ANALYTIC VALIDITY

Question 8: Is the test qualitative or quantitative?
Question 9: How often is a test positive when a mutation is present (analytic sensitivity)?
Question 10: How often is the test negative when a mutation is not present (analytic specificity)?
Question 11: Is an internal quality control program defined and externally monitored?
Question 12: Have repeated measurements been made on specimens?
Question 13. What is the within- and between-laboratory precision?
Question 14: If appropriate, how is confirmatory testing performed?
Question 15: What range of patient specimens have been tested?
Question 16: How often does the test fail to give a useable result?
Question 17: How similar are results obtained in multiple laboratories using the same, or different, technology?

ANALYTIC VALIDITY

Question 9: Is the test qualitative or quantitative?

The DNA test associated with HHC is qualitative (i.e., a mutation is reported as present or absent). Several mutations have been described, but when DNA analysis is proposed as a screening test for morbidity and mortality associated with iron overload in the setting of the general adult population, the only mutation of interest is C282Y. The genotype of interest is homozygosity for the C282Y mutation.

ANALYTIC VALIDITY

Question 9: How often is the test positive when a mutation is present (analytic sensitivity)?
Question 10: How often is the test negative when a mutation is not present (analytic specificity)?

Summary

External proficiency testing schemes are the only major reliable source currently available for computing analytic sensitivity and specificity for *HFE* testing. The following caveats should be kept in mind, however, when examining these estimates. First, external proficiency testing schemes are designed to be educational. For that reason, the types of challenges may not represent routine testing. Also, laboratories from outside the U.S. are included, and both research and clinical laboratories participate. In spite of these shortcomings, this source of data can be useful in establishing a baseline for laboratory performance.

Based on data from the American College of Medical Genetics and the College of American Pathologists (ACMG/CAP) Molecular Genetics Survey Set MGL from 1998 through 2002:

- The overall error rate for C282Y genotyping (analyzed by chromosome) is 1.0% (95 percent CI 0.6 to 1.5%).
- Analytic sensitivity for C282Y homozygotes is 98.4% (95 percent CI 95.9 to 99.5%).
- Analytic specificity for other non-homozygous genotypes is 99.8% (95 percent CI 99.4 to 99.9%).
- It is not possible to determine whether errors in the survey occurred in the pre-analytic, analytic or post-analytic phase of testing.
- The analytic performance (sensitivity and specificity) for the C282Y mutation is expected be consistent, regardless of the race/ethnicity of the population being tested. It is possible, however, that rare, unknown polymorphisms (that could cause false positive results) might vary by race/ethnicity
- Although the H63D mutation is not considered part of the screening test, the analytic sensitivity and specificity are similar to those described for the C282Y mutation, serving as further documentation of laboratory performance.

Definitions

Analytic performance is summarized by the sensitivity and specificity of the detection system. Generically, analytic sensitivity is defined the proportion of positive test results, when a detectable mutation is present (i.e., the test is designed to detect that mutation). Analytic sensitivity is equivalent to the analytic detection rate. Given that this report is focusing on DNA testing for morbidity and mortality due to iron overload in the setting of general adult population screening, only the C282Y mutation is of interest (Question 4, Question 18). More specifically, the main interest in such a screening program would be to identify C282Y homozygotes. Thus, analytic sensitivity will be defined in this document as the proportion of C282Y homozygotes correctly identified.

Generically, analytic specificity is the proportion of negative test results when no detectable mutation is present. Analytic specificity can also be expressed in terms of the analytic false positive rate. The false positive rate is the proportion of positive test results when no detectable mutations are present (1-analytic specificity). In keeping with the specific definition of analytic sensitivity above, analytic specificity is defined in this document as the proportion of individuals that are not C282Y homozygotes who are correctly identified as not being homozygous for C282Y.

Optimal source(s) of data

Few data sources exist for estimating analytic validity. Published reports of method comparisons and screening experiences provide limited information on only a few testing methodologies. The data are derived from a small number of laboratories, and the "true" genotypes of the tested samples are often undocumented (i.e., not confirmed by another methodology, laboratory consensus or sequencing). External proficiency testing programs such as the ACMG/CAP Molecular Genetics Laboratory (MGL) Surveys provide a source of data that has several advantages. The ACMG/CAP survey serves a large proportion of clinical testing laboratories in the U.S. Data provided by these laboratories represent the range of methodologies presently being used. In addition, the samples distributed for proficiency testing exercises have consensus genotypes. However, basing analytic performance estimates on external proficiency testing also has drawbacks, including:

- mixing of clinical and research laboratories and methodologies
- relatively few challenges
- reporting summary results in ways that do not allow a straightforward computation of analytic sensitivity and specificity
- challenges that do not represent the 'mix' of genotypes expected in a screening program (e.g., too few wild challenges and too many homozygotes).

Future analyses should be aimed at providing reliable, method-specific analytic performance estimates. One approach for collecting such data might include the following steps:

- An independent body (such as the College of American Pathologists, American College of Medical Genetics, Food and Drug Administration or the Coriell Institute of Medical Research, Camden, NJ) would develop a standard set of samples, most of which would be randomly selected from the general population. Included in the standard set, however, would be additional, less common genotypes.
- The sample set would then be available for method validation. Correct genotypes would be arrived at by consensus, or, if disagreements emerged, by a reference method (e.g., sequencing). The current validation practice of having a laboratory (or manufacturer) run a series of samples with unknown genotype is inadequate, since there is no 'gold standard' with which to compare. For example, how would a laboratory running an unknown sample determine whether a positive finding is a true, or a false, positive?
- Ideally, this blinded sample set would be available to manufacturers as part of the pre-market approval process, with the understanding that multiple laboratories using these commercial reagents would be asked by the manufacturer to analyze portions of the sample set independently. This initial assay validation process is distinct from assay control samples that are discussed later (Question 13).

Appropriate sample size for determining analytic sensitivity and specificity has been discussed in detail in an earlier ACCE review (Prenatal Cystic Fibrosis Screening via Carrier Testing – Question 11 and 12). In brief, a target sensitivity (or specificity) can be chosen, along with an acceptable lower limit (assumed to be the lower limit of the 95% confidence interval). Given these targets, the number of necessary samples can be derived. For example, if a laboratory chose a target specificity of 98% and wanted to rule out a specificity of 90%, it would need to correctly identify at least 49 of 50 known negative samples (estimated using the binomial distribution). When the estimates approach 100% and relatively tight confidence intervals are sought, it may not be economically feasible for laboratories to individually collect and analyze their data. However, this level of confidence could be attained by a consortium of laboratories using the same methodology, or by a manufacturer that forms a consortium of laboratories using its reagents. All of these suggested analyses could be done using a 2x2 table, and all rates could be accompanied by 95% confidence intervals (CI).

The ACMG/CAP external proficiency testing scheme

As part of ACMG/CAP external proficiency testing in the United States, purified DNA from established cell lines (derived from human cells with known mutations http://locus.umdnj.edu) is distributed to participating laboratories. Many of these laboratories are likely to be providing clinical services, but reagent manufacturers and research laboratories also participate. In late 2001, there were 90 participants reporting *HFE* results. In general, there are three types of errors. A false positive result occurs when the laboratory reports finding a mutation in the sample, when none is present. A false negative result occurs when a laboratory reports no mutation, but a mutation for which it tests is, in fact, present in the sample. A third type of error occurs when the laboratory accurately identifies that a mutation is present, but it is not the correct mutation. Given the nature of this scheme, it has not been possible to determine the phase of testing in which the error has occurred (e.g., pre-analytic, analytic or post-analytic). When considering the performance of identifying the C282Y mutation alone in the setting of general adult population screening, these errors need to be carefully redefined.

- A false negative result occurs when an individual who is homozygous for C282Y has a test result that is not homozygous for C282Y (i.e., wild/wild or C282Y/wild)
- A false positive result occurs when an individual who is not homozygous for C282Y has a test result falsely indicating homozygosity for this mutation.
- The third type of error, wrong mutation, is not considered in this analysis, since it is assumed that the test will only be directed at one mutation: C282Y.

