Noninvasive Fetal RHD Genotyping Using Cell-Free Fetal DNA

Policy # 00400
Original Effective Date: 02/19/2014
Current Effective Date: 02/15/2017

Applies to all products administered or underwritten by Blue Cross and Blue Shield of Louisiana and its subsidiary, HMO Louisiana, Inc. (collectively referred to as the "Company"), unless otherwise provided in the applicable contract. Medical technology is constantly evolving, and we reserve the right to review and update Medical Policy periodically.

Services Are Considered Investigational
Coverage is not available for investigational medical treatments or procedures, drugs, devices or biological products.

Based on review of available data, the Company considers noninvasive fetal RHD genotyping using cell-free fetal DNA to be investigational.*

Background/Overview
Alloimmunization refers to the development of antibodies in a patient whose blood type is Rhesus (Rh)—negative and who is exposed to Rh-positive red blood cells (RBCs). This most commonly occurs from fetal-placental hemorrhage and entry of fetal blood cells into maternal circulation. The management of an Rh-negative pregnant patient who is not alloimmunized and is carrying a known Rh-positive fetus, or if fetal Rh status is unknown, involves administration of Rh immune globulin at standardized times during the pregnancy to prevent formation of anti-Rh antibodies. If the patient is already alloimmunized, monitoring the levels of anti-Rh antibody titers and for the development of fetal anemia is performed. Both noninvasive and invasive tests to determine fetal Rh status exist.

Rh blood groups
The Rh system includes more than 100 antigen varieties found on RBCs. RhD is the most common and the most immunogenic. When people have the RhD antigen on their RBCs, they are considered to be RhD-positive; if their RBCs lack the antigen, they are considered to be RhD-negative. The RhD-antigen is inherited in an autosomally dominant fashion, and a person may be heterozygous (Dd) (~60% of Rh-positive people) or homozygous (DD) (~40% of Rh-positive people). Homozygotes always pass the RhD antigen to their offspring, whereas heterozygotes have a 50% chance of passing the antigen to their offspring. A person who is RhD-negative does not have the Rh antigen. Although nomenclature refers to RhD-negative as dd, there is no small d antigen (i.e., they lack the RHD gene and the corresponding RhD antigen).

RhD-negative status varies among ethnic group and is 15% in whites, 5 to 8% in African Americans, 5% to 8%, and 1 to 2% in Asians and Native Americans, respectively.

In the white population, almost all RhD-negative individuals are homozygous for a deletion of the RHD gene. However, in the African-American population, only 18% of RhD-negative individuals are homozygous for an RHD deletion, and 66% of RhD-negative African Americans have an inactive RH Dy. There are also numerous rare variants of the D antigen, which are recognized by weakness of expression of D and/or by absence of some of the epitopes of D. Some individuals with variant D antigens, if exposed to RhD-positive RBCs, can make antibodies to one or more epitopes of the D antigen.
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RhD-negative women can have a fetus that is RhD-positive if the fetus inherits the RhD-positive antigen from the paternal father.

Causes of alloimmunization
By 30 days of gestation, the RhD antigen is expressed on the RBC membrane, and alloimmunization can be caused when fetal Rh-positive RBCs enter maternal circulation, and the Rh-negative mother develops anti-D antibodies. Once anti-D antibodies are present in a pregnant woman's circulation, they can cross the placenta and cause destruction of fetal RBCs.

The production of anti-D antibodies in RhD-negative women is highly variable and significantly affected by several factors, including the volume of fetomaternal hemorrhage, the degree of maternal immune response, concurrent ABO incompatibility, and fetal homozygosity versus heterozygosity for the D antigen. Therefore, although ~10% of pregnancies are Rh-incompatible, <20% of Rh-incompatible pregnancies actually lead to maternal alloimmunization.

Small fetomaternal hemorrhages of RhD-positive fetal RBCs into the circulation of an RhD-negative woman occurs in nearly all pregnancies, and percentages of feto-maternal hemorrhage increase as the pregnancy progresses: 7% in the first trimester, 16% in the second trimester, and 29% in the third trimester, with the greatest risk of RhD alloimmunization occurring at birth (15 to 50%). Transplacental hemorrhage accounts for almost all cases of maternal RhD alloimmunization.

