

---

# Guidance for Industry

## Assay Development for Immunogenicity Testing of Therapeutic Proteins

### ***DRAFT GUIDANCE***

**This guidance document is being distributed for comment purposes only.**

Comments and suggestions regarding this draft document should be submitted within 60 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register*.

For questions regarding this draft document contact (CDER) Susan Kirshner at 301-827-1731, or (CBER) Office of Communication, Outreach, and Development at 301-827-1800.

**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)**

December 2009  
CMC

---

# Guidance for Industry

## Assay Development for Immunogenicity Testing of Therapeutic Proteins

*Additional copies are available from:*

*Office of Communication  
Division of Drug Information, WO51, Room 2201  
Center for Drug Evaluation and Research  
Food and Drug Administration  
10903 New Hampshire Ave.  
Silver Spring, MD 20993  
(Tel) 301-796-3400; (Fax) 301-847-8714  
druginfo@fda.hhs.gov*

<http://www.fda.gov/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/default.htm>

*Office of Communication, Outreach, and  
Development, HFM-40  
Center for Biologics Evaluation and Research  
Food and Drug Administration  
1401 Rockville Pike, Suite 200N, Rockville, MD 20852-1448  
ocod@fda.hhs.gov*

<http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm>

*(Tel) 800-835-4709 or 301-827-1800*

**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)**

December 2009  
CMC

*Contains Nonbinding Recommendations*

*Draft — Not for Implementation*

**TABLE OF CONTENTS**

<b>I.</b>	<b>INTRODUCTION.....</b>	<b>1</b>
<b>II.</b>	<b>DISCUSSION .....</b>	<b>1</b>
	<b>A. General.....</b>	<b>1</b>
	<b>B. Immunogenicity Testing During Product Development .....</b>	<b>2</b>
	<b>C. Principles of Immunogenicity Testing During Product Development .....</b>	<b>2</b>
<b>III.</b>	<b>APPROACH TO ASSAY DEVELOPMENT.....</b>	<b>3</b>
	<b>A. Overview of Design Elements.....</b>	<b>3</b>
	1. <i>Multi-tiered Approach.....</i>	<i>3</i>
	2. <i>Aspects of Assay Development .....</i>	<i>4</i>
	<b>B. Screening Assay .....</b>	<b>4</b>
	1. <i>Selection of Format.....</i>	<i>4</i>
	2. <i>Selection of Assay and Reagents.....</i>	<i>4</i>
	3. <i>Interference and Matrix .....</i>	<i>6</i>
	4. <i>Defining a Positive Result.....</i>	<i>7</i>
	<b>C. Neutralization Assay .....</b>	<b>7</b>
	1. <i>Selection of Format.....</i>	<i>7</i>
	2. <i>Activity Curve .....</i>	<i>7</i>
	3. <i>Interference.....</i>	<i>8</i>
	4. <i>Confirmation of Neutralizing Antibodies.....</i>	<i>9</i>
	5. <i>Cut Point of Neutralizing Antibodies Assays .....</i>	<i>9</i>
	6. <i>Multiple Functional Domains.....</i>	<i>9</i>
<b>IV.</b>	<b>CLINICAL ASPECTS OF ASSAY VALIDATION .....</b>	<b>9</b>
	<b>A. Critical Considerations and Caveats.....</b>	<b>10</b>
	<b>B. Determining the Minimal Dilution .....</b>	<b>10</b>
	1. <i>Importance.....</i>	<i>10</i>
	2. <i>Approach .....</i>	<i>10</i>
	3. <i>Recommendation .....</i>	<i>10</i>
	<b>C. Assay Cut Point .....</b>	<b>10</b>
	1. <i>Definition.....</i>	<i>11</i>
	2. <i>Determination.....</i>	<i>11</i>
	3. <i>Recommendation .....</i>	<i>11</i>
<b>V.</b>	<b>ASSAY VALIDATION.....</b>	<b>11</b>
	<b>A. Validation of Screening Assay .....</b>	<b>12</b>
	1. <i>Sensitivity .....</i>	<i>12</i>

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

2. <i>Specificity</i> .....	12
3. <i>Precision</i> .....	13
4. <i>Robustness and Sample Stability</i> .....	13
<b>B. Validation of Neutralizing Assay</b> .....	<b>14</b>
1. <i>Sensitivity</i> .....	14
2. <i>Specificity</i> .....	14
3. <i>Precision</i> .....	14
4. <i>Other Elements of Neutralizing Assay Validation</i> .....	14
<b>C. Validation of Immunodepletion/Competitive Confirmatory Assay</b> .....	<b>15</b>
<b>VI. IMPLEMENTATION OF ASSAY TESTING</b> .....	<b>15</b>
<b>A. Obtaining Patient Samples</b> .....	<b>15</b>
<b>B. Concurrent Positive and Negative Quality Controls</b> .....	<b>16</b>
<b>C. Cut Point Normalization</b> .....	<b>16</b>
<b>D. Reporting Patient Results</b> .....	<b>17</b>
<b>E. Pre-existing Antibodies</b> .....	<b>17</b>
<b>F. Specific Considerations</b> .....	<b>17</b>
1. <i>Monoclonal Antibodies</i> .....	17
2. <i>Rheumatoid Factor</i> .....	17
3. <i>Fusion Proteins</i> .....	18
4. <i>High Levels of Endogenous Protein in Sera</i> .....	18
<b>VII. OTHER ASPECTS OF IMMUNOGENICITY TESTING</b> .....	<b>18</b>
<b>A. Isotypes</b> .....	<b>18</b>
<b>B. Epitope Specificity</b> .....	<b>18</b>
<b>VIII. REFERENCES</b> .....	<b>19</b>

*Contains Nonbinding Recommendations*

*Draft — Not for Implementation*

1 **Guidance for Industry<sup>1</sup>**  
2 **Assay Development for Immunogenicity Testing**  
3 **of Therapeutic Proteins**  
4  
5

6 This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current  
7 thinking on this topic. It does not create or confer any rights for or on any person and does not operate to  
8 bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of  
9 the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA  
10 staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call  
11 the appropriate number listed on the title page of this guidance.

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

17 This guidance provides recommendations to facilitate industry's development of immune assays  
18 for assessment of the immunogenicity of therapeutic proteins during clinical trials.<sup>2</sup> This  
19 document includes guidance for binding assays, neutralizing assays, and confirmatory assays.  
20 While the document does not specifically discuss the development of immune assays for animal  
21 studies, the concepts discussed are relevant to the qualification and validation of immune studies  
22 for preclinical evaluation of data.

23  
24 This document does not discuss the product and patient risk factors that may contribute to  
25 immune response rates (immunogenicity).  
26

27 In addition, this document does not specifically discuss how results obtained from immunoassays  
28 relate to follow-on biologic therapeutic proteins. However, elements of assay validation may  
29 affect comparability determinations of immune responses. FDA guidance documents, including  
30 this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe  
31 the Agency's current thinking on a topic and should be viewed only as recommendations, unless  
32 specific regulatory or statutory requirements are cited. The use of the word *should* in Agency  
33 guidances means that something is suggested or recommended, but not required.  
34

35 **II. DISCUSSION**  
36

37 **A. General**  
38

---

<sup>1</sup> This guidance has been prepared by the Office Biotechnology Products in the Office of Pharmaceutical Science, Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration.

