, or antibody reagents used in the assay.
308 Similarly, for patient populations with a high incidence of RF, the sponsor should demonstrate
309 that RF does not interfere with the detection method. Host cell proteins and other product-
310 related impurities may interfere with demonstrating the assay specificity and selectivity as well.

311
312 A straightforward approach to addressing specificity and selectivity is to demonstrate that
313 binding can be blocked by soluble or unlabeled purified therapeutic protein product. One
314 approach is to incubate positive and negative control antibody samples with the purified
315 therapeutic protein product or its components under consideration. Inhibition of signal in the
316 presence of the relevant therapeutic protein product or its components demonstrates that the
317 response is specific and selective. For responses to mAb products, inclusion of another mAb
318 with the same Fc but different variable region can be critical. For responses to other proteins, an
319 unrelated protein of similar size and charge can be used. If the assay is specific and selective for
320 the protein in question, generally the addition of that protein in solution should reduce the
321 response to background or the cut point, whereas the addition of an unrelated protein of similar
322 size and charge should have no effect. Conversely, addition of the protein in question should
323 have little effect on antibodies specific to an unrelated protein. Selectivity should further be
324 evaluated by performing recovery studies, in which positive control antibodies are spiked into
325 matrix at defined concentrations, and the positive control antibody signal is compared to that
326 obtained from antibody spiked into assay buffer alone.

327 328 *1. Matrix Interference*

329
330 An important consideration is how interference from the assay matrix, which is composed of the
331 sample and the diluent, can affect assay performance. Components in the matrix other than
332 therapeutic protein product can interfere with assay results. For example, different
333 anticoagulants used during sample collection may have different effects in the assay, potentially
334 affecting the assay sensitivity and linearity. Sponsors should evaluate different salt anticoagulant
335 sample collection solutions for their effect on assay results.

336
337 Endogenous and exogenous components in serum or plasma may influence assay results, and it
338 is usually necessary to dilute patient samples for testing to minimize such effects. The sponsor
339 should examine the effect of such interferents by performing spike-and-recovery studies. The
340 sponsor should define the dilution factor that will be used for preparation of patient samples
341 before performing validation studies assessing potential interference of this matrix on assay
342 results (see section IV.D.2 on MRD).

343
344 Buffer components that are chemically related to the therapeutic protein product may also
345 interfere in the assay. For example, polysorbate is chemically similar to polyethylene glycol
346 (PEG) and therefore may interfere in the detection of anti-PEG antibodies. The chemical
347 composition of the buffer should be carefully considered during assay development.

348

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349 The sponsor may examine matrix interference by spiking different known amounts of purified
350 ADA into the assay buffer in the absence or presence of different matrix components.
351 Comparing the recovery of ADA in buffer alone with that in the matrix can provide input on the
352 degree of interference from matrix components. Furthermore, such analysis may guide decisions
353 on the MRD recommended for sample testing. In addition, the sponsor should examine other
354 parameters affecting patient samples, such as hemolysis, lipemia, presence of bilirubin, and
355 presence of concomitant medications that a patient population may be using. Samples that have
356 very high antibody titers may need additional testing, such as with different dilutions of the
357 competing product in the confirmatory assay, to ensure their identification.

2. *Minimal Required Dilution*

361 Matrix components can contribute to non-specific signal if undiluted, thereby obscuring positive
362 results. Therefore, there is frequently a need to dilute patient samples to maintain a reasonable
363 ability to detect ADA (sensitivity). Ideally, the MRD is the sample dilution that yields a signal
364 close to that of the assay diluent and allows for the highest signal-to-noise ratio. MRD typically
365 ranges from 1:5 to 1:100.

366
367 FDA recommends that the sponsor determine the MRD from a panel of appropriate number of
368 samples from treatment-naïve subjects. Determination of MRD usually involves serially diluting
369 treatment-naïve ADA-negative samples, as well as testing known amounts of purified antibody
370 (at high, medium, and low concentrations) in serially diluted matrix in comparison to the same
371 amount of antibody in buffer. This ensures a reasonable signal-to-noise ratio throughout the
372 range of the assay. The MRD should be calculated using at least 10 individual serum samples;
373 the appropriate number of samples will depend on various factors, including the variability of the
374 individual samples.

375
376 Although the MRD ultimately selected by the sponsor will depend on the assay design and
377 patient population, FDA recommends that dilutions not exceed 1:100. Higher dilution may
378 result in the spurious identification of a negative response when patients may actually possess
379 low levels of therapeutic protein product-specific antibodies, the occurrence of which can be
380 related to significantly altered pharmacokinetics, pharmacodynamics, safety, or efficacy profiles.
381 However, in some instances greater initial dilutions may be required, and the overall effect of
382 such dilutions on assay sensitivity and immunogenicity risk assessment should be considered.

E. Precision

384
385
386 Precision is a measure of the variability in a series of measurements for the same material run in
387 a method. Results should be reproducible within and between assay runs to assure adequate
388 precision.¹⁸ Demonstrating assay precision is critical to the assessment of ADA because assay
389 variability is the basis for determining the cut points and ensuring that low positive samples are

¹⁸ For more information on precision, see the guidance for industry *Bioanalytical Method Validation*. Also see the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

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390 detected as positive. To provide reliable estimates, the sponsor should evaluate both intra-assay
391 (repeatability) and inter-assay (intermediate precision) variability of assay responses.

F. Reproducibility

394
395 Reproducibility is an important consideration if an assay will be run by two or more independent
396 laboratories during a study, and a sponsor should establish the comparability of the data
397 produced by each laboratory.¹⁹ In addition, the assays should have the same precision between
398 different laboratories under the established assay operating conditions (for example, using the
399 same instrument platform).