A separate analysis of analytic sensitivity and specificity for both the C282Y and H63D mutations performed by chromosome can be found in Appendix 1 at the end of this section. A listing of other mutations in the HFE gene can be found in Appendix 2.

Error rates for the ACMG/CAP external proficiency testing scheme Table 2-1 shows how each of the two types of error are counted in the analyses of analytic performance. Column 1 shows those individuals who are actually homozygous for C282Y. The first entry in that column contains those receiving positive test results (i.e., true positives with a result of homozygous for C282Y). Any other result in this column (rows 2 and 3) is considered a false negative. Among true heterozygotes (Column 2), the finding of homozygosity for C282Y would be a false positive (first row). Any other test result would be considered negative. Column 3 shows the three

possible test results in individuals with no C282Y mutations. If the aim of testing is to correctly identify individuals who are homozygous for the C282Y mutation, the table could be collapsed according to the darkened lines.

Table 2-1. All Possible Combinations of Test Results with the Three Genotypes of Interest Assuming Testing is Limited to the C282Y Mutation

	Actual Genotype			
Test Result	C282Y/C282Y	C282Y/wild	wild/wild	**Total**
C282Y/C282Y	True Positive	False Positive	False Positive	Positive Tests
C282Y/wild	False Negative	Negative	Negative	Negative Tests
wild/wild	False Negative	Negative	True Negative	Negative Tests
Total	Homozygotes	Heterozygotes	No C282Y	All

Table 2-2 shows the results of the ACMG/CAP MGL survey for *HFE* mutations in the format described in Table 2-1. That survey did include several challenges of the H63D mutation. For this analysis, the H63D mutations and corresponding results are ignored, but the sample is still included. For example, a compound heterozygote challenge of C282Y/H63D is viewed as a C282Y heterozygote challenge. Overall, 20 of the 2,043 sample challenges were incorrectly genotyped for C282Y, for an overall error rate of 1.0% (95 percent CI 0.6 to 1.5%). As indicated earlier, the major goal of DNA screening for hemochromatosis is to correctly identify C282Y homozygotes. The "collapsed" table shows that 98.4% of the homozygous genotypes were correctly identified (243/247, 95 percent CI 95.9 to 99.5%). In addition all but four of the 1,796 negative (non-homozygous samples) were identified as non-homozygotes (99.78%). The error rate did not change appreciably over time, as shown in the summary of challenges and errors displayed in Figure 2-1.

Table 2-2. *HFE* Mutation Testing: Results of the ACMG/CAP Molecular Genetics Survey When the Analysis is Restricted to the C282Y Mutation

	Actual Genotype			
Test Result	**C282Y/C282Y**	**C282Y/wild**	**wild/wild**	**Total**
C282Y/C282Y	243	1	3	247
C282Y/wild	2	585	5	592
wild/wild	2	7	1,195	1,204
	247	593	1,203	2,043

Figure 2-1. Summary of Errors Reported in the ACMG/CAP MGL Survey, with Interpretation restricted to the C282Y mutation

- In 1998, three samples were distributed to 67 laboratories (201 laboratory challenges)
  No errors
  Error rate 0.0% (95 percent CI 0.0 to 1.8%)
- In 1999, two samples were distributed to 78 laboratories (156 laboratory challenges)
  One laboratory identified no C282Y mutations in a C282Y heterozygous sample
  One laboratory identified a C282Y mutation in a sample with no mutation present
  Error rate 1.3% (95 percent CI 0.2 to 4.6%)
- In 2000-A, three samples were distributed to 81 laboratories (243 laboratory challenges)
  One laboratory identified a heterozygote as being homozygous for C282Y
  Error rate 0.4% (95 percent CI 0.1 to 2.3%)
- In 2000-B, three samples were distributed to 90 laboratories (270 laboratory challenges)
  One identified a heterozygote when no C282Y mutations were present
  Two laboratories incorrectly identified a homozygote as having no mutations
  Error rate 1.1% (95 percent CI 0.2 to 3.2%)
- In 2001-A, three samples were distributed to 100 laboratories (300 laboratory challenges)
  One identified a heterozygote when no C282Y mutations were present
  One identified a homozygote for C282Y when no C282Y mutations were present
  One laboratory identified no C282Y mutations in a C282Y heterozygous sample
  Error rate 1.0% (95 percent CI 0.2 to 2.9%)
- In 2001-B, three samples were distributed to 90 laboratories (270 laboratory challenges)
  Two laboratories incorrectly identified a homozygote as being heterozygous
  Error rate 0.7% (95 percent CI 0.1to 2.7%)
- In 2002-A, three samples were distributed to 103 laboratories (309 laboratory challenges)
  In two instances, a homozygote was reported when no C282Y mutations were present
  One identified a heterozygote when no C282Y mutations were present
  Two laboratories reported no C282Y mutations in a heterozygote
  One laboratory reported homozygosity for an individual heterozygous for C282Y
  Error rate 1.9% (95 percent CI 0.7 To 4.2%)
- In 2002-B, three samples were distributed to 98 laboratories (294 laboratory challenges)
  One laboratory identified a heterozygote when non C282Y mutations were present
  Three laboratories reported no C282Y mutations in a heterozygote
  Error rate 1.4% (95 percent CI 0.4 to 3.5%)

Analytic sensitivity identifying C282Y homozygotes Only eight of the 20 errors identified in the proficiency testing samples influence the analytic sensitivity of identifying C282Y homozygotes (first column in Table 2-2). Overall, the analytic sensitivity is 243/247, or 98.4% (95 percent CI 95.9 to 99.5%). These confidence intervals could be considered pessimistic and optimistic extremes of the analytic sensitivity. Because of the relatively few challenges (and observed false negatives), it is not possible to determine whether analytic sensitivity varied over the four years.

Analytic specificity for identifying non-C282Y homozygotes The analytic specificity (computed using the second and third columns in Table 2-2) is 1,191/1,193 or 99.8% (95 percent CI 99.4 to 99.9%).

Genotyping Errors and Method of Testing According to the ACMG/CAP Participant Summary Reports, there was no correlation between genotyping error and the laboratory method. Errors were made by laboratories using restriction digestion and ASO analysis. The majority of laboratories (77% on the 2000-B survey) used PCR and restriction digestion. Other methods of analysis included ASO (9%), ARMS (8%), Light-cycler (3%), DNA sequencing (2%), and other (1%). In one survey (MGL 2001-A), seven errors were made that involved either C282Y or H63D. The Participant Summary Report notes that seven different laboratories made these errors, and that six of those laboratories provided clinical test results (only one was a research laboratory).

Recognition of a potential source of method-specific error As part of the ACMG/CAP Survey Program, concern was raised regarding the protocol validation of new laboratories, inexperienced in *HFE* testing. Laboratories using restriction analysis were encouraged to ensure that their assays contain internal controls to validate enzyme restriction. One other potential source for error is the use of the Feder primers for C282Y analysis, due to the G5569A polymorphism in the reverse primer. Laboratories were cautioned that they should use alternate primers that do not include this polymorphism and that decreased annealing temperatures of 50-55°C would decrease the stringency of the PCR reaction and thus control for non-amplification due to primer site polymorphisms. In the MGL 2000-B ACMG/CAP Participant Summary Report, participants reported that only 38/84 laboratories (45%) used the C282Y Feder primers, while 58/82 laboratories (70%) still used the H63D Feder primers. A more in-depth discussion of this topic follows in the next two paragraphs.