<sup>2</sup> This guidance does not pertain to immunogenicity assays for assessment of immune response to preventative and therapeutic vaccines for infectious disease indications.

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

39 The clinical effect of patient immune responses to therapeutic proteins has ranged from no effect  
40 at all to extreme harmful effects to patient health. The potential for such varied immune  
41 responses affect product safety and efficacy. Because this range exists, clinicians rely on the  
42 immunogenicity section of the package labeling that contains immunogenicity rates observed  
43 during clinical trials. This makes the development of valid, sensitive immune assays a key  
44 aspect of product development.  
45

46 For new products, the design of such assays poses many challenges to applicants and FDA  
47 supports an evolving approach to assay development and validation during product development.  
48 Because these assays are critical when immunogenicity poses a high-risk and real time data  
49 concerning patient responses are needed, the applicant should implement preliminary validated  
50 assays early (preclinical and phase 1). Therapeutic proteins are frequently immunogenic in  
51 animals. Immunogenicity in animal models is not predictive of immunogenicity in humans.  
52 However, assessment of immunogenicity in animals may be useful to interpret nonclinical  
53 toxicology and pharmacology data. In addition, immunogenicity in animal models may reveal  
54 potential antibody related toxicities that could be monitored in clinical trials.  
55

56 In other situations, FDA recommends the applicant bank patient samples so samples can be  
57 tested when suitable assays are available. FDA expects that the assays will be refined during  
58 product development and the suitability of the assays will be reassessed according to their use.  
59 For example, FDA does not require an applicant to establish interoperator precision early in  
60 clinical development if only a single operator is performing an assay. Nevertheless, at the time  
61 of license application, the applicant should provide data supporting full validation of the assays.  
62

### **B. Immunogenicity Testing During Product Development**

63  
64  
65 Even though different companies developing similar products employ fully validated assays to  
66 assess immunogenicity, such assays will differ in a number of parameters. These differences can  
67 make immunogenicity comparisons across products in the same class invalid. A true comparison  
68 of immunogenicity across different products in the same class can best be obtained by  
69 conducting head-to-head patient trials using a standardized assay that has equivalent sensitivity  
70 and specificity for both products. When such trials are not feasible, FDA recommends the  
71 applicant develop assays that are highly optimized for sensitivity, specificity, precision, and  
72 robustness.  
73

74 FDA believes that such assays enable a true understanding of the immunogenicity, safety, and  
75 efficacy of important therapeutic protein products. The detection of antibodies is dependent on  
76 key operating parameters of the assays (e.g., sensitivity, specificity, methodology, sample  
77 handling) which vary between assays. Therefore, in the product labeling, FDA does not  
78 recommend comparing the incidence of antibody formation between products when different  
79 assays are used.  
80

### **C. Principles of Immunogenicity Testing During Product Development**

81  
82

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

83 Multiple approaches may be appropriate for immunogenicity testing during clinical trials.  
84 However, when designing immunogenicity assays and their application to patient testing, the  
85 applicant should address the following:

- 86
- 87 • Sensitivity. The assays should have sufficient sensitivity to detect clinically relevant  
88 levels of antibodies.
- 89
- 90 • Interference. Assays results may be affected by interference from the matrix or from on-  
91 board product and this potential effect should be evaluated.
- 92
- 93 • Functional or physiological consequences. Immune responses may have multiple effects  
94 including neutralizing activity and ability to induce hypersensitivity responses, among  
95 others. Immunogenicity tests should be designed to detect such functional consequences.
- 96
- 97 • Risk based application. The risk to patients of mounting an immune response to product  
98 will vary with the product.
- 99

100 The applicant should provide a rationale for the immunogenicity testing paradigm. Further  
101 recommendations on assay development and validation are provided below. These  
102 recommendations are based on common issues encountered by the Agency upon review of  
103 immunogenicity submissions. In addition, other publications may also be consulted for  
104 additional insight (see section VIII, 1, 2).

### **105**

### **106 III. APPROACH TO ASSAY DEVELOPMENT**

#### **107**

#### **108 A. Overview of Design Elements**

##### *109*

##### *110 1. Multi-tiered Approach*

111

112 Because of the size of some clinical trials and the necessity of testing patient samples at several  
113 time-points, FDA recommends a multi-tiered approach to the testing of patient samples. In this  
114 approach, a rapid, sensitive screening assay should initially be used to assess samples. Samples  
115 testing positive in the screening assay should then be subjected to a confirmatory assay. For  
116 example, a competition assay could confirm that antibody is specifically binding to product and  
117 that the positive finding is not a result of non-specific interactions of the test serum or detection  
118 reagent with other materials in the assay milieu such as plastic or other proteins.

119

120 This approach should lead to a culling of samples that should then be tested in other assays, such  
121 as neutralizing assays, that are generally more laborious and time-consuming. Neutralizing  
122 antibodies (NAB) are generally of more concern than binding antibodies (BAB) that are not  
123 neutralizing, but both may have clinical consequences. Further, tests to assess the isotype of the  
124 antibodies and their epitope specificity may also be recommended once samples containing  
125 antibodies are identified by the screening assay.

126

127 Although results of patient sample testing are often reported as positive vs. negative, an  
128 assessment of antibody levels is informative. FDA, therefore, recommends that positive

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

129 antibody responses be reported as a titer (e.g., the reciprocal of the highest dilution that gives a  
130 value equivalent to the cut point of the assay). Values may also be reported as amount of drug  
131 (in mass units) neutralized per volume serum with the caveat that these are arbitrary in vitro  
132 assay units and cannot be used to directly assess drug availability in vivo. Antibody levels  
133 reported in mass units based on interpolation of data from standard curves generated with a  
134 positive control standard antibody are generally less informative because interpretation is based  
135 on the specific control antibody.

136

### *2. Aspects of Assay Development*

138

139 There are several important concepts to remember when using this multi-tiered approach to  
140 assess immunogenicity. First, the initial screening should be very sensitive. A low, but defined  
141 false positive rate is desirable because it maximizes detection of true positives. Other assays can  
142 be subsequently employed to exclude false positive results when determining the true incidence  
143 of immunogenicity.

144

145 Second, the assay should be able to detect all isotypes (particularly immunoglobulin M (IgM)  
146 and the different immunoglobulin G (IgG) isotypes).

147

148 Third, FDA recognizes that antibodies generated in patients may have varied avidities for the  
149 product, while the positive controls used to validate the assay and demonstrate data legitimacy  
150 may only represent a subset of potential avidities. Although this may be unavoidable, FDA  
151 recommends the applicant carefully consider the avidity of controls used to evaluate the assay.

152

153 A fourth consideration is how interference from biological materials (matrix, e.g., serum,  
154 plasma) will affect assay performance. The applicant should conduct assay performance tests in  
155 the same concentration of matrix as that used to assess patient samples. The applicant should  
156 also define the dilution factor that will be used for preparation of patient samples before  
157 validation studies assessing potential interference of matrix on assay results.