G. Robustness and Sample Stability

400
401
402 Assay robustness is an indication of the assay's reliability during normal usage²⁰ and is assessed
403 by the capacity of the assay to remain unaffected by small but deliberate variations in method
404 and instrument performance that would be expected under relevant, real-life circumstances in
405 routine laboratory practice. For example, changes in temperature, incubation times, or buffer
406 characteristics, such as pH and salt concentration, can all impact assay results. The complexity
407 of bioassays makes them particularly susceptible to variations in assay conditions, and it is
408 essential to evaluate and optimize parameters such as cell passage number, incubation times, and
409 culture media components. The sponsor should examine robustness during the development
410 phase, and if small changes in specific steps in the assay affect results, specific precautions
411 should be taken to control their variability. FDA recommends storing patient samples in a
412 manner that preserves antibody reactivity at the time of testing. FDA recommends that the
413 sponsor avoid freeze-thaw cycles because freezing and thawing patient samples may also affect
414 assay results. However, studies evaluating long-term stability of positive control antibodies may
415 be useful.²¹

H. Selection of Format

416
417
418
419 A number of different assay formats and instrumentation are available that can be employed for
420 detection of ADA. These include, but are not limited to, direct binding assays, bridging assays,
421 and equilibrium binding assays. Each assay format has advantages and disadvantages, including
422 rapidity of throughput, sensitivity, selectivity, dynamic range, ability to detect various Ig
423 isotypes, ability to detect rapidly dissociating antibodies, and availability of reagents. One of the
424 major differences between each of these assay formats is the number and vigor of washes, which
425

¹⁹ For more information on reproducibility, see the guidance for industry *Bioanalytical Method Validation*. Also see the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*, the USP General Chapter 1225 *Validation of Compendial Procedures*, and the ICH guidance for industry *Q2B Validation of Analytical Procedures: Methodology*.

²⁰ For more information on robustness, see the ICH guidance for industry *Q2B Validation of Analytical Procedures: Methodology*. Also see the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

²¹ For more information on stability studies, see the guidance for industry *Bioanalytical Method Validation*.

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426 can have an effect on assay sensitivity. All assays should be evaluated for their ability to detect
427 rapidly dissociating antibodies such as IgM, which are common in early immune responses.
428 Failure to detect such antibodies in early immune responses to therapeutic protein products may
429 result in under-detection of true-positive antibody samples. Epitope exposure is also important
430 to consider because binding to plastic or coupling to other agents, such as reporters (i.e.,
431 fluorochromes, enzymes, or biotin), can result in conformational changes of the antigen that can
432 obscure, expose, modify, or destroy relevant antibody binding sites on the therapeutic protein
433 product in question.

I. Selection of Reagents

434
435
436
437 Many components of the assays for ADA detection may be standard or obtained from
438 commercial sources, for example, commercially available reagents such as Protein A/G coated
439 resins used in the depletion approach for confirmatory assays. Other components, however,
440 including positive control antibodies, negative controls, and system suitability controls, may
441 need to be generated specifically for the particular assay.

1. Development of Positive Control Antibodies

442
443
444
445 Sponsors may use different or the same positive control antibodies to establish and monitor
446 system suitability during routine assessment of assay performance, as well as to determine that
447 the assay employed is fit for purpose. For system suitability controls, a positive control
448 antibody, either mono- or polyclonal, used at concentrations adjusted to control the cut point and
449 dynamic range levels, may be suitable.

450
451 Positive control antibodies frequently are generated by immunizing animals in the absence or
452 presence of adjuvants. FDA recommends that positive control antibodies generated by
453 immunizing animals be affinity purified using the therapeutic protein product. This approach
454 enriches the polyclonal antibody preparation for ADA, which enables a more accurate
455 interpretation of sensitivity assessment results. The selection of animal species when generating
456 positive control antibodies should be carefully considered. For example, if an anti-human Ig
457 reagent will be used as a secondary reagent to detect patient antibodies, the positive control
458 antibodies and quality control (QC) samples should be detectable by that same reagent. When
459 the positive control antibody is not detectable by that same reagent, an additional secondary
460 reagent to detect the positive control antibody may be needed. In those cases, an additional
461 positive control antibody for the secondary reagent used to detect human antibodies should be
462 implemented to ensure that the reagent performs as expected. In some instances, the sponsor
463 may be able to generate a positive control antibody from patient samples.²² Although such
464 antibodies can be very valuable, such samples are generally not available in early trials.
465 Alternatively, individual mAb or panels of mAb may be used for positive control antibodies.
466 Sponsors should discuss with FDA alternative approaches to assay development and validation
467 in the rare event that a sponsor is not able to generate a positive control antibody.
468

²² Proper informed consent from patients is needed and should be planned ahead of time.

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469 Ideally, the positive control antibody used to determine assay applicability for the purpose of the
470 respective assay should reflect the anticipated immune response that will occur in humans. For
471 therapeutic mAb, the sponsor should give special consideration to the selection of a positive
472 control antibody for the assay. When animals are immunized with a chimeric, humanized, or
473 human mAb to develop a positive control antibody, the humoral response may be against the
474 human Fc and not the variable region of the molecule. Such positive control antibodies may not
475 be relevant for the anticipated immune response in patients where the response is primarily
476 directed to the antigen-binding regions.

477
478 Once a source of a positive control antibody has been identified, the sponsor should use that
479 source to assess assay performance characteristics such as sensitivity, selectivity, specificity, and
480 reproducibility. FDA recommends that sponsors generate and reserve positive control antibody
481 solution for use as a quality or system suitability control. For assay development and validation,
482 dilutions should be representative of a high, medium, and low value in the assay. This is needed
483 even for qualitative assays to understand whether assay performance is acceptable across a broad
484 range of antibody concentrations. Although high- and low-value QC samples should be used,
485 medium-value QC samples for detection of ADA are generally not needed for monitoring system
486 suitability during routine assessment of assay performance.

2. Development of Negative Controls

487
488
489 For negative control samples, it is recommended that when possible, the control population
490 should have the same disease condition. The control samples should represent a similar gender,
491 age, and concomitant medications so that the sample matrix is representative of the study
492 population. Similarly, control samples should be collected and handled in the same manner as
493 study samples with respect to, for example, type of anticoagulant used, sample volume, and
494 sample preparation and storage, because these pre-analytical variables can impact the
495 performance of control samples in the assay. It is frequently the case that such control samples
496 are not available for use during development or pre-study validation exercises. In those
497 situations, it is acceptable to use purchased samples or samples from healthy donors, but
498 important parameters of assay performance such as cut point, sensitivity, and selectivity should
499 be confirmed when samples from treatment-naïve subjects from the appropriate target population
500 become available.
501

502
503 FDA recommends that the sponsor establish a negative control for validation studies and patient
504 sample testing. In this regard, a pool of sera from an appropriate number of treatment-naïve
505 subjects can serve as a useful negative control. Importantly, the value obtained for the negative
506 control should be below but close to the cut point determined for the assay in the patient
507 population being tested. Negative controls that yield values far below the mean value derived
508 from individual serum samples used to establish the cut point may not be useful in ensuring
509 proper assay performance.

3. Detection Reagent Consideration

510
511
512
513 The selection of a suitable detection reagent (i.e., reporter) depends on the assay format chosen.
514 It is critical to minimize the non-specific signal from the detection reagent. The detection

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515 reagent chosen should have the adequate sensitivity required for the particular assay. These
516 factors should be taken into consideration when deciding on the detection reagent.