In 1999, Jeffrey *et al.* reported that a previously described polymorphism, 5569A (Totaro *et al.*, 1997), was associated with misdiagnosis of 15 C282Y/5569A heterozygotes as C282Y homozygotes. Because this single base pair polymorphism is located in the primer binding site for the C282Y wild type allele in exon 4, Jeffrey *et al.* theorized that the Feder reverse primer might fail to anneal and thus prevent amplification of the wild type allele. Since only the mutant allele would then be amplified, this could result in the appearance of a C282Y homozygote, and a false positive result. Subsequently, two other laboratories reported misclassification of C282Y heterozygotes as homozygotes (Gomez *et al.*, 1999; Somerville *et al.*, 1999). Because this polymorphism is relatively common (allele frequencies as high as 13%), this report raised immediate concern about C282Y results in genotyping studies worldwide and led some laboratories to re-analyze previous results.

The ACMG/CAP Molecular Genetics Survey quickly responded that 67 U.S. laboratories (many using the Feder primers) had correctly genotyped a C282Y heterozygote sample that also carried the 5569A polymorphism (Noll *et al.*, 1999). Thorstensen *et al.* (2000) also reported no errors in genotyping in 433 patients tested using the Feder primers. These authors suggested that the difference in performance might relate to a change in a PCR reaction condition (i.e., primer annealing temperature), and that most laboratories had used conditions that did not affect result accuracy. The European Haemochromatosis Consortium reported that some laboratories had replaced the Feder reverse primer to remove the possibility of misclassification, but that previous

publications by member laboratories had not been compromised. Therefore, it appears that prevalence estimates of the C282Y mutation are unlikely to have been overestimated. However, clinical laboratories should avoid primers containing polymorphic sites in which primer binding could affect test outcome.

Other polymorphisms The DNA testing utilized for screening is aimed at identifying a specific mutation (C282Y) that, when found in the homozygous state, can be the cause of primary iron overload. The test is designed to identify this mutation in any DNA sample, regardless of the characteristics of the individual being tested (e.g., race or ethnicity). Although the prevalence of iron overload and the mix of mutations responsible for the disorder may vary by race, the test should reliably identify the target mutation. One exception to this might occur if the presence and/or frequency of unknown polymorphisms were found to vary by race/ethnicity (or some other factor). In reality, however, it would be difficult for laboratories to thoroughly examine this possibility in all populations to which testing may be offered.

Gap in Knowledge: Allele frequency by race/ethnicity. Variation in allele and polymorphism frequencies by race/ethnicity have been well described in the literature for some population groups, while others have much less information available. Laboratories should make efforts to report *HFE* allele frequencies as well as polymorphisms that could interfere with the analysis.

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Appendix 1. Data used to calculate analytic sensitivity and specificity

Analytic sensitivity and specificity by genotype

Tables 2-3 through 2-7 summarize the *HFE*-related external proficiency testing results obtained by ACMG/CAP for the years 1998 through 2002. Samples with known genotypes have been distributed to participants since 1998. For orientation, the first column of Table 2-4 contains the distribution label (98 MGL-16 indicates the 16th DNA sample distributed as part of the Molecular Genetics Laboratory survey in 1998). The second column contains the number of participating laboratories, and the third column lists the consensus genotype of the sample. The number of laboratories reporting specific genotypes is then provided, along with a tabulation of their 'correct' and 'incorrect' responses. The last two columns provide an adjusted interpretation by taking into account that some laboratories do not test for the H63D mutation. The last column also shows the type of error and is listed by chromosome. Some analyses use errors listed by genotype instead. For example, identifying a C282Y homozygote as having no identifiable mutations is considered two errors when counting by chromosome, but only one error when counting by genotype. The main analysis, which ignores H63D mutations and is performed by genotype, is shown at the end of each year's results and is labeled '282 performance measures'. Table 2-9 summarizes results from all five years.

Table 2-3. Computations for the 1998 ACMG/CAP Proficiency Testing Surveys

			Reported Alleles		Adjusted Report	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**	**Correct**	**Incorrect**
98 MGL-16	**67**	**C282Y/C282Y**				
	58	C282Y/C282Y	116	0	116	0
	9*	C282Y/C282Y	18	0	18	0
98 MGL-17	**67**	**C282Y/H63D**				
	58	C282Y/H63D	116	0	116	0
	9*	C282Y/N	9	9	18	0
98 MGL-18	**67**	**C282Y/N**				
	58	C282Y/N	116	0	116	0
	9*	C282Y/N	18	0	18	0
Totals 1998		**402 alleles**	**393**	**9**	**402**	**0**

282 Performance Measures For Homozygosity	**Genotype challenges**	**Errors**	**Rate (%)**
Sensitivity	67	0	100
Specificity	67+67=134	0	100

* These laboratories do not test for the H63D mutation

Table 2-4. Computations for the 1999 ACMG/CAP Proficiency Testing Survey

			Reported Alleles		Adjusted Report	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**	**Correct**	**Incorrect**
99 MGL-17	**78**	**C282Y/H63D**				
	70	C282Y/H63D	140	0	140	0
	7*	C282Y/N	7	7	14	0
	1	H63D/N	1	1	1	1 (fn 282)
99 MGL-18	**78**	**N/N**				
	69	N/N	138	0	138	0
	7*	N/N	14	0	14	0
	1	H63D/N	1	1	1	1 (fp 63)
	1	C282Y/N	1	1	1	1 (fp 282)
Totals 1999		**312 alleles**	**302**	**10**	**309**	**3**

282 Performance Measures For Homozygosity	**Genotype challenges**	**Errors**	**Rate (%)**
Sensitivity	0	0	-
Specificity	78+78=156	0	100

* These laboratories do not test for the H63D mutation

Table 2-5. Computations for the 2000 ACMG/CAP Proficiency Testing Survey

			Reported Alleles		Adjusted Report	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**	**Correct**	**Incorrect**
00 MGL-04	**81**	**N/N**				
	72	N/N	144	0	144	0
	9*	N/N	18	0	18	0
00 MGL-05	**81**	**N/N**				
	72	N/N	144	0	144	0
	9*	N/N	18	0	18	0
00 MGL-06	**81**	**282/N**				
	72	282/N	144	0	144	0
	8*	282/N	16	0	16	0
	1*	282/282	1	1	1	1 (fp 282)
00 MGL-16	**90**	**63/N**				
	79	63/N	158	0	158	0
	8*	N/N	8	8	16	0
	2	N/N	2	2	2	2 (fn 63)
	1	63/282	1	1	1	1 (fp 282)
00 MGL-17	**90**	**N/N**				
	82	N/N	164	0	164	0
	8*	N/N	16	0	16	0
00 MGL-18	**90**	**282/282**				
	80	282/282	160	0	160	0
	8*	282/282	16	0	16	0
	2	N/N	0	4	0	4 (fn 282)
Totals 2000		**1,026 alleles**	**1,010**	**16**	**1,008**	**8**

282 Performance Measures For Homozygosity	**Genotype challenges**	**Errors**	**Rate (%)**
Sensitivity	90	2	97.8
Specificity	81+81+81+90+90=423	1	99.8

* These laboratories do not test for the H63D mutation

Table 2-6. Computations for the 2001 ACMG/CAP Proficiency Testing Survey

			Reported Alleles		Adjusted Report	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**	**Correct**	**Incorrect**
01 MGL-04	**100**	**N/N**				
	90	N/N	180	0	180	0
	8*	N/N	16	0	16	0
	1	282/63	0	2	0	2 (fp 282, fp 63)
	1	63/N	1	1	1	1 (fp 63)
01 MGL-05	**100**	**63/63**				
	88	63/63	176	0	176	0
	8*	N/N	0	16	16	0
	2	N/N	0	4	0	4 (fn 63)
	1	63/N	1	1	1	1 (fn 63)
	1	282/282	0	2	0	2 (fp 282)
01 MGL-06	**100**	**282/63**				
	91	282/63	182	0	182	0
	8*	282/N	8	8	16	0
	1	63/N	1	1	1	1 (fn 282)
01 MGL-16	**90**	**N/N**				
	84	N/N	168	0	168	0
	5*	N/N	10	0	10	0
	1	63/N	1	1	1	1 (fp 63)
01 MGL-17	**90**	**N/N**				
	85	N/N	170	0	170	0
	5*	N/N	10	0	10	0
01 MGL-18	**90**	**282/282**				
	83	282/282	166	0	166	0
	5*	282/282	10	0	10	0
	2	282/N	2	2	2	2 (fn 282)
Totals 01		**1140 alleles**	**1,102**	**38**	**1,126**	**14**