158

## **B. Screening Assay**

160

### *1. Selection of Format*

162

163 A number of different formats are available for screening assays. These include, but are not  
164 limited to, direct binding enzyme-linked immuno sorbent assay (ELISA), bridging ELISA,  
165 radioimmunoprecipitation assays (RIPA), surface plasmon resonance (SPR), Bethesda Assay  
166 (for clotting factor inhibitors, see section VIII, 3), and bridging electrochemiluminescence  
167 assays. Each assay has its advantages and disadvantages as far as rapidity of throughput,  
168 sensitivity, and availability of reagents. One of the major differences between each of these  
169 assays is the number and vigor of washes, which can have an effect on assay sensitivity. Epitope  
170 exposure is also important to consider as binding to plastic or coupling to other agents (e.g.,  
171 fluorochromes) can obscure relevant antibody binding sites on the protein product in question.

172

### *2. Selection of Assay and Reagents*

174



## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

- Development of positive and negative controls

While many components of the screening assay may be standard (e.g., commercially available reagents) others may need to be generated specifically for the particular assay. The applicant should immunize animals (or hyperimmunize them with adjuvant) to generate a positive control. For the validation of immunogenicity assays, the positive control antibodies should be spiked into the matrix selected for routine assay performance (e.g., human serum diluted 1:10 in assay buffer). To prevent contamination of the assay matrix that could bias results, it is important to purify the positive control antibodies from the animal serum or plasma.

In addition, the applicant should carefully consider the selection of species when generating controls. For example, if an antihuman immunoglobulin reagent will be used to detect patient antibodies, the positive control and quality control samples should be detectable by that reagent (e.g., primate immune sera, humanized monoclonal). In some instances, the applicant may be able to generate a positive control antibody from patient samples. While such a reagent can be very valuable, such samples are generally not available in early trials. In addition, an applicant may not be able to generate such a reagent for therapeutic proteins with very low immunogenicity rates.

Once a source of antiproduct antibodies has been identified, the applicant should use it to assess assay validation parameters such as sensitivity, specificity, and reproducibility. FDA recommends the applicant generate and reserve specific dilutions of the sample for use as quality controls (QC). These dilutions should be representative of high, medium, and low values in the antibody assay. The applicant should use these samples for validation and patient sample testing to ensure the assay is operating within desired assay ranges at the time the assays are performed (system suitability testing).

FDA recommends the applicant establish a negative control for validation studies and patient sample testing. In this regard, a pool of sera from 5-10 non-exposed individuals can serve as a useful negative control. Importantly, the value obtained for the negative control should closely reflect the cut point determined for the assay in the patient population being tested. Negative controls that yield values far below that of the cut point may not be useful in ensuring proper assay performance.

For therapeutic monoclonal antibodies, the applicant should give special consideration to the selection of a positive control for the assay. If non-primate animals are immunized with a monoclonal antibody (mAb) containing a human immunoglobulin constant region (Fc) to develop a positive control, the antibody response is likely to be against the human Fc and not the variable region. Such a positive control may not be relevant for the anticipated immune response in human patients where the response to humanized mAb is primarily to the variable regions. Ideally, the positive control should reflect the anticipated immune response that will occur in humans.

- Detection reagent consideration

## *Contains Nonbinding Recommendations*

*Draft — Not for Implementation*

220 The nature of the detection agent is also critical. Reagents, such as Protein A are not optimal as  
221 they fail to detect all immunoglobulin isotypes. Although antibody bridging studies do not  
222 depend on isotype for detection, they can present specific concerns. In these assays, antigen is  
223 used to coat a surface, antibody containing samples are allowed to react with the antigen, and  
224 binding is detected by adding a labeled form of the antigen (product in question). Since  
225 multivalent binding of antiproduct antibody to the antigen on the plate can prevent binding of the  
226 detecting reagent, bridging assays are highly dependent on the product coating density and would  
227 be unable to detect lower affinity interactions. In these assays, the applicant should demonstrate  
228 that the labeling of the detection antigen does not significantly obscure critical antigenic  
229 determinants.

230

- 231 • Controlling nonspecific binding

232

233 Every reagent, from the plastic of the microtiter plate to developing agent, can affect assay  
234 sensitivity and non-specific binding. One of the most critical elements is the selection of the  
235 assay buffer and blocking reagents used to prevent non-specific binding to the solid surface.  
236 Since most assays require wash steps, the selection of specific detergents and concentrations is  
237 critical and should be optimized early. Similarly, the applicant should carefully consider the  
238 number of wash steps to reduce background noise, yet maintain sensitivity. A variety of proteins  
239 can be used as “blockers” in assays. However, these proteins may not all perform equivalently in  
240 specific immunoassays. For example, they may not bind well to the microtiter plate or may  
241 show unexpected cross reactivity with the detecting reagent. Therefore, the applicant may need  
242 to test several proteins to optimize results. Moreover, including uncoated wells is insufficient to  
243 control for non-specific binding. The capacity to bind to an unrelated protein may prove a better  
244 test of the binding specificity.

245

### 246 *3. Interference and Matrix*

247

248 Components in the matrix other than antibodies can interfere with assay results. Of greatest  
249 concern is the presence in the matrix of product or its endogenous counterpart. Specifically, if  
250 large quantities of product related material are present in sera/plasma, that material can prevent  
251 detection of antibodies in the test format. FDA recommends the applicant address such  
252 possibilities early (preclinical and phase 1 or early phase 2). The applicant should also examine  
253 this issue by deliberately adding known amounts of purified antibodies into assay buffer in the  
254 absence or presence of different quantities of the protein under consideration. This problem will  
255 also be minimized if the applicant collects patient samples at a time when the therapeutic protein  
256 has decayed to a level where it does not interfere with assay results. Data from pharmacokinetic  
257 studies are useful in establishing optimal sample collection times.

258

259 Other serum/plasma components may also influence assay results and it is usually necessary to  
260 dilute patient samples for testing to minimize such effects. The applicant should examine the  
261 effect of such interference by recovery studies in which positive control antibodies are spiked  
262 into serum at defined concentrations. Comparing results obtained in buffer alone with those in  
263 diluted serum can provide input on the degree of interference from matrix components and guide  
264 decisions on minimum starting dilutions recommended for sample testing.

265

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

### 266 4. *Defining a Positive Result*

267  
268 One generally defines positive results by using a cut point (section IV, C). FDA recommends the  
269 applicant perform confirmation assays at the screening level. The applicant could also include  
270 additional titrations, antibody depletion, and antibody blockade with excess product (section V,  
271 C).