Fetomaternal hemorrhage can also be associated with miscarriage, pregnancy termination, ectopic pregnancy, invasive in-utero procedures (e.g., amniocentesis), in utero fetal death, maternal abdominal trauma, antepartum maternal hemorrhage, and external cephalic version. Other causes of alloimmunization include inadvertent transfusion of RhD-positive blood and RhD-mismatched allogeneic hematopoietic stem-cell transplantation.

Consequences of alloimmunization
Immunoglobulin (Ig) G antibody–mediated hemolysis of fetal RBCs, known as hemolytic disease of the fetus and newborn, varies in severity and can have a variety of manifestations. The anemia can range from mild to severe with associated hyperbilirubinemia and jaundice. In severe cases, hemolysis may lead to extramedullary hematopoiesis and reticuloendothelial clearance of fetal RBCs, which may result in hepatosplenomegaly, decreased liver function, hypoproteinemia, ascites, and anasarca. When accompanied by high-output cardiac failure and pericardial effusion, this condition is known as hydrops fetalis, which without intervention, is often fatal. Intensive neonatal care, including emergent exchange transfusion, is required.

Cases of hemolysis in the newborn that do not result in fetal hydrops can still lead to kernicterus, a neurologic condition observed in infants with severe hyperbilirubinemia due to the deposition of unconjugated bilirubin in the brain. Symptoms that manifest several days after delivery can include poor feeding, inactivity, loss of the Moro reflex, bulging fontanelle, and seizures. The 10% of infants who survive may develop spastic choreoathetosis, deafness, and/or mental retardation.
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The result of disease from alloimmunization, hemolytic disease of the fetus or newborn, was once a major contributor to perinatal morbidity and mortality. However, with the widespread adoption of antenatal and postpartum use of Rh immune globulin in developed countries, the result has been a major decrease in frequency of this disease. In developing countries without prophylaxis programs, stillbirth occurs in 14% of affected pregnancies, and 50% of pregnancy survivors either die in the neonatal period or develop cerebral injury.

Prevention of alloimmunization
There are four currently in use Rh immune globulin products available in the U.S., all of which undergo micropore filtration to eliminate viral transmission. To date, no reported cases of viral infection related to Rh immune globulin administration have been reported in the U.S. Theoretically, the Creutzfeldt-Jakob disease (CJD) agent could be transmitted by use of Rh immune globulin. Local adverse reactions may occur, including redness, swelling, and mild pain at the site of injection, and hypersensitivity reactions have been reported.

The American College of Obstetricians and Gynecologists (ACOG) and the American Association of Blood Banks (AABB) recommend the first dose of Rh(D) immune globulin (e.g., RhoGAM®) be given at 28 weeks’ gestation, (or earlier if there’s been an invasive event), followed by a postpartum dose given within 72 hours of delivery.

Diagnosis of alloimmunization
The diagnosis of alloimmunization is based on detection of anti-RhD antibodies in the maternal serum.

The most common test for determining antibodies in serum is the indirect Coombs test. Maternal serum is incubated with known RhD-positive RBCs. Any anti-RhD antibody present in the maternal serum will adhere to the RBCs. The RBCs are then washed and suspended in Coombs serum, which is antihuman globulin. RBCs coated with maternal anti-RhD will agglutinate, which is referred to as a positive indirect Coombs test. The indirect Coombs titer is the value used to direct management of pregnant alloimmunized women.

Management of alloimmunization during pregnancy
A patient’s first alloimmunized pregnancy involves minimal fetal or neonatal disease. Subsequent pregnancies are associated with more severe degrees of fetal anemia. Treatment of an alloimmunized pregnancy requires monitoring of maternal anti-D antibody titers and serial ultrasound assessment of middle cerebral artery peak systolic velocity of the fetus.

If severe fetal anemia is present near term, delivery is performed. If severe anemia is detected remote from term, intrauterine fetal blood transfusions may be performed.

Determining fetal RhD status
ACOG recommends that all pregnant women should be tested at the time of their first prenatal visit for ABO blood group typing and Rh-D type and be screened for the presence of anti-RBC antibodies. These laboratory tests should be repeated for each subsequent pregnancy. The AABB also recommends that
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antibody screening be repeated before administration of anti-D immune globulin at 28 weeks’ gestation, postpartum, and at the time of any event during pregnancy.