282 Performance Measures For Homozygosity	**Genotype challenges**	**Errors**	**Rate (%)**
Sensitivity	90	2	97.8
Specificity	100+100+100+90+90=480	1	99.8

* These laboratories do not test for the H63D mutation

Table 2-7. Computations for the 2002 ACMG/CAP Proficiency Testing Survey

			Reported Alleles		Adjusted Report	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**	**Correct**	**Incorrect**
02 MGL-04	**104**[1]	**N/N**				
	94	N/N	188	0	188	0
	7*	N/N	14	0	14	0
	1	282/N	1	1	1	1 (fp 282)
	1	282/282	0	2	0	2 (2fp 282)
	1	63/N	1	1	1	1 (fp 63)
02 MGL-05	**103**[1]	**N/N**				
	94	N/N	188	0	188	0
	7*	N/N	14	0	14	0
	1	63/N	1	1	1	1 (fp 63)
	1	282/282	0	2	0	2 (fp 282)
02 MGL-06	**103**[1]	**282/N**				
	93	282/N	186	0	186	0
	6*	282/N	12	0	12	0
	1*	N/N	1	1	1	1 (fn 282)
	1	N/N	1	1	2[2]	0
	1	282/282	1	1	2[2]	0
	1	63/N	1	1	1	1 (wm)
02 MGL-10	**98**	**63/N**				
	89	63/N	178	0	178	0
	7*	N/N	7	7	14	0
	1	N/N	1	1	1	1 (fn 63)
	1	282/63	1	1	1	1 (fp 282)
02 MGL-11	**98**	**N/N**				
	91	N/N	182	0	182	0
	6*	N/N	12	0	12	0
	1	63/N	1	1	1	1 (fp 63)
02 MGL-12	**97**	**282/63**				
	86	282/63	172	0	172	0
	6*	282/N	6	6	12	0
	2	282/N	2	2	2	2 (fn 63)
	2	63/N	2	2	2	2 (fn 282)
	1	N/N	0	2	0	2 (fn 282 fn 63)
Totals 02		**1,206 alleles**	**1,173**	**33**	**1,188**	**18**

282 Performance Measures For Homozygosity	**Genotype challenges**	**Errors**	**Rate (%)**
Sensitivity	0	0	
Specificity	104+103+103+98+98+97=603	2	99.7

* These laboratories do not test for the H63D mutation

[1] Assumes the laboratory reporting incorrectly was actually testing for both C282Y and H63D

[2] Does not count a reporting error (each allele reported as a genotype)

Table 2-8. Computations for the ACMG/CAP Proficiency Testing Surveys for the Combined Years 1998-2002

			Reported Alleles		Adjusted Report	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**	**Correct**	**Incorrect**
Totals 98-02		**4,086 alleles**	**3,980**	**106**	**4,043**	**43**

282 Performance Measures For Homozygosity	**Genotype challenges**	**Errors**	**Rate (%)**
Sensitivity	67+0+90+90+0=247	4	98.4
Specificity	134+156+423+480+603=1,796	4	99.8

Analytic sensitivity and specificity for the C282Y mutation by chromosome

Table 2-9 shows the raw data used in the analysis of analytic sensitivity and specificity for the C282Y mutation, using data from the ACMG/CAP Survey. This analysis differs from that shown in Tables 2-3 through 2-8, in that each chromosome is analyzed separately. For example, there is no explicit analysis of the rate at which laboratories incorrectly identify homozygous C282Y individuals. Rather, the analytic sensitivity is a measure of how often a chromosome with a C282Y mutation is correctly identified as being positive. Analytic specificity is a measure of how often a chromosome without a C282Y mutation is correctly identified as being negative. Overall, analytic sensitivity by chromosome is 97.8% (95 percent CI 96.3 to 98.8%) and analytic specificity is 99.6% (95 percent CI 99.3 to 99.8%). The analytic performance when estimated by chromosome is less good than the analysis by genotype, because all errors are counted.

Table 2-9. Computation of Analytic Sensitivity and Specificity by Chromosome for the C282Y Mutation Using Data from the ACMG/CAP Molecular Genetics Laboratory External Proficiency Testing Surveys

ACMG/CAP	Sensitivity		Specificity	
MGL Sample	Chromosome 1	Chromosome 2	Chromosome 1	Chromosome 2
98 MGL-16	67/67	67/67	-	-
98 MGL-17	67/67	-	67/67	-
98 MGL-18	67/67	-	67/67	-
99 MGL-17	77/78 **(1 fn)**	-	78/78	-
99 MGL-18	-	-	77/78 (**1fp)**	78/78
00 MGL-04	-	-	81/81	81/81
00 MGL-05	-	-	81/81	81/81
00 MGL-06	81/81	-	80/81 **(1 fp)**	-
00 MGL-16		-	89/89 **(1fp)**	90/90
00 MGL-17		-	90/90	90/90
00 MGL-18	88/90 **(2 fn)**	88/90 **(2 fn)**		
01 MGL-04	-	-	100/100	99/100 **(1 fp)**
01 MGL-05			99/10 **(1 fp)**	99/100 **(1 fp)**
01 MGL-06	99/100 **(1 fn)**	-	100/100	-
01 MGL-16	-	-	90/90	90/90
01 MGL-17	-	-	90/90	90/90
01 MGL-18	90/90	88/90 **(2 fn)**		
02 MGL-04			102/103 (**1 fp)**	101/103 (**2 fp)**
02 MGL-05			102/103 (**1 fp)**	102/103 **(1 fp)**
02 MGL-06	101/103 (**2 fn)**		102/103 (**1 fp)**	
02 MGL-10			97/98 (**1 fp)**	98/98
02 MGL-11			98/98	98/98
02 MGL-12	95/98 (**3 fn)**		98/98	
All	**583/596 (13 fn)**		**3123/3136 (13 fp)**	
	97.8% (95% CI 96.3 – 98.8%)		**99.6% (95% CI 99.3 – 99.8%)**	

Analytic sensitivity and specificity for the H63D mutation by chromosome

Table 2-10 shows the raw data used in the analysis of analytic sensitivity and specificity for the H63D mutation using data from the Molecular Genetics Laboratory Survey sponsored by ACMG/CAP. In this analysis, each chromosome is analyzed separately. The analytic sensitivity is a measure of how often a chromosome with an H63D mutation is correctly identified as being positive. Analytic specificity is a measure of how often a chromosome without an H63D mutation is correctly identified as being negative. Overall, analytic sensitivity is 98.4% (95 percent CI 97.1 to 99.2%) and analytic specificity is 99.7% (95 percent CI 99.5 to 99.9%).

Table 2-10. Computation of Analytic Sensitivity and Specificity by Chromosome for the H63D Mutation Using Data from the ACMG/CAP Molecular Genetics Laboratory External Proficiency Testing Surveys for the Years 1998-2002

ACMG/CAP	Sensitivity		Specificity	
MGL Sample	Chromosome 1	Chromosome 2	Chromosome 1	Chromosome 2
98 MGL-16	-	-	58/58	58/58
98 MGL-17	58/58	-	58/58	-
98 MGL-18	-	-	58/58	58/58
99 MGL-17	71/71	-	71/71	-
99 MGL-18			71/71	70/71 **(1 fp)**
00 MGL-04	-	-	72/72	72/72
00 MGL-05	-	-	72/72	72/72
00 MGL-06	-	-	72/72	72/72
00 MGL-16	80/82 **(2 fn)**	-	82/82	-
00 MGL-17	-	-	82/82	82/82
00 MGL-18	-	-	82/82	82/82
01 MGL-04			92/92	90/92 **(2 fp)**
01 MGL-05	90/92 **(2 fn)**	89/92 **(3 fn)**	-	-
01 MGL-06	92/92	-	92/92	-
01 MGL-16	-	-	85/85	84/85 (**1fp**)
01 MGL-17	-	-	85/85	85/85
01 MGL-18	-	-	85/85	85/85
02 MGL-04			96/97 (**1 fp)**	97/97
02 MGL-05			96/97 (**1 fp)**	97/97
02 MGL-06			96/97 (**1 fp)**	97/97
02 MGL-10	90/91 (**1 fn)**		91/91	
02 MGL-11			90/91 (**1 fp)**	91/91
02 MGL-12	88/91 (**3 fn)**		91/91	
All	**658/669 (11 fn)**		**3069/3077 (8 fp)**	
	98.4% (95% CI 97.1 – 99.2%)		**99.7% (95% CI 99.5 – 99.9%)**	

Appendix 2.