517

518 4. *Controlling Non-Specific Binding*

519

520 Every reagent, from the plastic of the microtiter plates to the developing agent, can affect assay
521 sensitivity and non-specific binding. One of the most critical elements is the selection of the
522 proper assay buffer and blocking reagents used to prevent non-specific binding to the solid
523 surface. The sponsor should carefully consider the number and timing of wash steps as well as
524 the detergents added to the assay buffer (i.e., blocking or wash buffer) to reduce background
525 noise, but still maintain sensitivity. A variety of proteins can be used as blocking reagents to
526 provide acceptable signal-to-noise ratio. However, these proteins may not all perform
527 equivalently in specific immunoassays. For example, they may not bind well to all types of solid
528 phases or may show unexpected cross-reactivity with the detecting reagent. Therefore, the
529 sponsor may need to test several blocking agents to optimize assay performance. Moreover,
530 including uncoated wells is insufficient to assess non-specific binding. Rather, determining the
531 capacity of ADA to bind to an unrelated protein of similar size and charge that may be present in
532 the sample may prove to be a better test of binding specificity.

533

534 **J. Reporting Results for Qualitative and Semi-Quantitative Assays**

535

536 Several approaches may be used to report positive antibody responses, and the appropriateness of
537 the approach used should be evaluated on a case-by-case basis. The most common approach is
538 qualitative, with patients reported as having a positive or negative antibody response.

539

540 For patients who are confirmed to be ADA positive, determining antibody levels can be
541 informative because it allows for the stratified assessment of ADA levels and their impact on
542 safety and efficacy. These relationships may not be elucidated unless ADA levels are
543 determined. Positive antibody responses may be reported as a titer (e.g., the reciprocal of the
544 highest dilution that gives a readout at or just above the cut point of the assay), when appropriate.
545 The MRD should be factored in the calculations of titers and provided when reporting titers.
546 Reporting levels of antibodies in terms of titers is appropriate and generally understood by the
547 medical community. Values may also be reported as amount of mass units of therapeutic protein
548 product neutralized per volume serum with the caveat that these are arbitrary in vitro assay units
549 and cannot be used to directly assess therapeutic protein product availability in vivo.

550

551 Unless the assay method used allows for independent determination of mass, antibody levels
552 reported in mass units are generally not acceptable because they are based on interpolation of
553 data from standard curves generated with a positive control antibody, and parallelism between
554 the reference standard and test article cannot be assumed. Thus, FDA does not consider it
555 necessary nor desirable for the sponsor to report patient antibody results in terms of mass units
556 unless (1) the results are determined by quantitative means or (2) a universally accepted and
557 accessible source of validated antibody is available as a control and parallelism between the
558 dilution curves of the control antibody and patient samples has been demonstrated. Furthermore,
559 even if parallelism is demonstrated, because the reference standard and test articles are likely to

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560 contain different populations of antibodies, the absolute mass units cannot be calculated.
561 Therefore, FDA understands that the mass units reported are relative rather than absolute values.

K. Other Considerations for Assay Development

562
563
564
565 A myriad of factors can affect the assessment of antibody levels, such as patient sample
566 variability, therapeutic protein product-dose response of the cells used to generate the standard
567 curve in a cell-based neutralization bioassay, affinity and avidity of the ADA, and concentration
568 of competing product in confirmatory assays. Accounting for such factors is important to
569 understand and analyze assay variability and avoid errors. Common factors that should be
570 considered include the following:

1. Pre-Existing Antibodies

571
572
573
574 A growing body of evidence in the medical literature suggests that B-cells and T-cells with
575 specificity for a number of self-proteins exist naturally and may even be heightened in some
576 disease states, such as in patients subjected to cytokine therapy or suffering from a variety of
577 immunological or immunoinflammatory diseases (Coutinho, Kazatchkine, et al. 1995; van der
578 Meide and Schellekens 1997; Boes 2000). For example, antibodies to interferon can be found in
579 normal individuals (Ross, Hansen, et al. 1990; Turano, Balsari, et al. 1992; Caruso and Turano
580 1997). Less surprisingly, subjects may have pre-existing antibodies to foreign antigens, such as
581 bacterial products, most likely as a result of exposure to the organism or cross-reactivity. Pre-
582 existing antibodies may have clinical effects and may affect the efficacy of the therapeutic
583 protein product being tested. An alternative to the qualitative screening assay approach may be
584 needed to assess the quantity and quality of ADA when pre-existing antibodies are present. For
585 example, testing samples for an increase in ADA using a semi-quantitative assay type such as a
586 titrating assay (see sections V.C and VI.D) can provide information on the impact of a therapeutic
587 protein product on product immunogenicity that is not provided by a qualitative assay.

2. Rheumatoid Factor

588
589
590
591 Measuring immune responses to therapeutic protein products that possess Ig tails, such as mAb
592 and Fc-fusion proteins, may be particularly difficult when RF is present in serum or plasma. RF
593 is generally an IgM antibody that recognizes IgG, although other Ig specificities have been
594 noted. Consequently, RF will bind Fc regions, making it appear that specific antibody to the
595 therapeutic protein product exists. Several approaches for minimizing interference from RF have
596 proven useful, including treatment with aspartame (Ramsland, Movafagh, et al. 1999) and
597 careful optimization of reagent concentrations so as to reduce background binding. When
598 examining immune responses to Fc-fusion proteins in clinical settings where RF is present, FDA
599 recommends developing an assay specific for the non-Fc region of the proteins.

3. Monoclonal Antibodies

600
601
602
603 Some special considerations pertain to the detection of antibodies against mAb. Animal-derived
604 mAb, particularly those of rodent origin, are expected to be immunogenic with the immune
605 response directed against the whole mAb molecule. In the early days of the therapeutic mAb

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606 industry, this was a key reason for the failure of clinical trials (Kuus-Reichel, Grauer, et al.
607 1994).

608
609 Technologies reducing the presence of non-human sequences in mAb, such as chimerization and
610 humanization, have led to a dramatic reduction but not elimination of immunogenicity. In these
611 cases, the immune responses are directed largely against the variable regions of the mAb
612 (Harding, Stickler, et al. 2010; van Schouwenburg, Kruithof, et al. 2014). As immune responses
613 against the variable regions of human mAb are anticipated, FDA does not expect that the use of
614 human mAb will further reduce immunogenicity by a significant margin. The assays that can
615 detect the reactivity against variable regions are considered more appropriate to evaluate the
616 potential impact of antibodies against mAb-based therapeutics in patients. However, engineering
617 of Fc portion (e.g., modification of the levels of afucosylation) in human antibodies may affect
618 immunogenicity. Many of these concerns also pertain to Fc-fusion proteins containing a human
619 Fc region.