### 272 273 **C. Neutralization Assay**

#### 274 275 1. *Selection of Format*

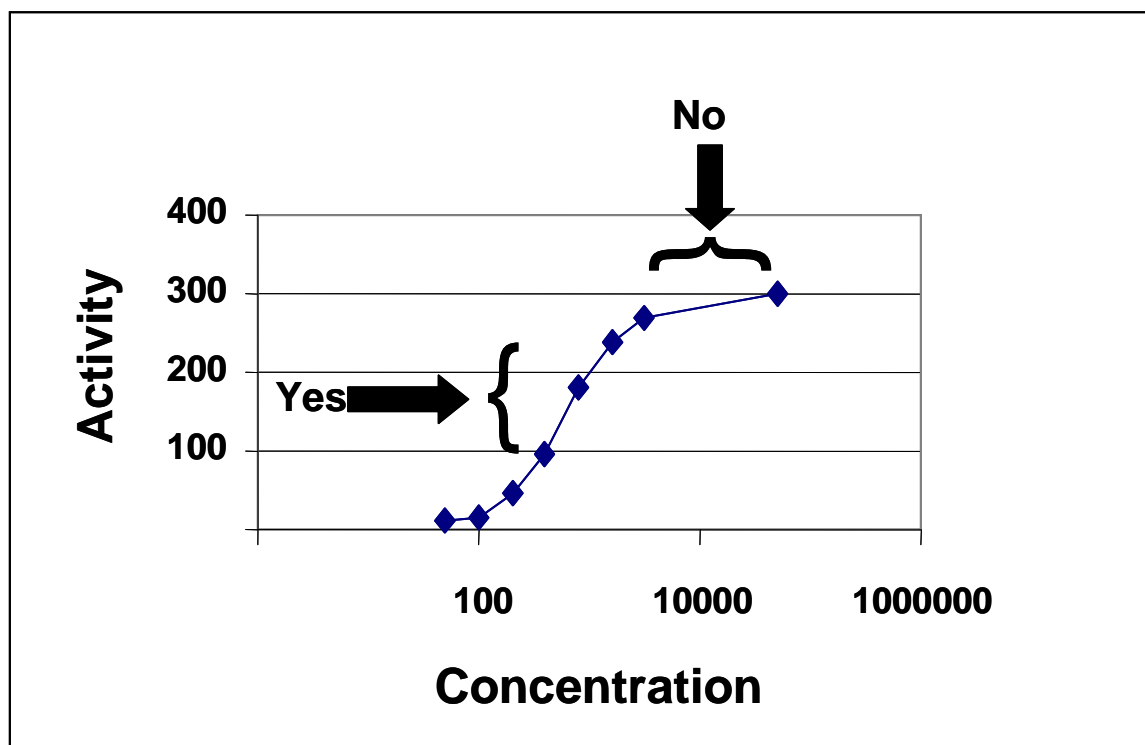
276  
277 Two types of assays have been used to measure neutralizing antibody activity: cell-based  
278 biologic assays and non cell-based competitive ligand-binding assays. While competitive ligand-  
279 binding assays may be the only alternative in some situations, generally FDA considers that  
280 bioassays are more reflective of the in vivo situation and are recommended. Because the cell-  
281 based (bioactivity) assays are often based on the potency assay, historically, the format of these  
282 assays has been extremely variable. These bioassays are generally based on a cell's ability to  
283 respond to the product in question. For NAB assays, the bioassay should be related to product  
284 mechanism of action, otherwise the assay will not be informative as to the effect of NAB on  
285 clinical results.

286  
287 The cellular responses potentially being measured in these bioassays are numerous and include  
288 outcomes such as phosphorylation of intracellular substrates, proliferation, calcium mobilization,  
289 and cell death. In some cases, the applicants have developed cell lines to express relevant  
290 receptors or reporter constructs. For many of these assays, there is a direct effect of neutralizing  
291 antibodies on the assay (e.g., inhibition of the cellular response). Alternatively, for monoclonal  
292 antibodies, the ability to block a response emanating from a receptor/ligand interaction may form  
293 the basis for a relevant potency assay. Therefore, the neutralizing assay may indirectly assess  
294 cell response by determining the "inhibition of inhibition." Generally, bioassays have significant  
295 variability and a limited dynamic range for their activity curves. Such problems can make  
296 development and validation of neutralization assays difficult and FDA understands such  
297 difficulties. Nonetheless, we will recommend such assays because they are critical to  
298 understanding the importance of patient immune responses to therapeutic proteins.

#### 299 300 2. *Activity Curve*

301  
302 The applicant should carefully consider the dose response curve (product concentration vs.  
303 activity) before examining other elements of neutralization assay validation. Assays with a small  
304 dynamic range may not prove useful for determination of neutralizing activity. Generally, the  
305 neutralization assay will employ a single concentration of product with different concentrations  
306 of antibody samples added to determine neutralizing capability. Consequently, the applicant  
307 should choose a product concentration whose activity readout is sensitive to inhibition. If the  
308 assay is performed at concentrations near the plateau of the curve; as shown in Figure 1, "No"; it  
309 may not be possible to discern neutralization. FDA recommends that the neutralization assay be  
310 performed at product concentrations that are on the linear range of the curve, as noted in Figure  
311 1, "Yes." The assay should also give reproducible results.

312



313  
314 Figure 1. Activity Curve for a Representative Therapeutic Protein.

315  
316 The X axis indicates a concentration of the therapeutic protein and the Y axis indicates resultant  
317 activity (e.g., concentration of cytokine secretion of a cell line upon stimulation with the  
318 therapeutic protein). The curve demonstrates a steep response to a product that plateaus at  
319 approximately 300. The “No” arrow indicates a concentration of a product that would be  
320 inappropriate to use in a single dose neutralization assay because it would represent a  
321 concentration relatively insensitive to inhibition by neutralizing antibodies. The “Yes” arrow  
322 represents an area on the linear part of the curve where the activity induced by that concentration  
323 of therapeutic protein would be sensitive to neutralization by antibody.

324  
325 *3. Interference*

326  
327 The matrix can also cause interference with neutralizing assays, particularly as sera or plasma  
328 components (apart from antibodies) may enhance or inhibit the activity of a therapeutic protein  
329 in bioactivity assays. For example, sera from patients with particular diseases may contain  
330 elevated levels of cytokines. These cytokines might serve to activate cells in the bioassay and  
331 obscure the presence of NAB. Therefore, the applicant should understand matrix effects in these  
332 assays. For some situations, approaches such as enriching antibodies from sera/plasma samples  
333 may be appropriate. However, this approach may result in the loss of antibodies. Consequently,  
334 such approaches will need to be thoroughly examined and validated by the applicant.

335

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

### *4. Confirmation of Neutralizing Antibodies*

Because of the complexity of these biologic assays, confirmatory approaches are critical during assay development and validation and may be useful in determining whether patients have mounted a true neutralizing antibody response. The applicant should consider the following approaches:

- a. As discussed above, performing antibody depletion assays to confirm the neutralizing activity is truly due to antibodies and not due to other inhibitory molecules could be useful.
- b. In many instances, a cell may be responsive to multiple stimuli other than the product under study. In such cases, the presence of neutralizing antibodies can be examined in the presence of the product (which should be blocked by a specific NAB response) vs. alternative stimuli (which should not be blocked by a specific NAB response).
- c. In some instances, serum may contain components that may yield false results in the NAB assay (soluble receptors, endogenous product counterpart). In such instances, adding test serum/plasma samples directly to the bioassay in the absence of product can be useful in understanding assay results.
- d. Finally, confirmation of neutralizing activity may be achieved by examining neutralizing activity in the presence of additional product versus an irrelevant protein (immunocompetition). Reduced neutralization should be observed in the presence of the specific product but not with an unrelated molecule.

