
Guidance for Industry

Assay Development for Immunogenicity Testing of Therapeutic Proteins

DRAFT GUIDANCE

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**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

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Assay Development for Immunogenicity Testing of Therapeutic Proteins

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**U.S. Department of Health and Human Services
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1 **Guidance for Industry¹**
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6 This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current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.² 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

¹ 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.

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

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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

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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

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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

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- 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

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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.

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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*

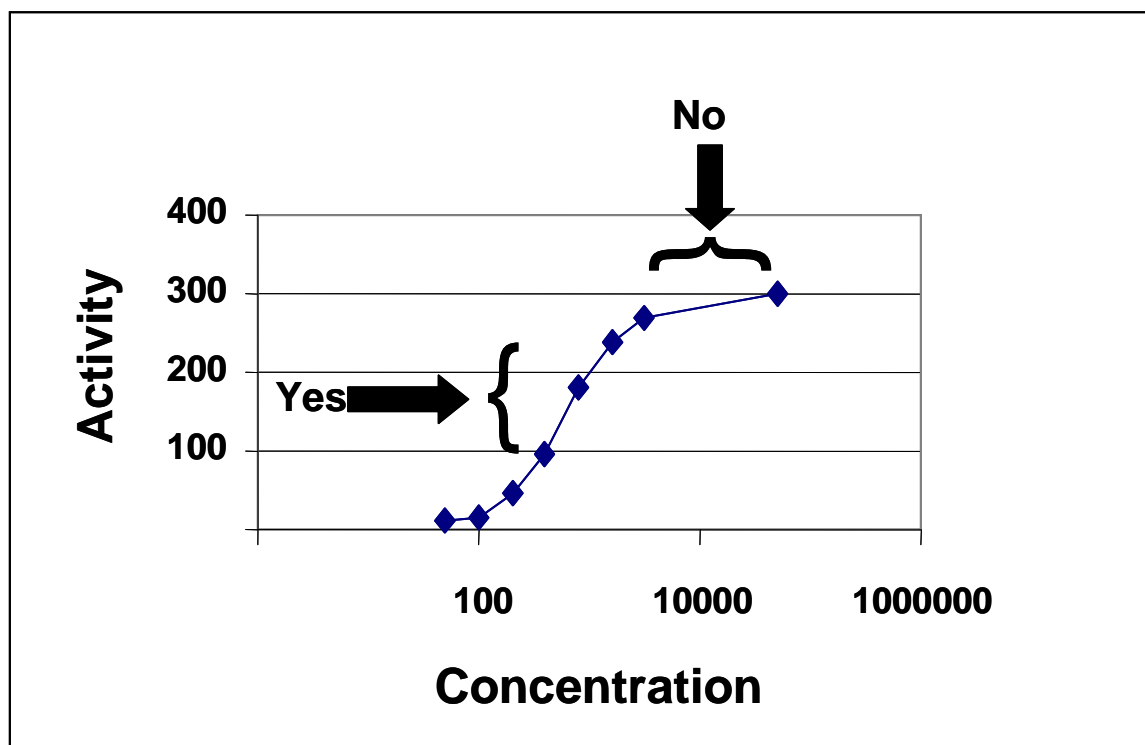
276
277 Two types of assays have been used to measure neutralizing antibody activity: cell-based
278 biologic assays and non cell-based competitive ligand-binding assays. While competitive ligand-
279 binding assays may be the only alternative in some situations, generally FDA considers that
280 bioassays 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.

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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

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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**

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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 95th 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

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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.

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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

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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

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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 competed³ 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

³ "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.

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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

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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.

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D. Reporting Patient Results

695
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.

E. Pre-existing Antibodies

705
706
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.

F. Specific Considerations

1. Monoclonal Antibodies

716
717
718
719
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.

2. Rheumatoid Factor

734
735
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

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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

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

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

VII. OTHER ASPECTS OF IMMUNOGENICITY TESTING

766

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

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

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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

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