4. Conjugated Proteins

620
621
622
623 Because antibody-drug conjugates (ADCs) are antibodies conjugated with small molecule drugs,
624 they represent a classic hapten-carrier molecule. Therefore, the immunogenicity assays should
625 be able to measure the responses to all components of the ADC therapeutic protein product,
626 including the antibody, linker-drug, and new epitopes that may result from conjugation. When
627 ADCs need to be labeled for immunogenicity assays, the conjugation should be performed
628 carefully because ADCs are already modified. The potential for increased hydrophobicity of the
629 labeled molecules may cause aggregation, and therefore the stability and solubility of these
630 capture reagents should be adequately characterized.

5. Products With Multiple Functional Domains

631
632
633
634 Some proteins possess multiple domains that function in different ways to mediate clinical
635 efficacy. An immune response to one domain may inhibit a specific function while leaving
636 others intact. Examination of immune responses to therapeutic protein products with multiple
637 functional domains may require development of multiple assays to measure immune responses to
638 different domains of the molecules.

V. ASSAY DEVELOPMENT

639
640
641
642
643 Information specific to development of respective assay types is provided in sections A through
644 D below. These sections supplement information relevant to all assay types provided in
645 section IV.

A. Development of Screening Assay

646
647
648
649 Based on the multi-tiered approach discussed previously in section IV.A, the first assay to be
650 employed for detection of ADA should be a highly sensitive screening assay that detects low-
651 and high-affinity ADA. Approximately 10 individual samples may be used to estimate the cut

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652 point early in assay development; however, this may need to be adjusted when treatment-naïve
653 samples from the target population become available. A low but defined false-positive rate is
654 desirable for the initial screening assay because it maximizes detection of true positives.
655 Subsequent assays can be employed to exclude false-positive results when determining the true
656 incidence of immunogenicity.

657

B. Development of Confirmatory Assay

658

659 Because the screening assay is designed to broadly detect the presence of antibodies that bind
660 product in serum samples with a defined false-positive rate, FDA recommends that the sponsor
661 develop assays to confirm the binding of antibodies that are specific to the therapeutic protein
662 product. Implementation of a suitable confirmatory assay is important to prevent data on ADA
663 false-positive patients from confounding the analyses of the impact of ADA on safety and
664 efficacy.
665

666

1. Selection of Format for Confirmatory Assay

667

668 It is expected that the selected confirmatory assay will be at least as sensitive as the screening
669 assay but have higher specificity and at least as good selectivity in order to identify any false-
670 positive samples. The method and instrument platform selected may be similar to or different
671 from those used for the screening assay. Frequently, both screening and confirmatory assays use
672 the same method and instrument platform. In such cases, the sensitivity of each assay will need
673 to be determined in mass units and confirmed using system suitability controls to ensure that the
674 assay is sensitive to the presence of binding antibody. When using a binding competition assay,
675 the concentration of competing product should be optimized to confirm the presence of
676 antibodies throughout and above the range of the assay.
677

678

2. Cut Point of Confirmatory Assay

679

680 If a competitive inhibition format is selected, a recommended approach to determining the cut
681 point uses the data from the binding of antibody-negative treatment-naïve patient samples in the
682 presence of the competitor, which is usually the therapeutic protein product. In this case, the
683 amount of therapeutic protein product used to establish the cut point should be the same as the
684 amount of therapeutic protein product that will be used as a competitive inhibitor in the assay.
685 However, this approach may not be appropriate when dealing with samples where pre-existing
686 antibodies are present in the treatment-naïve population. In those cases, the sponsor should
687 exclude true positives from the cut point assessment. In rare cases when baseline negative
688 samples are not available, sponsors may evaluate changes in titer or use an orthogonal method to
689 confirm samples that screen positive.
690

691

C. Development of Titering Assay

692

1. Titer Determination

693

694 Titers are defined as the maximal dilution where a sample gives a value above the screening cut
695 point. Titers are often informative and can be linked to clinical impact of the ADA. Titering
696
697

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698 assays can be particularly informative when patients have pre-existing antibodies. Titering
699 assays most often are performed using the same platform as the screening assay. Sera are tested
700 in sequential dilutions. Alternatively, titer may be determined by extrapolating the dilution to the
701 assay cut point using the linear portion of the dose response curve.

702

703 **2. *Cut Point of Titering Assay***

704

705 When patients have pre-existing ADA, treatment-boosted ADA responses may be identified by
706 post-treatment increases in titer. A cut point for defining the treatment-emergent or boosted
707 responses is needed. Frequently this cut point is determined as a titer that is two dilution steps
708 greater than the pre-treatment titer, when twofold dilutions are used to determine the titer. If titer
709 is established by extrapolating the dilution curve to the assay cut point, treatment-emergent
710 responses may be determined using estimates of assay variability.

711

712 **D. *Development of Neutralization Assay***

713

714 In vitro neutralization assays provide an indication of the potential of the ADA to inhibit the
715 biological activity of the product. Such NAb can interfere with the clinical activity of a
716 therapeutic protein product by preventing the product from reaching its target or by interfering
717 with receptor-ligand interactions. The testing method selected to assess neutralizing potential for
718 ADA-positive samples should be based on the mechanism of action of the therapeutic protein
719 product.

720

721 **1. *Selection of Format for Neutralization Assay***

722

723 Two formats of assays have been used to measure NAb activity: cell-based bioassays and non-
724 cell-based competitive ligand-binding assays. Selection of the appropriate assay format depends
725 on various factors. These factors include, but are not limited to, the mechanism of action of the
726 therapeutic protein product, its ability to reflect the in vivo situation most closely, and the
727 selectivity, sensitivity, precision, and robustness of the assay. FDA recommends that
728 neutralization assays use a cell-based bioassay format depending on the therapeutic protein
729 product's mechanism of action because, frequently, cell-based bioassays more closely reflect the
730 in vivo situation and therefore provide more relevant information than ligand-binding assays.
731 Because the cell-based bioassays are often based on the product's potency, historically the
732 format of these assays has been extremely variable. The choice and design of potency bioassays
733 are generally based on a cell line's ability to respond to the product in question and the potency
734 bioassay's relevance to the therapeutic protein product's mechanism of action.