### *5. Cut Point of Neutralizing Antibodies Assays*

Determination of assay cut point has historically posed a great challenge for NAB assays. Specifically, FDA recognizes the difficulty determining the degree of inhibition that is accurately indicative of neutralizing antibodies in a sample. As for all assays (see below), the determination should be statistically based and derived from assays using samples from patients not exposed to the product. If the degree of sample variation makes it difficult to assess neutralizing activity, other approaches may be considered but should be discussed with FDA before implementation. Alternatively, exploring other assay formats that lead to less variability and provide a more accurate assignment of cut point may be necessary.

### *6. Multiple Functional Domains*

Some proteins possess multiple domains that function in different ways to mediate clinical efficacy. An immune response to one domain may inhibit a specific function while leaving others intact. In such cases, the applicant should develop several neutralization assays to truly evaluate the implication of a neutralizing antibody response.

## **IV. CLINICAL ASPECTS OF ASSAY VALIDATION**

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

### 382 **A. Critical Considerations and Caveats**

383  
384 An extremely important consideration for assay selection is whether the assay can perform  
385 adequately in the relevant clinical setting (e.g., with actual human samples representing the  
386 patient population under study). This fact is often not given adequate consideration early and  
387 leads to problems when assay validation studies are attempted. For example, patients with  
388 rheumatoid arthritis express appreciable amounts of rheumatoid factor (RF), IgM, anti-IgG.  
389 When the product under consideration possesses an immunoglobulin “tail,” such as with  
390 monoclonal antibodies or Ig-fusion proteins, RF can interfere significantly with assay results. As  
391 a result, the applicant should carefully consider their ability to define reasonable assay cut points,  
392 problems with potential pre-existing antibodies, and the presence of analogous product/product-  
393 related material in the matrix early on in assay development.

### 395 **B. Determining the Minimal Dilution**

#### 397 *1. Importance*

398  
399 Matrix components can contribute to high assay background if undiluted, obscuring positive  
400 results. Therefore, there is almost always a need to dilute patient samples to maintain a  
401 reasonable ability to detect anti-product antibodies (sensitivity). Ideally, the minimum dilution is  
402 the dilution that yields a signal close to the signal of non-specific binding of assay diluent.  
403 However, there are exceptions where background remains high. Such a situation may necessitate  
404 careful analysis of pre-dose samples and determination of positivity as a significant increase over  
405 predose values. The applicant should carefully conduct statistical analyses that consider  
406 intersample variability to determine whether there has been a significant increase in antibody  
407 titer.

#### 409 *2. Approach*

410  
411 FDA recommends the applicant determine the minimum dilution from a panel of at least 10  
412 samples from the untreated patient population (or healthy donors if these samples are not readily  
413 available). The minimum dilution also should involve the use of a dilution series for each of the  
414 samples. Greater numbers of samples may be recommended by FDA and will depend on the  
415 variability of the data.

#### 417 *3. Recommendation*

418  
419 While the minimum dilution ultimately selected by the applicant will depend on the assay design  
420 and patient population, FDA recommends that dilutions not exceed 1:100. Higher dilution may  
421 result in the spurious identification of a negative response when patients may actually possess  
422 low, but clinically relevant, levels of antibodies. However, we appreciate that in some instances  
423 greater initial dilutions may be required, and the overall effect on assay sensitivity and  
424 immunogenicity risk should be kept in mind.

### 426 **C. Assay Cut Point**

427

## *Contains Nonbinding Recommendations*

*Draft — Not for Implementation*

### 428 1. *Definition*

429  
430 The cut point of the assay is the level of response of the assay at or above which a sample is  
431 defined to be positive and below which it is defined to be negative.

### 432 433 2. *Determination*

434  
435 The cut point should be statistically determined by using negative control samples (e.g., samples  
436 from patients not exposed to product). A small number of samples (5-10 samples from untreated  
437 individuals) may initially be used during assay development. However, assay validation with a  
438 sample size of 50-100 is statistically more reliable for determining the variability of the assay to  
439 effectively define the cut point. By performing several runs of negative samples, the variability  
440 of the assay can be determined. It may also be necessary to determine the cut point for different  
441 populations of patients. Depending on disease states and interfering components in  
442 serum/plasma, the cut point value may vary.

443  
444 When establishing the cut point, the applicant should also consider the removal of statistically  
445 determined outlier values. These values may derive from non-specific serum factors or the  
446 presence of pre-existing (“natural”) antibodies in patient samples (see section VIII, 4-9). While  
447 such natural antibodies to a variety of endogenous proteins exist even in normal individuals, they  
448 can be much higher in some disease states. Using immunodepletion approaches, the applicant  
449 should identify those samples with pre-existing antibodies and remove them from the analysis.  
450 If the presence of pre-existing antibodies is a confounding factor, it may be necessary to assign  
451 positive responses or a cut point based on the difference between individual patient results before  
452 and after exposure. Through careful design consideration such as minimal dilution, removal of  
453 outliers from analyses and appreciation of the natural antibody incidence, arriving at a reasonable  
454 value to define assay cut point should be possible.

### 455 456 3. *Recommendation*

457  
458 FDA recommends that the cut point have an upper negative limit of approximately 95 percent.  
459 While this value yields a 5 percent false positive rate, it improves the probability that the assay  
460 will identify all patients who developed antibodies. This sensitivity is particularly important in  
461 the initial screening assay as these results dictate the further analysis of the sample for NAB.  
462 Several approaches can be used. For example, parametric approaches using the mean plus 1.645  
463 standard deviation (SD), where 1.645 is the 95<sup>th</sup> percentile of the normal distribution may be  
464 appropriate. Other approaches include use of median and median absolute deviation value  
465 instead of mean and SD. Whatever approach is used, data must be presented to support the  
466 conclusion and the conclusion statistically justified. The specific approach employed will  
467 depend on various factors and FDA recommends that the method be discussed with FDA before  
468 implementation.

## 469 470 **V. ASSAY VALIDATION**

471

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

### 472 **A. Validation of Screening Assay**

473

#### 474 *1. Sensitivity*

475

476 The applicant should determine the sensitivity of the assay to have confidence when reporting  
477 immunogenicity rates. A purified preparation of antibodies specific to the product should be  
478 used to determine the sensitivity of the assay so assay sensitivity can be reported in mass  
479 units/ml of matrix. Antibodies used to assess sensitivity can take the form of affinity purified  
480 polyclonal preparations, or monoclonal antibodies. FDA recognizes that the purification process  
481 may result in loss of low avidity antibodies. Therefore, the applicant should evaluate antibody  
482 avidity before and after purification as part of reagent characterization.

483

484 Assay sensitivity represents the lowest concentration at which the antibody preparation  
485 consistently produces either a positive result or a readout equal to the cut point (defined below)  
486 determined for that particular assay. As assessment of patient antibody levels will occur in the  
487 presence of biological matrix, testing of assay sensitivity should be performed with the relevant  
488 dilution of the same biological matrix (e.g., normal human serum, plasma). The final sensitivity  
489 should be expressed as mass of antibody detectable/ml of matrix. Based on data from completed  
490 clinical trials, FDA recommends that screening assays achieve a sensitivity of approximately 250  
491 - 500 ng/ml as such antibody concentrations have been associated with clinical events.