Mutations in the *HFE* gene The two most common mutations are C282Y and H63D, and the majority of clinical laboratories test for both of these mutations, since at least some samples are submitted primarily for diagnostic testing. Recently, the S65C variant has been described and tentatively linked to a mild form of iron overload, although this variant is not associated with increased transferrin saturation in voluntary blood donors (Arya *et al.*, 1999) and has not been included in the ACMG/CAP proficiency testing program. Other mutations have been identified in the *HFE* gene (Table 2-11), but are less frequent and/or of low penetrance. Based on the extensive published literature of more than 12,000 individuals genotyped (including more than 2,000 C282Y homozygotes), it is clear that the two common mutations, C282Y and H63D, are very rarely observed in the 'cis' phase (both mutations on the same chromosome). Two patients, apparently unrelated and of different ethnic backgrounds, have been described who are homozygous for the C282Y mutation and heterozygous for the H63D mutation (Thorstensen *et al.*, 2000; Best *et al.*, 2001). This finding of C282Y and H63D in the 'cis' phase is likely to represent two independent recombination events and probably occurs at a frequency much less than 1 in 1,000 (Best *et al.*, 2001).

Table 2-11. *HFE* Gene Mutations Reported in the Literature

***HFE* Mutation**	**Location (Exon)**	**Allele Frequency (%)**	**Reference**
Missense Mutations			
C282Y (845G→A)	4	~ 7	Feder *et al.*, 1996
H63D (187C→G)	2	~ 20	Feder *et al.*, 1996
S65C (193A→T)	2	~ 2	Henz *et al.*, 1997
I105T (314T→C)	2	V Low/Private	Barton *et al.*, 1999
G93R (277G→C)	2	V Low/Private	Barton *et al.*, 1999
Q127H (381A→C)	3	V Low/Private	De Villiers *et al.*, 1999
V53M (157A→G)	2	V Low/Private	De Villiers *et al.*, 1999
V59M (175G→A)	2	V Low/Private	De Villiers *et al.*, 1999
E168Q (502G→C)	3	Private	Oberkanins *et al.*, 2000
V212V (636G→C)	4	???	Bradbury *et al.*, 1999
V272L (814G→T)	4	Private	Worwood *et al.*, 1999
E277K (829G→A)	4	Private	Bradbury *et al.*, 1999
R330M (989G→T)	5	V Low/Private	De Villiers *et al.*, 1999
Nonsense Mutations			
E168X (502G→T)	3	Rare	Piperno *et al.*, 2000
W169X (506G→A)	3	Rare	Piperno *et al.*, 2000
E74X (211C→T)	2	Private	Beutler *et al.*, 2002
Frameshift Mutations			
V68delT (203delT)	2	Private	Liechti-Gallati *et al.*, 1999
P160delC (478delC)	3	Private	Pointon *et al.*, 2000
Splice Site Mutation			
IVS3+1G→T	Intron 3	Private	Wallace *et al.*, 1999

ANALYTIC VALIDITY

Question 11: Is an internal quality control program defined and externally monitored?

Summary

- Internal quality control procedures are well described in several published sources
- External monitoring of these procedures is provided through inspections conducted by accrediting organizations such as CLIA, CAP or New York State

Definition

Internal quality control is a set of laboratory procedures designed to ensure that the test method is working properly. An internal quality control program includes documentation that high standards are being practiced to ensure that:

- reagents used in all aspects of genetic testing are of high quality to allow successful test completion,
- all equipment is properly calibrated and maintained,
- good laboratory practices are being applied at every level of the genetic testing process

Quality control procedures

Techniques that are used for analyzing DNA in screening for HHC are the same as those used for other molecular testing. These techniques are widely applied and well understood. As a result, it has been possible to design and publish generic internal quality control procedures, which many molecular laboratories already have in place. Table 2-12 lists published guidelines that, among other topics, describe reagent quality control, equipment calibration and maintenance, education of the technical staff, and other internal quality control procedures. The purpose of the quality control procedures is to rigorously control all steps of the DNA testing process to minimize the potential for test failure. Given that the internal procedures for establishing and maintaining good laboratory practice are readily available (Neumaier *et al*., 1998), the important next step will be to encourage, assist, and require laboratories to apply and document appropriate quality control procedures.

Table 2-12. Guidelines, Recommendations, and Checklists that Address Internal Quality Control Issues and Requirements.

Guidelines, Recommendations and Checklists	Source / Reference
Clinical Laboratory Improvement Amendments of 1988	Federal Register 1992;57:7002-3
Genetic Testing Under CLIA	Federal Register 2000;65: 25928-24934
New York State Laboratory Standards (9/00)	www.wadsworth.org/labcert/download.htm
Molecular Diagnostic Methods for Genetic Diseases: Approved Guidelines	National Committee for Clinical Laboratory Standards MM1-A Vol 20 #7
College of American Pathologists Checklist	www.cap.org
Standards and Guidelines for Clinical Genetics Testing	American College of Medical Genetics www.faseb.org/genetics/acmg/stds
Technical Standards and Guidelines for Hereditary Hemochromatosis	Supplement to the ACMG Standards and Guidelines for Clinical Genetics Laboratories (in preparation by QA Committee)

External monitoring

All clinical laboratories performing genetic testing must comply with general regulations under the Clinical Laboratory Improvement Amendments (CLIA), and a CLIA certification should be considered the minimum acceptable level of external monitoring. One shortcoming of having only a CLIA certification is that CLIA inspectors often have less experience in evaluating genetic testing laboratories than other certifying organizations. CLIA is in the process of upgrading its regulations regarding genetic testing. The Task Force on Genetic Testing concluded that the current CLIA requirements are insufficient to ensure quality of molecular genetic testing. Laboratories certified by the College of American Pathologists or by New York State Health Department will have undergone a more rigorous external monitoring that requires specific procedures and documentation.

Positive *HFE* assay controls

Positive controls for *HFE* mutations must be utilized to validate the assay and each lot of reagents. Positive controls are recommended to be routinely included in each assay run. *HFE* controls are readily available through the American Type Culture Collection (ATCC, Rockville, MD www.atcc.org) or the Coriell Institute for Medical Research (Camden, NJ http:://arginine.umdnj.edu) repositories.

References:

Holtzman NA, Watson MS. 1997. Promoting Safe and Effective Genetic Testing in the United States. Final report of the Task Force on Genetic Testing. http://www.nhgri.nih.gov /ELSI/TFGT_final/, accessed June 30, 2003.

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ANALYTIC VALIDITY

Question 12: Have repeated measurements been made on specimens?

Summary

- Having information about repeated measurements on the same specimen is important for determining the type and rate of errors in detecting *HFE* mutations
- External proficiency testing programs are the only available source of data for repeated measurements on the same specimen by multiple laboratories
- All clinical laboratories measure individual control samples repeatedly, but results are not usually reported

Measurements made on the same specimen in different laboratories

Multiple laboratories have made repeated measurements on the same specimen, utilizing a variety of technologies. A collaborative external proficiency testing program, jointly administered by the ACMG/CAP provides up to six *HFE* challenges each year, along with a summary report of the results. Earlier sections in Analytic Validity (Questions 10 and 11) provide more details about the results of this program. In summary, the between-laboratory replication of a single specimen's C282Y genotype is between 98.7% and 100% (Figure 2-1).