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736
737 The cellular responses measured in these bioassays are numerous and can include outcomes such
738 as phosphorylation of intracellular substrates, calcium mobilization, proliferation, and cell death.
739 In some cases, sponsors have developed cell lines to express relevant receptors or reporter
740 constructs. When therapeutic protein products directly stimulate a cellular response, the direct
741 effect of NAb on reducing bioactivity in the bioassay can be measured. When therapeutic
742 protein products indirectly impact cellular activity; for example, by blocking a receptor-ligand
743 interaction, the indirect effect of the NAb on restoring bioactivity in a bioassay can be measured.
744 Generally, bioassays have significant variability and a limited dynamic range for their activity
745 curves. Such problems can make development and validation of neutralization assays difficult.
746

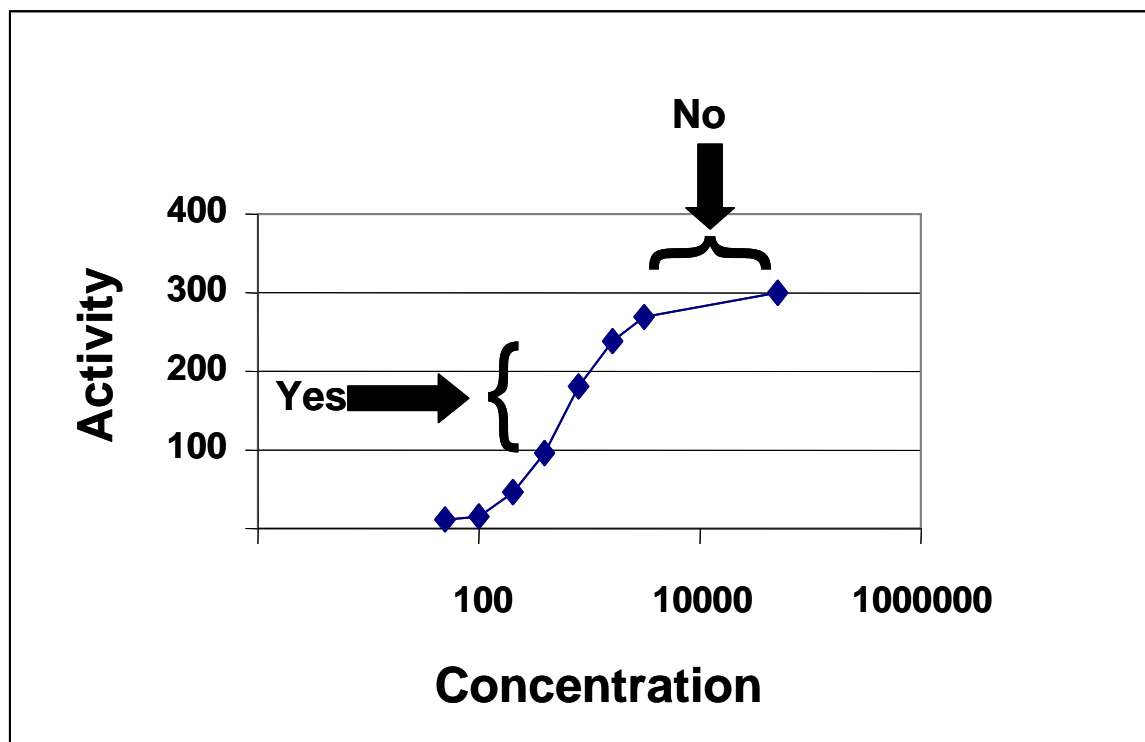
747 There are cases when ligand-binding assay formats may be used. One such case is when
748 sufficiently sensitive or selective cell-based bioassays cannot be developed. Another case is
749 when the therapeutic protein product does not have a cell-based mechanism of action; for
750 example, enzyme therapeutic protein products that target serum proteins. Ligand-binding assays
751 may also be appropriate for therapeutic protein products that bind serum ligands, preventing
752 them from interacting with their receptor. However, cell-based bioassays may still be more
753 appropriate for such therapeutic protein products to demonstrate that ADA are inhibiting cellular
754 activity. Sponsors should discuss using ligand-binding assays with FDA in such cases.
755

2. *Activity Curve of Neutralization Assay*

756
757
758 The sponsor should carefully consider the dose response curve (product concentration versus
759 activity) before examining other elements of neutralization assay validation. Assays with a small
760 dynamic range may not prove useful for determination of neutralizing activity. Generally, the
761 neutralization assay will employ a single concentration of therapeutic protein product with a
762 single dilution of antibody. Consequently, the sponsor should choose a therapeutic protein
763 product concentration whose activity readout is sensitive to inhibition. If the assay is performed
764 at concentrations near the plateau of the dose-response curve (marked “No” in Figure 1, below),
765 it may not be possible to discern samples with low amounts of NAb. FDA recommends that the
766 neutralization assay be performed at therapeutic protein product concentrations that are on the
767 linear range of the curve (marked “Yes” in Figure 1). The assay should also give reproducible
768 results.
769

770

771 **Figure 1. Activity Curve for a Representative Therapeutic Protein Product**



772

773 The x-axis (Concentration) indicates a concentration of the therapeutic protein product, and the
 774 y-axis (Activity) indicates resultant activity; for example, the concentration of cytokine secretion
 775 of a cell line upon stimulation with the therapeutic protein product. The curve demonstrates a
 776 steep response to a therapeutic protein product that plateaus at approximately 300. The “No”
 777 arrow indicates a concentration of a therapeutic protein product that would be inappropriate to
 778 use in a single dose neutralization assay because it would represent a range of concentrations
 779 where the activity induced by the therapeutic protein product would be relatively insensitive to inhibition
 780 by NAb. The “Yes” arrow represents a range of concentrations on the linear part of the curve
 781 where the activity induced by the therapeutic protein product would be sensitive to neutralization
 782 by antibody.

783

784 3. *Considerations for Matrix Interference for Neutralization Assay*

785

786 The matrix can cause interference with neutralization assays, particularly as serum or plasma
 787 components may enhance or inhibit the activity of a therapeutic protein product in bioassays.
 788 For example, sera from patients with particular diseases may contain elevated levels of one or
 789 more cytokines that might serve to activate cells in the bioassay and obscure the presence of
 790 NAb by increasing the response to the original stimulatory factor or therapeutic protein product.
 791 Therefore, the sponsor should understand matrix effects in these assays. Approaches such as
 792 enriching for ADA from serum or plasma samples may be appropriate for these types of
 793 situations. However, this approach may result in the loss of NAb, and consequently will require
 794 careful examination and validation by the sponsor.

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795
796 The concentration of therapeutic protein product employed in the neutralization assay has a
797 critical impact on assay sensitivity. FDA recognizes that although the use of low concentrations
798 of therapeutic protein product may lead to a neutralization assay that is more sensitive to
799 inhibition by antibodies, very low concentrations of therapeutic protein product may result in
800 poor precision of the assay. Also see section IV.D.1 for general information on matrix
801 interference.