If the mother is determined to be Rh-negative, the paternal Rh status should also be determined at the initial management of a pregnancy. If paternity is certain and the father is Rh-negative, the fetus will be Rh-negative, and further assessment and intervention are unnecessary. If the father is RhD-positive, he can be either homozygous or heterozygous for the D allele. If he is homozygous for the D allele (i.e., D/D), then the fetus is RhD-positive. If the paternal genotype is heterozygous for Rh status or is unknown, determination of the Rh-status of the fetus is the next step.

Invasive and noninvasive testing methods to determine the Rh status of a fetus are available.

Invasive procedures use polymerase chain reaction (PCR) assays to assess the fetal cellular elements in amniotic fluid by amniocentesis or by chorionic villus sampling (CVS). Although CVS can be performed earlier in a pregnancy, amniocentesis is the preferred method because CVS is associated with disruption of the villi and the potential for larger fetomaternal hemorrhage and worsening alloimmunization if the fetus if RhD-positive. The sensitivity and specificity of fetal RHD typing by PCR are reported as 98.7% and 100%, respectively, with positive and negative predictive values of 100% and 96.9%, respectively.

Noninvasive testing involves molecular analysis of cell-free fetal DNA (cffDNA) in the maternal plasma or serum. In 1998, Lo et al. showed that about 3% of cell-free DNA in the plasma of first trimester pregnant women is of fetal origin, with this percentage rising to 6% in the third trimester. Fetal DNA cannot be separated from maternal DNA, but if the pregnant woman is RhD-negative, the presence of specific exons of the RHD gene, which are not normally present in the circulation of an RhD-negative patient, predicts an RhD-positive fetus. cffDNA has been proposed as a noninvasive alternative to obtaining fetal tissue by invasive methods, which are associated with a risk of miscarriage.

The large quantity of maternal DNA compared to fetal DNA in the maternal circulation complicates the inclusion of satisfactory internal controls to test for successful amplification of fetal DNA. Therefore, reactions to detect Y chromosome-linked gene(s) can be included in the test, which will be positive when the fetus is a male. When Y chromosome-linked genes are not detected, tests for polymorphisms may be performed to determine whether the result is derived from fetal but not maternal DNA.

cffDNA testing to determine the fetal RHD genotype is standard of practice in many European countries.

FDA or Other Governmental Regulatory Approval

U.S. Food and Drug Administration (FDA)

Clinical laboratories may develop and validate tests in-house and market them as a laboratory service; laboratory-developed tests (LDTs) must meet the general regulatory standards of the Clinical Laboratory Improvement Act (CLIA). No genotyping tests were found. Laboratories that offer LDTs must be licensed by CLIA for high-complexity testing. To date, the FDA has chosen not to require any regulatory review of this test.
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Sequenom offers SensiGene™ Fetal RHD Genotyping test, performed by proprietary SEQureDx™ technology. The assay targets exons 4, 5, and 7 of the \( RHD \) gene located on chromosome 1, psi (\( y \)) pseudogene in exon 4, and assay controls which are 3 targets on the Y chromosome (SRY, TTTY, DBY).

The company claims that the uses of its test include:
- Clarify fetal RHD status without testing the father, avoiding the cost of paternity testing and paternal genotyping
- Clarify fetal RHD status when maternal anti-D titers are unclear
- Identify the RHD (-) fetus in mothers who are opposed to immunization(s) and vaccines
- RhD (-) sensitized patients
- Avoid invasive testing by CVS or genetic amniocentesis

Centers for Medicare and Medicaid Services (CMS)
There is no national coverage determination (NCD). In the absence of an NCD, coverage decisions are left to the discretion of local Medicare carriers.

**Rationale/Source**
This policy was recently update using MEDLINE database through October 6, 2015.

Assessment of this diagnostic technology focuses on 3 parameters: (1) technical performance; (2) diagnostic performance (sensitivity, specificity, and positive and negative predictive values) in appropriate populations of patients; and (3) demonstration that the diagnostic information can be used to improve patient outcomes (clinical utility).