Measurements made repeatedly on the same sample within a laboratory

It is common practice for repeated measurements to be made on the same specimen within a laboratory. For each assay, a positive control is usually included for each mutation tested. This internal documentation will remain within the laboratory but will be available for on-site inspections by certifying agencies. Thus, one avenue for collection of these data would be to use laboratory survey instruments. Nearly all laboratories will have these data available, even though they may not be routinely collated and analyzed.

ANALYTIC VALIDITY

Question 13. What is the within- and between-laboratory precision?

This question is not applicable to the use of DNA tests in screening for HHC, since such testing is qualitative. This question is relevant to quantitative measurements such as transferrin saturation, an alternative strategy for this type of screening.

ANALYTIC VALIDITY

Question 14: If appropriate, how is confirmatory testing performed?

Summary

- Confirmatory testing is additional testing to corroborate the finding of a mutation/genotype
- The type of confirmatory testing depends on the clinical circumstances, sample type and testing methodology
- Given that the prevalence of C282Y might be much lower among individuals with certain racial/ethnic heritages, the analytic positive predictive value (aPPV) will vary
  - aPPV could be as high as 80%, but might be 50% or lower in some groups
  - If confirmatory testing can identify 90% of the false positive results, aPPV would improve to 97%, but might be 90% or lower in some groups.
- Confirmatory testing should be considered when a positive result (C282Y homozygosity) is identified, as it is likely to be useful in identifying false positive results

Definition

Confirmatory testing is defined here as any additional testing performed after two C282Y mutations are identified in an individual, to ensure that the original result is correct. As seen in Table 2-2 (Questions 10 and 11), the four false negative results would not have been corrected by confirmatory testing, since they were initially reported as negative. It would not be feasible to retest all individuals with negative test results to try to identify false negatives. However, by performing confirmatory testing of the relatively small number of individuals identified as being homozygous (less than 1%), the two false positive results shown in the table might have been identified and corrected.

Four distinct types of confirmatory testing could be utilized, depending on the testing protocols in place and the circumstances in which the positive test result is obtained.

- Repeating the same test protocol on another aliquot of the same specimen
- Repeating the same test protocol on a different specimen
- Performing a different test protocol on another aliquot of the same specimen
- Performing a different test protocol on a different specimen

Importance of confirmatory testing

The analytic specificity is currently estimated to be 99.9% (Question 11). It is important, therefore, to determine how often 'false positive' results will be identified upon confirmatory testing. If the error is due to clerical or laboratory sample mix-up, simple retesting of an additional aliquot may be sufficient to identify and correct the error. Given that proficiency testing in Europe found a portion of the errors to be of this type (Dequeker and Cassiman, 2000), confirmatory testing might eliminate this type of false positive result. This issue is dealt with in more detail under Clinical Performance (Questions 18 and 19).

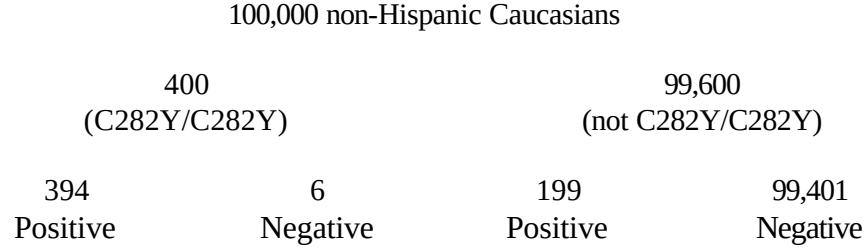
Gap in Knowledge: Proportion of Laboratories Performing Confirmatory Testing
Little or no information is available on whether clinical laboratories routinely perform confirmatory testing on samples found to be homozygous (or compound heterozygous).

These data could be collected as part of the ACMG/CAP external proficiency testing program.

Gap in Knowledge: Performance of Confirmatory Testing
Little or no information has been found on the ability of confirmatory testing to identify false positive test results in a clinical setting. According to proficiency testing data, false positive results will occur and might be identified as part of routine confirmatory testing of individuals found to be homozygous for C282Y.

Analytic positive predictive value (aPPV)
Figure 2-2 shows the aPPV of testing for C282Y homozygosity, in a population of non-Hispanic Caucasians who have a prevalence of homozygosity of about 4/1000 (corresponding to an allele frequency of about 7%). In addition, the analytic sensitivity is 98.4% (Question 10), the analytic specificity is 99.8% (Table 2-2, column 2).

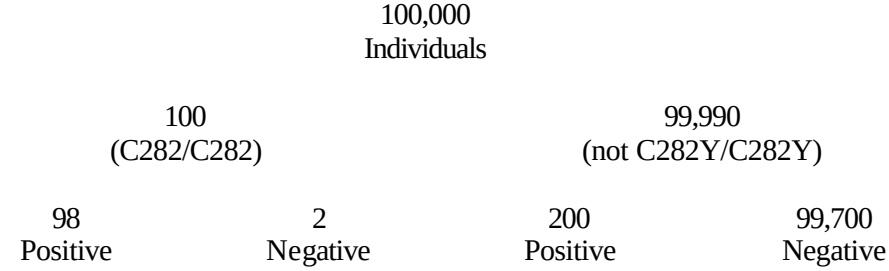
Figure 2-2. Analytic Positive Predictive Value for C282Y Homozygote Testing in Non-Hispanic Caucasians With a Prevalence of Homozygosity of Five per 1000



Among the 593 individuals identified as homozygous for C282Y, 394 (66%) are true positives. Thus, the aPPV is 66%. If confirmatory testing were routine, and if it were able to identify 90% of the false positive test results, the aPPV might be as high as 95% (388/408). An additional six true homozygotes may also be reclassified as negative.

In some populations, the prevalence of the C282Y genotype is much lower, and it should be expected that fewer of the positive test results would be true positives. Figure 2-3 shows a similar calculation to that shown in Figure 2-2, except that the prevalence of homozygosity is reduced to 1 per 1000.

Figure 2-3. Analytic Positive Predictive Power for C282Y Homozygote Testing in a Population with a Prevalence of Homozygosity of One per 1000



Among the 298 individuals identified as homozygous for C282Y, 98 (33%) are true positives. Thus, the aPPV is 33% in this population. If confirmatory testing were routine, and if it were able to identify 90% of the false positive test results, the aPPV might be as high as 76% (96/126). An additional two true homozygotes may also be reclassified as negative.

> **Gap in Knowledge: Analytic specificity among samples with one or no C282Y mutations.** The estimates of analytic specificity among these two groups are expected to be high, and, therefore, errors are relatively rare. For this reason, many challenges are necessary to have confidence in the estimates. Analytic specificity estimates are based on a small number of errors, underscoring the preliminary nature of the current estimates.

Gene frequencies in different racial/ethnic groups

Many reports document differences in HFE gene frequencies, based on racial/ethnic groups and/or geography. A recent study (Steinberg *et al.*, 2001) examined the prevalence of C282Y and H63D in the U.S. population, using samples from the Third National Health and Nutrition Examination Survey (NHANES III, 1992-1994). Samples were genotyped from 5,171 participants and analyzed with respect to race/ethnicity. The allele frequencies for C282Y and H63D are shown in Table 2-13. For a more complete analysis of allele and genotype frequencies by race/ethnicity, see Questions 18 and 19 in Clinical Validity.

Table 2-13. *HFE* Allele Frequencies in Selected Racial/Ethnic Groups in the United States

Race/Ethnic Group	Number	Allele Frequency (%)	
		C282Y	H63D
White	2,016	6.4	15.4
Mexican	1,555	1.6	11.4
Black	1,600	1.3	3.2

ANALYTIC VALIDITY

Question 15: What types of patient samples have been tested?