4. Cut Point of Neutralization Assay

802
803
804
805 Determination of assay cut point has historically posed a great challenge for neutralization
806 assays. As with all assays, the cut point should be determined based on the assay variability
807 established using samples from treatment-naïve subjects. If neutralization assays are performed
808 on samples that tested positive in screening and confirmatory assays, a 1% false-positive rate is
809 acceptable. If neutralization assays are used for screening, a 5% false-positive rate should be
810 used (see section VI.B.2). If the degree of sample variation makes it difficult to assess NAb
811 activity, other approaches may be considered but should be discussed with FDA before
812 implementation. Alternatively, exploring other assay formats that lead to less variability and
813 provide a more accurate assignment of cut point may be necessary. Also see section IV.B for
814 general information on assay cut point.

5. Additional Considerations for Neutralization Assay

815
816
817
818 Because neutralization assays are most commonly performed only on samples that are confirmed
819 to have antigen-specific ADA, confirmatory approaches are not usually necessary. However,
820 because of the complexity of bioassays, confirmation of assay specificity may be useful in
821 determining whether patients have mounted a true NAb response. The sponsor should consider
822 the following approaches:

- 823
824 a. Unrelated inhibitory molecules may cause neutralizing activity, and sometimes it may
825 be unclear whether the observed neutralizing activity is caused by neutralizing
826 antibodies or by other inhibitory molecules. Test results from baseline pre-exposure
827 samples may be informative. When there is concern that there is non-specific
828 inhibition, antibody depletion assays should be performed to evaluate whether the
829 neutralizing activity is truly caused by ADA and not caused by other inhibitory
830 molecules.
- 831
832 b. Cell lines may be responsive to multiple stimuli other than the therapeutic protein
833 product under study. In such cases, the presence of NAb can be examined in the
834 presence of the therapeutic protein product, which should be blocked by a specific
835 NAb response, versus alternative stimuli, which should not be blocked by a specific
836 NAb response.
- 837
838 c. Serum may contain components such as soluble receptors or endogenous product
839 counterparts that may yield false results in the neutralization assay. In such instances,

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840 adding test serum or plasma samples directly to the bioassay in the absence of
841 therapeutic protein product may be useful in understanding assay results.

VI. ASSAY VALIDATION

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845
846 Assay validation is a process of demonstrating, by the use of specific laboratory investigations,
847 that the performance characteristics of the ADA assay employed are suitable for its intended
848 use.²³ The level of validation depends on the stage of product development and the risks of
849 consequences of immunogenicity to patients associated with the therapeutic protein product. A
850 partial validation involving assessments of assay sensitivity, specificity, and precision
851 requirements with less emphasis on robustness, reproducibility, and stability may be adequate for
852 the earlier stages of clinical development such as phase 1 and phase 2 studies. However, as a
853 scientific matter, as stated in section VI.A, fully validated assays should be used for pivotal and
854 postmarketing studies.

855
856 Information specific to validation of respective assay types is provided in sections VI.B
857 through E. These sections supplement information relevant to all assay types provided in
858 sections IV and VI.A.

A. General Considerations for Assay Validation

859
860
861
862 Samples derived from pivotal studies should be tested with fully validated assays. At the time of
863 license application, the sponsor should provide data supporting full validation of the assays.
864 Validation includes all of the procedures that demonstrate that a particular assay used for
865 quantitative measurement of ADA in a given sample is reliable and reproducible for the intended
866 use. The fundamental parameters for validation include (1) cut point, (2) sensitivity,
867 (3) specificity and selectivity, (4) precision, (5) reproducibility when relevant, and (6) robustness
868 of some assay features and stability of reagents and control samples. The acceptability of
869 clinical data generated by an assay corresponds directly to the criteria used to validate the assay.

870
871 Determination of cut point is a fundamental aspect of assay validation. If treatment-naïve
872 samples from the appropriate patient population are not available for the pre-study validation
873 exercise, alternative samples may be used. Frequently these are samples from commercial
874 sources. When alternative samples are used to determine the cut point in the validation exercise,
875 the cut point should be determined again once samples from the appropriate population (e.g.
876 treatment-naïve patients) are available. The cut point validated using the appropriate samples
877 should be used to determine whether samples are positive for ADA.

878
879 For validation of the fundamental assay parameters, FDA recommends, at the minimum, that
880 inter-assay precision be evaluated on at least 3 different days with two analysts each preparing a

²³ See the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*. Also see the guidance for industry *Bioanalytical Method Validation*, the USP General Chapter 1225 *Validation of Compendial Procedures*, and the ICH guidance for industry *Q2(R1) Validation of Analytical Procedures: Text and Methodology*.

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881 minimum of six otherwise independent preparations of the same sample using the same
882 instrument platform and model. Intra-assay precision should be evaluated with a minimum of
883 six independent preparations of the same sample per plate independently prepared by the same
884 analyst. In cases where intra-assay or inter-assay precision has a coefficient of variance (%CV)
885 greater than 20%, sponsors should consider the need to refine the assay parameters to optimize
886 the assay precision to the extent possible or provide justification to explain why higher %CV
887 should be acceptable. Alternatively, in assays with low throughput (e.g., titer assay) when it may
888 not be possible to run six independent preparations of the same sample on a plate, intra-assay
889 precision should be evaluated with a minimum of three independent preparations of the same
890 sample per plate and at least nine total independent preparations of the same samples. Samples
891 should include negative controls and positive samples whose testing yields values in the low,
892 medium, and high levels of the assay dynamic range. The sponsor should evaluate inter-
893 instrument and inter-operator precision when relevant. Assays should have comparable precision
894 between different operators under the same operating conditions.

895
896 When changes are made to a previously validated method, the sponsor should exercise judgment
897 as to how much additional validation is needed. During the course of a typical product
898 development program, a defined ADA assay may undergo modifications. Occasionally, samples
899 may need to be re-tested with the optimized validated assay; therefore, provisions should be
900 made to preserve sufficient sample volume under conditions that allow for re-testing until the
901 assays have been completely validated and evaluated by the Agency.²⁴

902
903 Critical method parameters, for example, incubation times and temperatures, should be validated
904 to demonstrate that the assay performs as expected within predetermined ranges for these
905 parameters. Generally, the low, middle, and high values of the allowed range are tested in the
906 validation exercise.