492

#### 493 *2. Specificity*

494

495 Demonstrating assay specificity is critical to the interpretation of immunogenicity assay results.  
496 This can be challenging because of the presence of product and process related impurities (such  
497 as host cell proteins) and serum factors. When the therapeutic protein represents an endogenous  
498 human protein, the applicant should assess cross reactivity with the native human protein.  
499 Similarly, when the therapeutic protein is a member of a family of homologous proteins, the  
500 applicant should assess cross reactivity with multiple family members. Demonstrating the  
501 specificity of antibody responses to monoclonal antibodies and Ig-fusion proteins poses  
502 particular challenges. The applicant should clearly demonstrate that the assay method  
503 specifically detects anti-monoclonal antibodies and not the monoclonal antibody product itself,  
504 non-specific endogenous antibodies, or antibody reagents used in the assay. Similarly, for  
505 patient populations with a high incidence of RF, the applicant should demonstrate that RF does  
506 not interfere with the detection method.

507

508 Perhaps the most straightforward approach to addressing specificity is to demonstrate that  
509 binding can be blocked by soluble or unlabeled purified product. Specifically, positive and  
510 negative control antibody samples should be incubated with the purified protein under  
511 consideration or an irrelevant protein. The reduction in response can then be determined. For  
512 responses to monoclonal antibody products, inclusion of another monoclonal with the same Fc  
513 but different variable region can be critical. If the assay is specific for the protein in question,  
514 the addition of specific soluble protein should reduce the response to background or the cut point  
515 whereas the addition of an unrelated protein of similar size and charge should have no effect.  
516 Conversely, addition of specific protein should have little effect on negative antibody control  
517 samples.



## *Contains Nonbinding Recommendations*

*Draft — Not for Implementation*

518  
519 Other approaches to demonstrating specificity include the use of antibodies of irrelevant  
520 specificities to show that antibody binding is specific and not mediated by non-specific  
521 interactions with the substrate, blocking protein, or vessel. The issue of assay specificity is  
522 closely linked to the issue of assay interference from components in the matrix. Such  
523 interference can obscure the ability to detect samples that possess antibodies to the product. The  
524 presence of the drug itself or its endogenous counterpart in the matrix has the greatest potential  
525 to interfere with results.

526  
527 The potential for interference by the drug present in the serum should be assessed by testing the  
528 effect of various concentrations of study drug on the high, medium, and low QC positive  
529 controls. Therefore, the applicant should dilute antibody samples with varying concentrations of  
530 drug to assess how much drug is required to eliminate or reduce detection in the assay. There  
531 should be a relationship between the quantity of antibody and amount of drug required for a  
532 specified degree of inhibition (e.g., the high positive control should be inhibited less by a given  
533 concentration of product than the low positive control). Further discussion on this important  
534 aspect of antibody testing is addressed below.

### 535 536 *3. Precision*

537  
538 Demonstrating assay reproducibility (precision) is critical to the assessment of immunogenicity.  
539 This determination is particularly important when assessing changes in immunogenicity  
540 following changes in product manufacture, because such changes might only subtly alter  
541 immune response. The applicant should evaluate both intra-assay (repeatability) and inter-assay  
542 (intermediate precision) variability of assay responses. FDA recommends that inter-assay  
543 precision be evaluated on a minimum of three different days with a minimum of three replicates  
544 of the same sample in each assay. Intra-assay precision should be evaluated with a minimum of  
545 six replicates per plate. Samples should include negative controls and positive samples whose  
546 testing yields values in the low, medium and high levels of the assay dynamic range. The  
547 applicant should evaluate inter-operator precision when more than one operator will be running  
548 the assay.

549  
550 FDA acknowledges that samples with a low concentration of antibodies are likely to have a  
551 higher variability than samples with high antibody concentrations. Nonetheless this  
552 determination for low concentration samples will be important for understanding patient samples  
553 that may truly possess low levels or low avidity antibodies vs. those that yield false positive  
554 results. Positional effects (e.g., location on the microtiter plate) are a major contributor to assay  
555 variability and the applicant should evaluate such effects in the course of evaluating assay  
556 precision.

### 557 558 *4. Robustness and Sample Stability*

559  
560 The applicant should assess robustness as an indication of the assay's reliability during normal  
561 usage by examining the impact of small but deliberate changes in method parameters. For  
562 example, changes in temperature, pH, buffer, or incubation times can all impact results. FDA  
563 recommends storing patient samples in a manner that preserves antibody reactivity at the time of

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

564 testing. Freezing and thawing patient samples may also affect assay results and those assay  
565 results should be evaluated. In addition, the applicant should examine other parameters affecting  
566 patient samples such as state of hemolysis and specific anticoagulants. Other considerations may  
567 include state of lipemia, presence of bilirubin, and presence of concomitant medications that a  
568 patient population may be using. The applicant should examine robustness during the  
569 development phase and if small changes in specific steps in the assay affect results, specific  
570 precautions should be taken to control their variability.

571

### **B. Validation of Neutralizing Assay**

572

#### *1. Sensitivity*

573

574 The approach to demonstrating the sensitivity of the neutralization assay is similar to that of the  
575 binding assay. The applicant should report the sensitivity in mass units. FDA recognizes that  
576 not all anti-product antibodies are neutralizing and it can be difficult to identify positive control  
577 antibodies with neutralizing capacity. Nonetheless, such reagents are critical for demonstrating  
578 assay sensitivity.

579

580 The concentration of product employed in the neutralizing assay is also critical as discussed  
581 above. FDA recommends that the concentration of product used be on the linear region of the  
582 dose response curve for the product. FDA recognizes that while the use of low concentrations of  
583 product may lead to a neutralizing assay that is more sensitive to inhibition by antibodies, very  
584 low concentration of product may result in poor precision of the assay. Another feature of  
585 neutralizing assays is that they are often less sensitive than binding assay. While this limitation  
586 is noted, sponsors are encouraged to develop the most sensitive assays possible.

587

#### *2. Specificity*

588

589 Applicants should demonstrate assay specificity for cell based neutralizing assays. As  
590 mentioned above, for cells that may be responsive to stimuli other than the specific therapeutic  
591 protein, the ability to demonstrate that NAB only inhibit the response to product and not to other  
592 stimuli is a good indication of assay specificity. In such studies FDA recommends that the other  
593 stimuli be employed at a concentration that yields an outcome similar to that of the therapeutic  
594 protein. The applicant should also confirm the absence of alternative stimuli in patient serum.

595

#### *3. Precision*

596

597 Assay precision can also be more problematic for neutralizing assays than binding assays.  
598 Biologic responses can be inherently more variable than carefully controlled binding studies.  
599 Consequently, the applicant should perform more replicates for assessment of precision and  
600 assessment of patient responses than for the screening assay.

601

#### *4. Other Elements of Neutralizing Assay Validation*

602

603

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

608 The applicant should validate both specificity and robustness of the neutralizing assay during  
609 development. Approaches such as those described above for confirmatory approaches for  
610 neutralizing assays can support the specificity of the assay during validation. Many elements of  
611 assay validation of NAB are similar to those used for validation of the screening assay. The  
612 complexity of bioassays makes them particularly susceptible to changes in assay conditions and  
613 it is essential to control parameters such as cell passage number, incubation times, and media  
614 components.