Summary
- Both whole blood and buccal lysates are acceptable for screening
- Blood samples are more expensive and require collection at a medical facility, but are associated with larger amounts of higher quality DNA.
- Buccal lysates are less expensive and can be collected at home, but are associated with smaller amounts of lower quality DNA.

HFE mutation analysis has been successfully performed in a variety of types of specimens using available methodologies.

Screening can be performed on:
- whole blood (purified DNA and lysates),
- buccal lysates (cheekbrush, swab and mouthwash), or
- dried blood spots.

Blood samples are the most reliable method of collecting large amounts of higher quality DNA, but a trained phlebotomist is needed, thereby increasing costs and requiring that specimens be collected at a medical facility. Buccal cells obtained by scraping, brushing or mouthwash yield adequate amounts of DNA for screening purposes, as documented in prenatal cystic fibrosis programs (Doherty *et al.*, 1996; Loader *et al.*, 1996; Witt *et al.*, 1996; Grody *et al.*, 1997). This technique can be used to collect samples at the physician's office or at home. Buccal samples have the disadvantage of less DNA, higher failure rates, and less documentation of chain of custody. Buccal lysates can be frozen and stored for years and still be tested successfully (Bradley *et al.*, 1998). A comparison of test results from blood and buccal mouthwash samples showed consistent results (Baty *et al.*, 1998). Dried blood spots can also be used for PCR-based testing. Guthrie cards from the New York State Newborn Screening Program have been used to amplify multiple genes to detect mutations that impact public health (Caggana *et al.*, 1998). However, they have not routinely been used in hemochromatosis pilot screening programs. An informal survey of several commercial laboratories offering *HFE* testing determined that none accepted blood spots (Gasparini *et al.*, 1999; S Richards, personal communication).

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ANALYTIC VALIDITY

Question 16: How often does the test fail to give a useable result?

Summary

- Laboratory testing for *HFE* mutations can be divided into pre-analytic, analytic and post-analytic phases
- In the pre-analytic phase, generally agreed upon criteria are in use to determine the appropriateness of testing. If these criteria are not met, the test can be canceled
- In the analytic phase, samples fail for multiple reasons, and these failures are routinely documented in clinical laboratories but are not generally available for outside review
- When analytic failures do occur, repeating the analysis will often yield useable results
- Types of failures and their associated rates are rarely reported as part of pilot trials or method comparisons

Test 'failures' in the pre-analytic phase of testing

In the pre-analytic phase, it may be determined that the sample is not suitable for testing because specific clinical criteria are not met, or because the sample is considered inadequate. While programs often monitor pre-analytic test cancellation rates as part of an overall quality assurance plan, these events are usually not considered a laboratory or methodologic 'failure'. Table 2-14 lists criteria commonly used for deciding whether to reject a sample in the pre-analytic phase.

Table 2-14. Common Pre-analytic Criteria for Rejecting Samples Submitted for *HFE* C282Y Homozygote Testing as Part of Population Screening

Rejection Criteria Based on Clinical Information
Sample submitted for diagnostic testing (i.e., more than the C282Y mutation should be tested)
Rejection Criteria Based on Submitted Sample
Inadequate specimen quality (e.g., hemolyzed blood, dried buccal sample or obvious contamination)
Inappropriate sample (e.g., whole blood with no anticoagulant or wrong anticoagulant)
Inadequate specimen labeling
Inappropriate handling prior to laboratory receipt (e.g., sample too long in transit or exposed to extreme temperature)

Test failures during the analytic phase of testing

Failures of individual samples or assays occur when preset quality control standards are not met and test results are not reportable. Failures can arise for a number of reasons, such as improperly processed samples, problems with component reagents, or equipment malfunction. Many assay failures within the clinical molecular genetic laboratory are due to operator error. Automation and programs to properly train laboratory personnel can avoid most of these problems. Only a few medical technology programs, however, currently provide adequate molecular components in their programs. Documentation of failures and subsequent corrective action is required by regulatory agencies such as CLIA and the College of American Pathologists. Unfortunately, failure rates and other information on assay robustness are often not published as part of pilot trials or method evaluations. Available data suggest, however, that repeating the initial unsatisfactory analysis of an individual sample or assay run can often yield a satisfactory result.

An irretrievable assay failure occurs when an apparently suitable specimen is submitted and approved for testing, but the assay yields a result that is clinically uninterpretable. Failures of this type are most often related to the quality of the original sample. Procedural problems during specimen processing and DNA extraction can also be responsible. Success rates for obtaining clinically interpretable results are close to 100% for blood samples. Buccal samples have a somewhat lower success rate (98% to over 99%) as a result of poor sampling (inadequate number of cells), sample contamination, desiccation (exposure to extreme heat), or inadequate sensitivity of the testing methodology to account for the lower concentration and quality of the sample.

Test failure during the post-analytic phase of testing

Post-analytic failures, such as incorrect or inadequately interpreted results, are considered separately from analytic test failures, as part of a review of overall quality assurance in the Clinical Utility Section (Question 34).

Gap in Knowledge: Overall, and method-specific, failure rates

Clinical laboratories are required to document test failures, as described above. For this reason, this type of information should be readily available from laboratories participating in external proficiency testing administered by the ACMG/CAP. This could be accomplished though the use of a supplemental question attached to a routine distribution or, alternatively, the data could be collected via an externally funded, independent project.

ANALYTIC VALIDITY

Question 17: How similar are results obtained in different laboratories?

Summary

- Data derived from external proficiency testing can be used to judge the consistency of results from different *HFE* testing laboratories
- Stratification of results by methodology does not currently yield reliable information because of the small number of laboratories participating in proficiency testing and the large number of methodologies
- Overall, the results from multiple laboratories appear to be similar, regardless of the methodology used

Comparing results from different laboratories using the same or similar methodologies

The only potential source of data for evaluating differences in *HFE* test results from multiple laboratories using the same (or a similar) method would be derived from external proficiency testing. Method-specific comparisons are complicated, because laboratories in the same methodological category may be using different home-brew reagent components and protocols. For example, although three laboratories might be grouped under the ARMS methodology, one might use a prepared kit, a second might use commercially prepared analyte specific reagents (ASR), and the third might use in-house reagents. To help in comparing methodologies, the ACMG/CAP MGL Survey Participant Summary Reports have stratified results into broad methodological categories.

Comparing results from the same laboratory using different methodologies

Baty *et al*. (1998) compared the results of testing 46 samples using the ARMS test versus the restriction digestion method and found identical results. Similarly, Jackson *et al*. (1997) demonstrated the use of heteroduplex analysis for *HFE* genotyping and showed 100% concordance with results obtained by restriction digestion methods. Guttridge *et al*. (1998) described a method of sequence specific primers for PCR (PCR-SSP) for *HFE* analysis, tested 185 individuals previously typed using PCR-RFLP, and found complete agreement of results. Bernard *et al*. (1998) described a new method using fluorescent hybridization probes for *HFE* genotyping and compared it to the standard method of restriction enzyme digestion and gel electrophoresis. Of 117 patients and 56 controls tested, no discrepancies were noted. SSCP and capillary electrophoresis were also used to perform *HFE* testing on 85 patients with liver disease, and RFLP analysis was used to confirm the results (Bosserhoff *et al.*, 1999; Wenz *et al.*, 1999).

Neoh *et al*. (1999) reported a method based on fluorescence resonance energy transfer (FRET) and real time polymerase chain reaction (PCR) to identify *HFE* genotypes in 112 individuals. The results were compared to restriction digestion of PCR products. Agreement was found in 244 of 246 samples tested. Sequence analysis determined that the FRET analysis result was correct. Parks *et al*. (2001) reported a similar study in which 450 patients were tested for *HFE* (C282Y) using FRET analysis. Their results were compared with standard PCR and RFLP analysis, with 100% concordance. Steffensen *et al*. (1998) tested 200 Danish individuals for the C282Y and H63D mutations, using a sequence-specific primer method for PCR analysis (PCR-

SSP) and compared their results to a standard method of PCR-RFLP with complete agreement of methods for analysis.