**Technical Performance**
No studies were identified that provide direct evidence of the analytic validity of \( RHD \) genotyping. The commercially available test uses next-generation sequencing. Neither the routine quality control procedures used for the test, nor the analytic performance metrics have been published.

**Diagnostic Performance**
In 2014, Zhu et al published a meta-analysis of studies on the diagnostic accuracy of noninvasive fetal RHD genotyping using cell-free fetal DNA. The investigators identified 37 studies conducted in RhD-negative pregnant women that were published by the end of 2013. The studies included a total of 11,129 samples, and 352 inconclusive samples were excluded. When all data were pooled, the sensitivity of fetal RhD genotyping was 99% and the specificity was 98%. Diagnostic accuracy was higher in samples collected in the first trimester (99.0%) than those collected in the second (98.3%) or third (96.4%) trimesters.

Also in 2014, Chitty et al published a prospective study from the U.K. that was not included in the Zhu meta-analysis. Samples from 2288 Rh-negative women who initiated prenatal care before 24 weeks of gestation were analyzed using \( RhD \) genotyping. Overall, the sensitivity of the test was 99.34% and the specificity was 94.91%. The likelihood of correctly detecting RhD status in the fetus increased with gestational age, with high levels of accuracy after 11 weeks. For example, for samples taken before 11 completed weeks of gestation, the sensitivity was 96.85% and the specificity was 94.40%, and at 14 to 17 weeks’ gestation,
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sensitivity was 99.67% and specificity was 95.34%. The finding in the Chitty study of increased accuracy as pregnancies advanced differs from that of the Zhu meta-analysis, which found highest diagnostic accuracy in the first trimester.

Two key studies reporting the clinical validity of fetal RHD genotyping with the Sequenom assay, which is commercially available in the United States, are detailed next, and findings are summarized in Table 1.

Table 1. Sequenom SensiGene Clinical Validation Studies

<table>
<thead>
<tr>
<th>Author</th>
<th>Accuracy for RhD Status Determination, %</th>
<th>False Negative Rate RhD Determination, %</th>
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<tbody>
<tr>
<td>Moise (2012)</td>
<td>98.1-99.1, depending on trimester during which it was performed</td>
<td>.45</td>
</tr>
<tr>
<td>Bombard (2011)</td>
<td></td>
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<tr>
<td>Cohort 1</td>
<td>97.1</td>
<td>1.9</td>
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<tr>
<td>Cohort 2</td>
<td>99.5</td>
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In 2012, Moise and colleagues analyzed samples from 120 patients who were enrolled prospectively between May 2009 and July 2010 from multiple centers. All patients were Rh-negative pregnant patients with no evidence of alloimmunization. Race/ethnicity was Caucasian/white (72.5%), African-American/black (12.5%), Hispanic/Latino (12.5%), Asian (0.8%), and other (1.7%). The samples were analyzed using the SensiGENE RHD test using MALDI-TOF mass spectrometry to detect control and fetal-specific DNA signals. The determination of fetal sex was: 3 Y-chromosome markers=male fetus, 2 markers=inconclusive, and 1 or no markers=female fetus. The algorithm for RHD determination was: pseudogene present=inconclusive, 3 RHD markers present=RHD-positive fetus, 2 markers present=inconclusive, 1 or no markers=RHD-negative fetus. If the results were RHD-positive and male, the fetus was determined to be RHD-positive and male, and if RHD-negative and male results were noted, the fetus was determined to be RHD-negative and male. If the results were RHD-positive and female, the fetus was determined to be RHD-positive and female. If an RHD-negative and female result was noted, reflex testing was performed with a panel of 92 single-nucleotide polymorphisms (SNPs). If a minimum of 6 informative paternal alleles (uniquely and unambiguously fetal in nature) were detected, the result was an RHD-negative, female fetus. If less than 6 alleles were detected, the sample was reported as inconclusive. Cord blood was obtained at delivery and RhD typing was determined using standard serologic methods, and phenotype assessment of the newborns was used to assign gender. The pregnant patients underwent planned venipunctures during 3 time periods in gestation: 11–13 weeks, 16–19 weeks, and 28–29 weeks. At the second blood draw, 2 patients were not evaluated because they did not return during the prescribed gestational age window; and at the time of the third trimester blood draw, 7 patients did not have a sample obtained.