615

### **C. Validation of Immunodepletion/Competitive Confirmatory Assay**

617

618 While immunodepletion/competition assays are employed to confirm results of neutralizing  
619 assays, they are most often employed as an adjunct to antibody binding assays. While  
620 confirmatory assays need to be fully validated in a manner similar to binding and neutralizing  
621 assays (above), these assays raise some specific issues. In these assays, antibodies are  
622 specifically removed or competed<sup>3</sup> from patient samples and the loss of response is determined.  
623 The most difficult issue is identifying the degree of inhibition or depletion that will be used to  
624 ascribe positivity to a sample. In the past, 50 percent inhibition has been used, but this number is  
625 arbitrary and is unlikely to be relevant for all assays. FDA recommends that sponsors carefully  
626 address this issue during assay development and base determinations on meaningful data. In this  
627 regard, examining percent inhibition of QC samples (high, medium, and low) before and after  
628 immunodepletion/competition with specific vs. irrelevant proteins can help to identify  
629 meaningful values.

630

## **VI. IMPLEMENTATION OF ASSAY TESTING**

632

### **A. Obtaining Patient Samples**

634

635 FDA recommends the applicant obtain pre-exposure samples from all patients. The potential for  
636 pre-existing antibodies or confounding components in the matrix make it essential for one to  
637 understand the degree of reactivity before treatment. The applicant should then obtain  
638 subsequent samples, and the timing will depend on the frequency of dosing. Optimally, samples  
639 taken 7-14 days after exposure can help elucidate an early IgM predominant response. Samples  
640 taken at 4 to 6 weeks following exposure are generally optimal for determining IgG responses.  
641 For individuals receiving a single dose of product, the above time may be adequate. However,  
642 for patients receiving product at multiple times during the trial, the applicant should obtain  
643 samples at appropriate intervals throughout the trial.

644

645 The timing for obtaining these samples may be complicated and FDA recommends the applicant  
646 coordinate the sampling visits with visits to assess other aspects of the clinical trial. However,  
647 obtaining samples is essential and the applicant should obtain samples at a time when there will  
648 be minimal interference from product present in the serum. An applicant should consider the

---

<sup>3</sup> "Competed" 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.

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

649 product's half-life to help determine appropriate times for sampling. This is especially important  
650 for monoclonal antibody products because these products can have half-lives of several weeks or  
651 more and, depending on the dosing regimen, the therapeutic monoclonal antibody itself could  
652 remain present in the serum for months.

653  
654 The level of product that interferes with the assay, as determined by immune competition, may  
655 also help define meaningful time points for sampling. If drug-free samples cannot be obtained  
656 during the treatment phase or the trial, then the applicant should take additional samples after an  
657 appropriate washout period (e.g., five drug half-lives). Obtaining samples to test for meaningful  
658 antibody results can also be complicated if the product in question is itself an immune  
659 suppressant. In such instances, the applicant should obtain samples from patients who have  
660 undergone a washout period either because the treatment phase has ended or because the patient  
661 has dropped out of the study.

### **B. Concurrent Positive and Negative Quality Controls**

662  
663  
664  
665 If the applicant completes the proper validation work and makes the cut point determinations, the  
666 immunogenicity status of patients should be straightforward to determine. However, FDA  
667 believes positive control or QC samples are critical and should be run concurrently with patient  
668 samples. We recommend that these samples span a level of positivity with QC samples having a  
669 known negative, low, medium, and high reactivity in the assay. More importantly, the samples  
670 should be diluted in the matrix in which patient samples will be examined (e.g., same percent  
671 serum/plasma). In this way, the applicant ensures that the assay is performing to its required  
672 degree of accuracy and that patient samples are correctly evaluated. For the low positive sample,  
673 we recommend that a concentration be selected that, upon statistical analysis, would lead to the  
674 rejection of an assay run 1 percent of the time. Such an approach would ensure the appropriate  
675 sensitivity of the assay when performed on actual patient samples.

676  
677 FDA also recommends that these QC samples be obtained from humans or animals possessing  
678 antibodies that are detected by the secondary detecting reagent, to ensure that negative results  
679 that might be observed are truly due to lack of antigen reactivity and not due to failure of the  
680 secondary reagent. This issue is not a problem for antigen bridging studies (as labeled antigen is  
681 used for detection), although other considerations may apply in these assays.

### **C. Cut Point Normalization**

682  
683  
684  
685 FDA appreciates that there will be some degree of variability in an assay. Consequently, FDA  
686 recommends the applicant develop a predetermined method for normalization of data obtained at  
687 different times. During assay validation, the applicant should identify a negative or low QC  
688 sample and determine a normalization factor. The normalization factor is the difference in the  
689 readout value of the control and the value of the 95 percent limit obtained for the initial cut point  
690 determinations. The normalization factor can be added to the value obtained for the negative QC  
691 sample to normalize for the cut point of the assay performed at different times. Other  
692 approaches may also be appropriate such as normalizing all values against those obtained with a  
693 negative control sample or in extreme cases establishing plate specific cut points.

694

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

### 695 **D. Reporting Patient Results**

696  
697 As discussed, unless a universally accepted and accessible source of validated antibody is  
698 available as a control and parallelism between the dilution curves of the control antibody and  
699 patient samples has been demonstrated, FDA believes it is neither necessary, nor desirable for  
700 the applicant to report patient antibody results in terms of mass units. Reporting in terms of titers  
701 (e.g., reciprocal of the dilution able to yield a background just at or above the cut point) is more  
702 appropriate and is well understood by the medical community. We believe attempts to convert  
703 such data into mass units by using standard curves or other data conversion methods are  
704 generally confusing and inaccurate.

### 705 706 **E. Pre-existing Antibodies**

707  
708 The ability to test patient samples for antiproduct antibodies can serve as a critical safety  
709 assessment throughout clinical trial development. Early hints about risks of immunogenicity  
710 may be obtained from the measurement of pre-existing or natural antibodies. A growing body of  
711 evidence in the medical literature suggests that B cells and T cells with specificity for a number  
712 of self proteins exist naturally and may even be heightened in some disease states. For example,  
713 antibodies to IFN can be found in normal individuals (see section VIII, 7-9). Less surprisingly,  
714 pre-existing antibodies to foreign antigens, such as bacterial products, have also been found in  
715 normal individuals, possibly as a result of previous exposure to the organism or cross reactivity.

### 716 717 **F. Specific Considerations**

#### 718 719 *1. Monoclonal Antibodies*

720  
721 Some special considerations pertain to the detection of antibodies against monoclonal antibody  
722 therapeutics and in vivo diagnostics. Animal-derived monoclonal antibodies, particularly those  
723 of rodent origin, are expected to be immunogenic with the immune response directed primarily  
724 against the Fc portion of the molecule. In the early days of the therapeutic mAb industry, this  
725 was a primary reason for the failure of clinical trials.