907
908 Additional parameters may need to be validated depending on the method (or technology) and
909 instrument platform used for the assay. For example, surface plasmon resonance assays should
910 be validated for surface stability upon regeneration, and criteria should be set for baseline
911 performance of the chip. The efficiency and stability of the labeled²⁵ reagents should be
912 established. The sponsor should examine robustness during the development phase, and if small
913 changes in specific steps in the assay affect results, specific precautions should be taken to
914 control their variability.

915

²⁴ See the guidance for industry *Bioanalytical Method Validation* for different types and levels of validation. Also see the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

²⁵ A reagent is considered *labeled* if it is conjugated or fused to a moiety that will aid in its capture or visualization; for example, conjugation to biotin, streptavidin, or a fluorochrome. *Unlabeled* reagent is a reagent (for example, a drug) that is not *labeled*.

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916 **B. Validation of Screening Assay**

917

918 *1. Sensitivity of Screening Assay*

919

920 All the general considerations for assay validation discussed previously apply to validation of
921 screening assay. As noted earlier, the sensitivity is particularly important in the initial screening
922 assay because these results dictate the further analysis of the sample.

923

924 *2. Cut Point of Screening Assay*

925

926 The cut point should be determined statistically with a minimum of 50 samples tested on at least
927 3 different days by at least two analysts using suitable statistical methods. FDA recommends
928 that the cut point for screening assays be determined by a 90% one-sided lower confidence
929 interval for the 95th percentile of the negative control population (Shen, Dong, et al. 2015). This
930 will assure at least a 5% false-positive rate with a 90% confidence level. This approach
931 improves the probability of the assay identifying all patients who may develop antibodies. The
932 statistical method used to determine the cut point should be based on the statistical distribution of
933 the data. For example, the 95th percentile of the normal distribution is estimated by the mean
934 plus 1.645 standard deviation. Other approaches may be used for estimating 95th percentile,
935 including the use of median and median absolute deviation value instead of mean and standard
936 deviation.

937

938 The mean response of negative control samples may be constant or may vary between assays,
939 plates, or analysts. When the mean is constant, a cut point may be established during assay
940 validation that can be applied to the assay in-study. This is frequently called a fixed cut point.
941 When the mean varies between assays, plates, or analysts but the variance around the mean is
942 constant, a normalization factor can be statistically determined and applied in-study. This is also
943 known as a floating cut point. When both the mean and variance vary, a cut point must be
944 established for each assay, plate, or analyst. This is known as a dynamic cut point. One
945 drawback of the dynamic cut point is the need to have more replicates of the negative control in
946 the assay. Dynamic cut points should not be used to compensate for deficient assay
947 optimization.

948

949 **C. Validation of Confirmatory Assay**

950

951 Confirmatory assays should be fully validated in a manner similar to screening and neutralization
952 assays because these assays raise some specific issues. As a scientific matter, the studies to
953 validate the assay will depend on the assay format and instrumentation chosen. If these assays
954 are based on competition for antigen binding²⁶ by the antibodies in patient samples and the
955 measurement is loss of response, it is critical to identify the degree of inhibition or depletion that
956 will be used to ascribe positivity to a sample. In the past, fixed percentages of binding reduction
957 were used, but these numbers were often arbitrary and are unlikely to be relevant for all assays.

²⁶ *Competition for antigen binding* refers to a competition assay where the ability of antigen-specific antibodies to bind to either labeled or plate-bound antigen is inhibited by unlabeled or soluble antigen.

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958 FDA recommends establishing a cut point based on the assessment of the binding changes
959 observed in samples that are known to lack the antibodies when competing antigen is added.
960 FDA also recommends that the sensitivity of the confirmatory assay be confirmed using a low
961 concentration of the positive control antibody.

962
963 For the estimation of the confirmatory assay cut point, an 80% one-sided lower confidence
964 interval for the 99th percentile is recommended. Because the purpose of this assay is to eliminate
965 false-positive samples arising as a result of non-specific binding, it is adequate to use a 1% false-
966 positive rate for the calculation of the confirmatory cut point. The use of tighter false-positive
967 rates such as 0.1% is not recommended because it will lead to an increased risk of false-negative
968 results. See section IV.B for general information on assay cut point.

969
970 If the confirmatory assay format is a competitor assay in which a competitor, usually unlabeled
971 therapeutic protein product,²⁷ will be added to the reaction mixture to inhibit ADA binding to the
972 capture reagent for the cut point assay, the same concentration of unlabeled therapeutic protein
973 product should be added to the samples when determining the confirmatory cut point.

D. Validation of Titering Assay

974
975
976 The principles of assay validation described in section VI.A apply in general to validation of
977 titering assays. The cut point of the titration assay may be the same as or different from that of
978 the screening assay. When the titering assay is not used for screening and the cut point is
979 different than that of the screening assay, the validation of the separate titration method cut point
980 can become necessary; for example, when the signal from the assay diluent or matrix causes
981 higher results than the screening assay cut point because of a blocking effect of serum or if
982 samples at a dilution higher than the MRD do not generate consistently negative results, i.e.,
983 when the screening cut point falls on the lower plateau of the positive-control dilution curve.²⁸

E. Validation of Neutralization Assay

984
985
986 A minimum of 30 samples tested on at least 3 different days by at least two analysts should be
987 used to determine the cut point, using suitable statistical methods.

988
989
990 FDA recognizes that not all ADA are neutralizing, and it can be difficult to identify positive
991 control antibodies with neutralizing capacity. Further, if an affinity purified polyclonal positive
992 control antibody preparation is used, it is likely that only a portion of the antibodies are
993 neutralizing, which can make the assay appear less sensitive. Therefore, it is important to
994 validate assay sensitivity.

995
996
997 Sponsors should validate assay specificity for cell-based neutralization bioassays. As mentioned,
998 for cells that may be responsive to stimuli other than the specific therapeutic protein product, the

²⁷ See footnote 25.

²⁸ See the USP General Chapter 1106 *Immunogenicity Assays – Design and Validation of Immunoassays to Detect Anti-Drug Antibodies*.