Median gestational age of the first, second and third trimester samplings was 12.4 (range, 10.6–13.9) weeks, 17.6 (16–20.9) weeks and 28.7 (27.9–33.9) weeks, respectively. There were 3 samples in the first trimester and 2 samples in the second trimester insufficient in the quantity of samples to perform the DNA assay (1.4% of the total samples). Twenty-two samples (6.3% of the total samples; 2.5% of the patients) were deemed inconclusive. In 23% of these inclusive cases, there was an RHD-negative, female result, but there were an insufficient number of paternal SNPs detected to confirm the presence of fetal DNA. In the
removing 77% of the inconclusive results (4.8% of the total samples), the \textit{RHD} psi (\textit{y})-pseudogene was detected, and the sample was deemed inconclusive. Erroneous results were observed for 6 of the samples (1.7%), and included discrepancies in 4 \textit{RHD} typings (1.1%) and 2 fetal sex determinations (0.6%) following data unblinding. Three cases of RhD typing were false positives (ccfDNA was \textit{RHD}-positive but neonatal serology RhD-negative) and one case was a false negative (ccfDNA: \textit{RHD}-negative but neonatal serology RhD-positive). Accuracy for determination of the RhD status of the fetus was 99.1%, 99.1%, and 98.1%, respectively for each of the 3 consecutive trimesters of pregnancy, and accuracy of fetal sex determination was 99.1%, 99.1%, and 100%, respectively.

In 2011, Bombard and colleagues analyzed the performance of the SensiGene Fetal RHD test in 2 cohorts. Cohort 1 used as a reference point the clinical RhD serotype obtained from cord blood at delivery. Samples from cohort 2 were originally genotyped at the Sequenom Center in Grand Rapids, Michigan and results were used for clinical validation of genotyping performed at the Sequenom Center in San Diego, California.

In cohort 1, \textit{RHD} genotyping was performed on 236 maternal plasma samples from singleton, nonsensitized pregnancies with documented fetal RhD serology. The samples were obtained at 11-13 weeks’ gestation. Ethnic origin of the pregnant women was Caucasian 77.1%, African 19.1%, mixed race 3.4% and South Asian (0.4%). Neonatal RhD phenotype, determined by serology at the time of birth, was positive in 69.1% of samples and negative in 30.9% of samples. In 2 (0.9%) of the 236 samples, there the results were classified as invalid. In the 234 (99.1%) samples with sufficient DNA extraction, the result was conclusive in 207 samples (88.5%); inconclusive in 16 samples (6.8%); and psi (+)/\textit{RHD} variant in 11 samples (4.7%). In the 207 samples with a conclusive result, the neonatal RhD phenotype was positive in 142 samples (68.6%) and negative in 65 samples (31.4%). The \textit{RHD} Genotyping test correctly predicted the neonatal RhD phenotype in 201 of 207 samples for an accuracy of 97.1% (95% confidence interval [CI], 93.5 to 98.8). In the 142 samples with RhD-positive fetuses, the test predicted that the fetus was positive in 138 and in 4 that it was negative, for a sensitivity of prediction of RhD positivity of 97.2% (95% CI, 93.0 to 98.9). In 63 of the 65 samples with RhD-negative fetuses, the \textit{RHD} Genotyping test predicted that the fetus was negative and, in the remaining 2, that it was positive, for a specificity for the prediction of RhD positivity of 96.9% (95% CI, 89.5 to 99.1). The test predicted that the fetus was RhD-positive in 140 samples, of which, in 138 of these the prediction was correct, for a positive predictive value of 98.6% (95% CI, 94.9 to 99.6). The test predicted that the fetus was RhD-negative in 67 samples, of which, in 63 of these the prediction was correct, for a negative predictive value for RhD-positive fetuses of 94.0% (95% CI, 85.6 to 97.6).

Cohort 2 consisted of 205 samples from 6-30 weeks’ gestation. Testing was for the presence of \textit{RHD} exon sequences 4, 5, 7, the psi-pseudogene, and 3 Y-chromosome sequences (SRY, DBY and TTTY2), using MALDI-TOF MS-(the RHD Genotyping laboratory developed test). The laboratory performing the assays for both cohorts was blinded to the sex and fetal RhD genotype. In cohort 2, the test correctly classified 198 of 199 patients, for a test accuracy of 99.5%, with a sensitivity and specificity for prediction of \textit{RHD} genotype of 100.0% and 98.3%, respectively.