Other methods for testing for *HFE* include dHPLC analysis, with or without single-base extension (Devaney *et al.*, 2001; Liang *et al.*, 2001), Lightcycler (Kyger *et al.*, 1998), and the DNA capillary array electrophoresis chips (Woolley *et al.*, 1997), although less information is available regarding assay validation studies.

Comparing results from different laboratories using different methodologies

As part of the 2000 ACMG/CAP Molecular Genetics Laboratory external proficiency testing survey, laboratories were queried about their methodology for performing *HFE* mutation analysis (Table 2-15, Appendix 2). Overall, the reported methodologies were used to detect one or two mutations (with the majority of laboratories testing both C282Y and H63D). During the four years of proficiency testing (1998 through 2001) there was a high level of agreement between laboratories for detecting mutations that were targeted by their specific method, no matter which method was being used.

Gap in Knowledge: Comparison of Methods for *HFE* Mutation Detection In order to compare analytic validity for various testing methodologies, proficiency testing data from ACMG/CAP have been stratified by methodological category. It would also be useful to identify subsets using the same commercially available reagents (e.g., in-house reagents versus ASR). Alternatively, a previously described method for validation (Question 9 – Optimal Sources of Data) could be employed that would provide not only analytic performance for a methodology, but also comparative data between methodologies.

Appendix 2. Analytic Methodologies used for *HFE* Mutation Analysis

Introduction

Table 2-15 lists categories of methodologies that are used to detect *HFE* mutations by laboratories participating in proficiency testing programs in the United States (ACMG/CAP MGL Survey), along with the proportions using each method. Because many laboratories utilize "home brew" assays, these categories are no*t necessarily h*omogeneous. Some methodologies are relatively labor intensive, making them more suitable for research than diagnostic laboratories. When large numbers of specimens must be tested with short turn-around times, other methodologies are needed. Commercial suppliers can provide "kits" to European or Canadian-based clinical laboratories. U.S. laboratories are not allowed to use "kits" for clinical testing but may use analyte specific reagents (ASR).

Table 2-15. Testing Methods Utilized by 90 U.S. Laboratories According to the 2002 ACMG/CAP MGL External Proficiency Testing Survey

Testing Method	Proportion of Laboratories (%)
Electrophoresis for RFLP and size analysis	64
Allele Specific Oligonucleotide (ASO)	11
Allele-specific PCR/ARMS	6
LightCycler	8
Sequencing	3
Other/Not specified	8
Total	100

HFE testing methodologies for screening ought to include the following characteristics:

- ability to selectively identify only the C282Y mutation
- a low to moderate level of technical expertise
- a short turn-around time (one or two days)
- a high throughput (ideally, on an automated platform)
- a relatively low cost

These requirements might appear ambitious, but the evolution of other tests now used for screening in the clinical laboratory shows that these goals are achievable. For example, immunoassays that are now routinely performed were originally developed in the 1960s by investigators with in-depth knowledge of immunochemistry and radiation detection methods. Over the ensuing years, these assays were revised and streamlined by manufacturers to meet the needs of clinical laboratories, including the development of automated immunoassay systems that minimize the chance for error. For FDA approved kits, the responsibility for ensuring reagent quality and instrument performance now rests primarily with the manufacturer. The laboratory's responsibility is to monitor the quality control measures set by the manufacturer to verify that assay performance meets specifications. A further development is a computer link to

the instrument that automatically transfers test results to a patient record system for reporting. Automation is more expensive than manual assays in terms of reagents and instrument rental or purchase, but the overall cost per test can be the same or lower because of the reduced labor costs. This same development is beginning to occur for HHC screening. Several commercially prepared reagents have emerged, and their attributes are summarized in Table 2-16.

[Table 2-16 and the following notes are still under construction]

Table 2-16. Characteristics of Commercial Analyte Specific Reagents (ASR) to Detect *HFE* Mutations

	Commercial *HFE* Mutation Detection System			
Characteristic	**Bio-Rad**	**Nanogen**	**LightCycler**	**Orchid**
1. Method Type				
2. Company				
3. Mutations				
4. Robustness				
5. Special equipment				
6. Total time (days)				
7. Cost per patient				
8. Advantages				
9. Disadvantages				
For more information				

Notes pertaining to Table 2-16:

1 Method type: Methods displayed are those that are most commonly used and that are suitable for large-scale hemochromatosis screening. These include the allele specific oligonucleotide assay (ASO). The ASO assay uses reverse ASO technology, with the oligonucleotides bound to the microplate surface. Biotinylated DNA is bound to the ASOs; Streptavidin horseradish peroxidase is used bound to the biotin, oxidizes a substrate, and results in a colorimetric change. Detection is done by colorimetric analysis using a plate reader. Two wells are required for each allele analyzed. Genotype is determined by a ratio of absorbance. An alternate means of amplification of DNA is available in this ASR format and is termed LLA or linked linear amplification (Linked Linear Amplification: A New Method for Amplification of DNA. Clin Chem 47:31-40 (2001). Both standard PCR and LLA ASRs are available. This assay has been validated by the manufacturer against other molecular methods for performing hemochromatosis testing. Whole blood was the only sample type that was tested, according to the manufacturer. For more information about methodologies, including a description and set of references, see www.bio-rad.com.

2. Company: Commercial reagents have not been approved by the FDA. However, reagents have had one level of QC/QA performed by the manufacturer, as specified by the FDA's Analyte Specific Reagent (ASR) rule. ASRs are produced using GMP and have undergone rigorous quality control testing in house. As Bio-Rad owns the patent for hereditary hemochromatosis, there are no other commercially available manufactured reagents for this test.

3. Mutation(s): This is the hereditary hemochromatosis mutation(s) that can be detected by the testing protocol. This ASR is currently designed to test for both C282Y and H63D. Currently, a laboratory that uses this reagent must test for both alleles. The S65C mutation may be added to the ASR. Initially there was some concern of interference with ASO binding in H63D/S65C compound heterozygotes, as the ASOs were not designed to distinguish these two alleles. Thus, an H63D/S65C compound heterozygote could appear as an H63D homozygote. The current ASR does not include the S65C mutation.

4. Robustness: Robustness describes how consistently and reliably a set of reagents performs when used by different laboratories, under varying conditions, and on different sample types (e.g., blood, buccal smears).

5. Special equipment: Some manufacturers require that specialized equipment be used to perform their assays. Although initially more costly, the equipment may allow more samples to be tested. The Bio-Rad ASRs require a plate reader for the colorimetric analysis detection. Currently, there is no specific software associated with the interpretation of genotype, and laboratories are left to design their own system. Some use Excel spreadsheets. A more automated system with the flexibility to set cutoffs would be desirable.

6. Total time: Estimated time to complete assay, including sample processing and reporting. This method only requires one day, but laboratories may choose to extend the process to a second day for more convenient scheduling.

7. Cost per patient: Costs for the reagents and licenses to perform hereditary hemochromatosis testing are extremely variable. Some laboratories perform 'in-house' assays with relatively low reagent costs. In such cases, the cost of technical time for reagent preparation and QC/QA must also be considered. Costs of analyte specific reagents (ASR) can be relatively high, compared to traditional biochemical assays. However, the savings in technical staff time for preparation and QC/QA can offset reagent costs. For screening, the relevant figure is the cost per patient tested, rather than the cost per mutation tested.

8. Advantages: Reagents for hereditary hemochromatosis screening should have high throughput with relatively low labor costs. Assays that can be efficiently automated can be cost effective. Peer-reviewed analytic validity data are helpful for validation.

Newer testing technology platforms with high potential for hereditary hemochromatosis testing include various hybridization strategies (Roche and Luminex), arrayed primer extension (Orchid), mass spectrometry (Sequenom), and sequence analysis (Pyrosequencing). However, there are no existing data that accurately compare these technologies with currently utilized methodologies or with each other.

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