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999 ability to demonstrate that NAb only inhibit the response to therapeutic protein product and not
1000 the response to other stimuli is a good indication of assay specificity. In such studies, FDA
1001 recommends that the other stimuli be employed at a concentration that yields an outcome similar
1002 to that of the therapeutic protein product. The sponsor should also confirm the absence of
1003 alternative stimuli in patient serum (see sections IV.C and D).
1004

1005 Cell-based neutralization bioassays frequently have reduced precision when compared to ligand-
1006 binding assays because biologic responses can be inherently more variable than carefully
1007 controlled binding studies. Consequently, the sponsor should perform more replicates for
1008 assessment of precision and assessment of patient responses than for the screening assay (see
1009 section IV.E).
1010

1011 Additional parameters that should be validated are assay performance when cells at the low,
1012 middle, and high range of the allowed passage numbers, cell density, and cell viability are used
1013 (see section IV.G).
1014

1015

VII. IMPLEMENTATION OF ASSAY TESTING

1017

A. Obtaining Patient Samples

1018

1019
1020 FDA recommends that the sponsor obtain pre-exposure samples from all patients. Because there
1021 is the potential for pre-existing antibodies or confounding components in the matrix,
1022 understanding the degree of reactivity before treatment is essential. The sponsor should obtain
1023 subsequent samples, with the timing depending on the frequency of dosing. Optimally, samples
1024 taken 7 to 14 days after the first exposure can help elucidate an early IgM response. Samples
1025 taken at 4 to 6 weeks after the first exposure are generally optimal for determining IgG
1026 responses. For individuals receiving a single dose of therapeutic protein product, the above time
1027 frame may be adequate. However, for patients receiving a therapeutic protein product at
1028 multiple times during the trial, the sponsor should obtain samples at appropriate intervals
1029 throughout the trial and also obtain a sample approximately 30 days after the last exposure.
1030

1031 Obtaining samples at a time when there will be minimal interference from the therapeutic protein
1032 product present in the serum is essential. A sponsor should consider the therapeutic protein
1033 product's half-life to help determine appropriate times for sampling. This is especially important
1034 for mAb products because these products can have half-lives of several weeks or more; and
1035 depending on the dosing regimen, the therapeutic mAb itself could remain present in the serum
1036 for months. Under circumstances when testing for IgE is needed, the timing of sample collection
1037 should be discussed with FDA.
1038

1039 The level of therapeutic protein product that interferes with the assay, as determined by immune
1040 competition, may also help define meaningful time points for sampling. If therapeutic protein
1041 product-free samples cannot be obtained during the treatment phase of the trial, the sponsor
1042 should take additional samples after an appropriate washout period (e.g., five half-lives).
1043 Obtaining samples to test for meaningful antibody results can also be complicated if the
1044 therapeutic protein product in question is itself an immune suppressant. In such instances, the

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1045 sponsor should obtain samples from patients who have undergone a washout period either
1046 because the treatment phase has ended or because the patient has dropped out of the study.

1047
1048 Samples to determine serum concentrations of therapeutic protein product should be obtained at
1049 the same time as immunogenicity samples. Testing such samples can provide information on
1050 whether the therapeutic protein product in the samples may be interfering with ADA testing and
1051 whether ADA may be altering the therapeutic protein product's pharmacokinetics.

1052

B. Concurrent Positive and Negative Quality Controls

1053

1054
1055 If the sponsor completes the proper validation work and makes the cut point determinations, the
1056 immunogenicity status of patients should be straightforward to determine. However, positive
1057 control or QC samples are critical and should be run concurrently with patient samples. We
1058 recommend that these samples span a level of positivity with QC samples having a known
1059 negative, low, and high reactivity in the assay. More important, the QC samples should be
1060 diluted in the matrix in which patient samples will be examined; for example, the same percent
1061 serum or plasma (specify salt anticoagulant used). In this way, the sponsor ensures that the assay
1062 is performing to its optimal degree of accuracy and that patient samples are correctly evaluated.
1063 For the low-positive QC sample, we recommend that a concentration be selected that, upon
1064 statistical analysis, would lead to the rejection of an assay run 1% of the time. Such an approach
1065 would ensure the appropriate sensitivity of the assay when performed on actual patient samples.
1066 The concentration of high-positive QC samples should be set to monitor prozone effects.²⁹

1067

1068 FDA also recommends that these QC samples be obtained from humans or animals possessing
1069 antibodies that are detected by the secondary detecting reagent, to ensure that negative results
1070 that might be observed are truly caused by lack of antigen reactivity and not caused by failure of
1071 the secondary reagent. This issue is not a problem for antigen bridging assays because labeled
1072 antigen is used for detection.

1073

C. Confirmation of Cut Point in the Target Population

1074

1075
1076 Samples from different populations can have different background activity in ADA assays.
1077 Therefore, it is necessary to confirm that the cut point determined during assay validation is
1078 suitable for the population being studied. Similarly, if samples used to determine the cut point
1079 during assay validation were not obtained and handled in a manner that represents how samples
1080 will be obtained and handled in-study, the cut point should also be confirmed with appropriate
1081 samples in-study. A sufficient number of samples from the target population should be used, and
1082 justification for the number used should be provided. If sufficient numbers of samples are not
1083 available, agreement with the Agency should be sought for the number of samples to be used.

1084

1085

²⁹ Prozone effects (also referred to as hook effects) are a reduction in signal that may occur as a result of the presence of a high concentration of a particular analyte or antibody and may cause false-negative results.

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1086 **VIII. DOCUMENTATION**

1087
1088 The rationale and information for the immunogenicity testing paradigm should be provided in
1089 module 5.3.1.4 of the electronic common technical document (eCTD) on *Reports of*
1090 *Bioanalytical and Analytical Methods for Human Studies*.³⁰ The standard operating procedure of
1091 the respective assay being used should be provided to the FDA, together with the results of the
1092 validation studies and relevant assay development information for parameters that were not
1093 validated, such as the MRD, the stimulatory concentration of therapeutic protein product used in
1094 the NAb assay, and some robustness parameters that are critical for assay performance (see
1095 section VII. Documentation in the draft guidance for industry *Bioanalytical Method*
1096 *Validation*.)³¹
1097
1098

³⁰ See the FDA Web site for further information on eCTD submissions, available at <http://www.fda.gov/Drugs/DevelopmentApprovalProcess/FormsSubmissionRequirements/ElectronicSubmissions/ucm153574.htm>. For more information about the agreed-upon common format for the preparation of a well-structured Efficacy section of the CTD for applications that will be submitted to regulatory authorities, see the ICH guidance for industry *M4E: The CTD — Efficacy*. For more information on how sponsors and applicants must organize the content they submit to the Agency electronically for all submission types under section 745A(a) of the FD&C Act, see the guidance for industry (and the technical specification documents it incorporates by reference) *Providing Regulatory Submissions in Electronic Format — Certain Human Pharmaceutical Product Applications and Related Submissions Using the eCTD Specifications*.

³¹When final, this guidance will represent the FDA's current thinking on this topic. To make sure you have the most recent version of a guidance, check the FDA guidance Web page at <http://www.fda.gov/RegulatoryInformation/Guidances/default.htm>.

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