**Clinical Utility**
The possible clinical utility of cffDNA \textit{RHD} genotyping includes the following scenarios:
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In the Rh-negative, nonalloimmunized pregnant patient:
- Avoidance of unnecessary anti-D immune globulin if the fetus is Rh-negative.
- Avoidance of invasive procedure to obtain fetal tissue when the paternity is unknown or the father is heterozygous for the D antigen.

In the Rh-negative, alloimmunized pregnant patient:
- Avoidance of invasive procedure to obtain fetal tissue if Rh-negative pregnant woman is alloimmunized to determine fetal Rh status.
- Avoidance of serial antibody testing in the mother and middle cerebral artery surveillance of the fetus if the fetus is determined to be Rh-negative.

No published data were identified showing that fetal \textit{RHD} genotyping leads to improved health outcomes. This type of testing could lead to the avoidance of the use of anti-D immune globulin (eg, RhoGAM) in Rh-negative mothers with Rh-negative fetuses. However, the false-negative rate of the test, which is low, is not zero, and a certain percentage of Rh-negative women will develop alloimmunization to Rh-positive fetuses. Other issues that still need to be defined include the optimal timing of testing during the pregnancy.

A search of ClinicalTrials.gov in November 2015 did not identify any ongoing or unpublished phase 3 trials that would likely influence this review.

Summary
The evidence for the use of fetal \textit{RHD} genotyping using cell-free DNA in women who are pregnant and have RhD-negative blood type includes: for clinical validity, a meta-analysis and additional prospective studies, and for analytic validity, no direct evidence. Relevant outcomes are test accuracy and validity, morbid events, medication use, and treatment-related morbidity. Clinical validity studies have demonstrated that the sensitivity and specificity of the test are high; however, the false-negative rate of the test, which is low, is not zero, potentially leading to alloimmunization of the Rh-negative mothers in these cases. It is uncertain whether \textit{RHD} genotyping using cell-free fetal DNA will lead to improved health outcomes. The evidence is insufficient to determine the effects of the technology on health outcomes.

References
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02/06/2014 Medical Policy Committee review
02/19/2014 Medical Policy Implementation Committee approval. New policy
02/05/2015 Medical Policy Committee review
02/18/2015 Medical Policy Implementation Committee approval. No change to coverage.
08/03/2015 Coding update: ICD10 Diagnosis code section added; ICD9 Procedure code section removed.
02/04/2016 Medical Policy Committee review
02/17/2016 Medical Policy Implementation Committee approval. No change to coverage.
01/01/2017 Coding update: Removing ICD-9 Diagnosis Codes
02/02/2017 Medical Policy Committee review
02/15/2017 Medical Policy Implementation Committee approval. Title changed. “Maternal plasma” replaced with “cell-free fetal DNA” in the policy statement.
10/01/2017 Coding update
Next Scheduled Review Date: 02/2018

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*Investigational – A medical treatment, procedure, drug, device, or biological product is Investigational if the effectiveness has not been clearly tested and it has not been incorporated into standard medical practice. Any determination we make that a medical treatment, procedure, drug, device, or biological product is Investigational will be based on a consideration of the following:

A. Whether the medical treatment, procedure, drug, device, or biological product can be lawfully marketed without approval of the U.S. FDA and whether such approval has been granted at the time the medical treatment, procedure, drug, device, or biological product is sought to be furnished; or

B. Whether the medical treatment, procedure, drug, device, or biological product requires further studies or clinical trials to determine its maximum tolerated dose, toxicity, safety, effectiveness, or effectiveness as compared with the standard means of treatment or diagnosis, must improve health outcomes, according to the consensus of opinion among experts as shown by reliable evidence, including:
   1. Consultation with the Blue Cross and Blue Shield Association technology assessment program (TEC) or other nonaffiliated technology evaluation center(s);
   2. Credible scientific evidence published in peer-reviewed medical literature generally recognized by the relevant medical community; or
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