726  
727 Technologies reducing the presence of non-human sequences in monoclonal antibodies  
728 (chimerization and humanization) have led to a dramatic reduction but not elimination of  
729 immunogenicity. In these cases, the immune responses are directed largely against the variable  
730 regions of the monoclonal antibody. As immune responses against the variable regions of fully  
731 human monoclonal antibody are also anticipated, FDA does not expect that the use of fully  
732 human monoclonal antibodies will further reduce immunogenicity by a significant margin.  
733 Many of these concerns also pertain to Fc fusion proteins containing a human Fc region.

#### 734 735 *2. Rheumatoid Factor*

736  
737 Measuring immune responses to products that possess immunoglobulin tails (monoclonal  
738 antibodies, Fc fusion proteins) is particularly difficult when RF is present in serum/plasma. RF  
739 is generally an IgM antibody that recognizes IgG (although other Ig specificities have been  
740 noted). Consequently, RF will bind Ig regions, making it appear that specific antibody to the

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

741 product exists. Several approaches for minimizing interference from RF have proven useful,  
742 including treatment with aspartame (see section VIII, 10) and careful optimization of reagent  
743 concentrations so as to reduce background binding. FDA recommends examining immune  
744 responses to Fc fusion proteins in clinical settings where RF is present to develop an antigenic  
745 moiety that corresponds to the non-Fc region of the molecule and assess whether patient serum  
746 binds the truncated product. For example, for a cytokine-Fc fusion protein, measuring antibody  
747 responses to the purified cytokine can help in assessing the specific immunogenicity of the  
748 fusion protein.

749

### 750 *3. Fusion Proteins*

751

752 Examination of immune responses to fusion proteins can be challenging and may require  
753 development of multiple assays to measure immune responses to both domains of the molecules  
754 as well as to the neoantigen formed at the junction of the components.

755

### 756 *4. High Levels of Endogenous Protein in Sera*

757

758 If serum/plasma possess high levels of protein that are analogous to the product under study,  
759 developing traditional antibody binding assays to measure relevant antibodies can be particularly  
760 challenging. For example, studies looking at immune response to albumin can be confounded by  
761 large quantities of serum albumin. In these instances, other approaches for measuring  
762 immunogenicity may be warranted, such as enzyme-linked immunosorbent spot (ELISPOT) or  
763 plaque type assays, to measure numbers of antigen-specific antibody secreting cells.

764

## 765 **VII. OTHER ASPECTS OF IMMUNOGENICITY TESTING**

766

### 767 **A. Isotypes**

768

769 While the initial screening assay should be able to detect all isotypes, in some circumstances the  
770 applicant should develop assays that discriminate between antibodies of specific isotypes. For  
771 example, for products that induce allergic responses, assays that can specifically measure levels  
772 of IgE may be important for helping predict and prepare for anaphylactic reactions in the clinic.  
773 In addition, the generation of immunoglobulin G4 (IgG4) antibodies has been associated with  
774 immune responses generated under conditions of chronic antigen exposure such as with factor  
775 VIII treatment. IgG4 antibodies have also been shown to be less pathogenic as they fail to fix  
776 complement and are associated with blocking of allergic responses (section VIII, 11).  
777 Consequently, determining if antibody responses occurring upon prolonged exposure to  
778 therapeutic proteins are associated with this isotype may be useful.

779

### 780 **B. Epitope Specificity**

781

782 FDA recommends the applicant direct initial screening tests against the whole molecule and its  
783 endogenous counterpart. However, for product development, the applicant should investigate the  
784 regions or “epitopes” to which immune responses are specifically generated. This determination  
785 may be particularly important with fusion molecules in which two proteins are genetically or  
786 physically fused. In these circumstances, the region where the two molecules join may form a

## ***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

787 neoantigen and immune responses to this region may arise. Because of epitope spreading,  
788 immune responses to other parts of the molecule may ensue, leading to generation of neutralizing  
789 antibodies to the product or its endogenous counterpart. For these products, FDA encourages  
790 sponsors to investigate the initiating event in the immune cascade. This knowledge may allow  
791 for modification to the protein to reduce its potential immunogenicity.

792

### **VIII. REFERENCES**

793

- 794
- 795 1. Mire-Sluis AR, Barrett YC, Devanarayan V, Koren E, Liu H, Maia M, et al., 2004,  
796 Recommendations for the Design and Optimization of Immunoassays used in the Detection  
797 of Host Antibodies Against Biotechnology Products, *Immunol Methods*, 289(1-2):1-16.  
798
  - 799 2. Gupta S, Indelicato S, Jethwa V, Kawabata T, Kelley M, Mire-Sluis AR, et al., 2007,  
800 Recommendation for the Design, Optimization, and Qualification of Cell-based Assays used  
801 for the Detection of Neutralizing Antibody Responses Elicited to Biological Therapeutic. *J*  
802 *Immunol Methods*, 321:1-18.  
803
  - 804 3. Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, 1995, The Nijmegen  
805 Modification of the Bethesda Assay for Factor VIII:C Inhibitors: Improved Specificity and  
806 Reliability, *Thromb Haemostas*, 73:247-251.  
807
  - 808 4. Coutinho, A, Kazatchkine MD, and Avrameas S, 1995, Natural Autoantibodies, *Current*  
809 *Biology*, 7:812-818.  
810
  - 811 5. van der Meide PH and Schellekens H, 1997, Anti-cytokine Autoantibodies: Epiphenomenon  
812 or Critical Modulators of Cytokine Action. *Biotherapy*, 10: 39-48.  
813
  - 814 6. Boes M. Role of Natural and Immune IgM Antibodies in Immune Response, 2000, *Mol*  
815 *Immunol*, 37: 1141-1149.  
816
  - 817 7. Turano A, Balsari A, Viani, E, Landolfo S, Zanoni L, Gargiulo F, and Caruso A, 1992,  
818 Natural Human Antibodies to Interferon Interfere with the Immunomodulating Activity of  
819 the Lymphokine, *Proc Natl Acad Sci* 89:4447-4451.  
820
  - 821 8. Ross C, Hansen MB, Schyberg T, and Berg K, 1990, Autoantibodies to Crude Human  
822 Leucocyte Interferon (IFN), Native Human IFN, Recombinant Human IFN-alpha 2b and  
823 Human IFN-gamma in Healthy Blood Donors, *Clin Exp Immunol*, 82: 57-62.  
824
  - 825 9. Caruso A and Turano A, 1997, Natural Antibodies to Interferon-gamma, *Biotherapy*, 10: 29-  
826 37.  
827
  - 828 10. Ramsland PA, Movafagh BF, Reichlin M, and Edmundson AB, 1999, Interference of  
829 Rheumatoid Factor Activity by Aspartame, a Dipeptide Methyls Ester, *J of Mol Recognition*,  
830 12: 249-257.  
831
  - 832 11. Aalberse RC and Schuurman J., 2002, IgG4 Breaking the Rules, *Immunol*, 105:9-19.

***Contains Nonbinding Recommendations***

*Draft — Not for Implementation*

- 833  
834 12. Shankar G, Devanarayan V, Amaravadi L, Barret YC, Bowsher R, Finco-Kent D, et al. 2008  
835 Epub ahead of print, Recommendations for the Validation of Immunoassays used for  
836 Detection of Host Antibodies Against Biotechnology Products, J Pharm Biomed Anal.  
837