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Development of a Genomic DNA Reference Material Panel for Rett Syndrome (*MECP2*-Related Disorders) Genetic Testing

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Rett syndrome is a dominant X-linked disorder caused by point mutations (approximately 80%) or by deletions or insertions (approximately 15% to 18%) in the *MECP2* gene. It is most common in females but lethal in males, with a distinctly different phenotype. Rett syndrome patients have severe neurological and behavioral problems. Clinical genetic testing laboratories commonly use characterized genomic DNA reference materials to assure the quality of the testing process; however, none are commercially available for *MECP2* genetic testing. The Centers for Disease Control and Prevention's Genetic Testing Reference Material Coordination Program, in collaboration with the genetic testing community and the Coriell Cell Repositories, established 27 new cell lines and characterized the *MECP2* mutations in these and in 8 previously available cell lines. DNA samples from the 35 cell lines were tested by eight clinical genetic testing laboratories using DNA sequence analysis and methods to assess copy number (multiplex ligation-dependent probe amplification, semiquantitative PCR, or array-based comparative genomic hybridization). The eight common point mutations known to cause approximately 60% of Rett syndrome cases were identified, as were other *MECP2* variants, including deletions, duplications, and frame shift and splice-site mutations. Two of the 35 samples were from males with *MECP2* duplications. These *MECP2* and other characterized genomic DNA samples are publicly available from the NIGMS Repository at the Coriell Cell Repositories. *(J Mol Diagn 2014, 16: 273—279; http://dx.doi.org/10.1016/j.jmoldx.2013.11.004)*

Rett syndrome is a dominant X-linked disorder usually caused by point mutations (approximately 80% in classical and 40% in atypical cases) and deletions or insertions (approximately 15% to 18% in classical and 3% in atypical cases) in the *MECP2* gene, although patients with mutations in two other genes, *CDKL5* and *FOXG1*, may also exhibit a Rett-like phenotype.[1,2] *MECP2*, located on Xq28 and comprising four exons, encodes methyl-CpG binding protein 2

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(MeCP2). In males, Rett syndrome is usually lethal, because of abnormal MeCP2 function from the single X chromosome and severe neonatal encephalopathy, although Rett syndrome in males with an XXY karyotype has been reported.[3] Duplications in *MECP2* have also been observed, and these can cause severe neurodevelopmental disability in males.[4] The prevalence of Rett syndrome in females is approximately 1:10,000.[5]

Girls with classic Rett syndrome (OMIM #312750) exhibit a rapid decline in language and motor skills at approximately 1 year of age. These patients exhibit a loss of acquired purposeful hand use, loss of communication, gait ataxia and apraxia, and stereotypic hand movements. They may also exhibit additional symptoms, including bruxism, seizures, episodic apnea or hyperpnea, abnormal muscle tone, and often acquired microcephaly.[4,6,7]

Patients with Rett syndrome can present with various phenotypes. Those diagnosed with atypical Rett syndrome may have either more mild or more severe presentation than patients with the classical form. These patients share some of the same symptoms with classical Rett syndrome cases, but must also have some of the additional symptoms listed above.[4,6] Some patients present with only mild learning disabilities (females) or intellectual disability (males).[4,8]

Molecular diagnosis of Rett syndrome is performed by examination of the patient's DNA for *MECP2* mutations, using a variety of molecular diagnostic methods. Most *MECP2* mutations are sporadic, and testing is performed on the proband, although predictive prenatal and preimplantation genetic testing may also be performed. DNA sequence analysis may detect point mutations and small insertions and deletions (indels). Larger deletions and duplications are detected using multiplex ligation-dependent probe amplification (MLPA), quantitative PCR, and array-based comparative genomic hybridization (CGH). To date, there are no US Food and Drug Administration (FDA)—approved assays for Rett syndrome. All testing is performed using laboratory-developed tests.

Reference materials are needed by laboratories to comply with regulatory and accreditation requirements for assay development, assay validation, and quality control, and their use is recommended by professional guidelines for clinical laboratories [eg, *http://www.acmg.net/Pages/ACMG_Activities/stds-2002/g.htm*, last accessed October 16, 2013; Washington State Legislature, *http://www.doh.wa.gov/hsqa/fsl/lqa_home.htm*, last accessed January 11, 2013; College of American Pathologists *http://www.cap.org/apps/cap.portal*, last accessed January 11, 2013 (registration required); New York State Clinical Laboratory Evaluation Program, *http://www.wadsworth.org/clep*, last accessed January 11, 2013).[9–15] Clinical genetic testing laboratories commonly use characterized genomic DNA reference materials to assure the quality of the testing process. Ideally, these reference materials should closely resemble patient samples containing variants and types of variants common to the disorder and should be thoroughly characterized using methods different from those used in the user's laboratory.[15] For Rett syndrome, genomic DNA reference materials derived from females (and, if possible, males) containing common point mutations, indels, and larger deletions and duplications should be used. Careful use of a well-characterized and comprehensive set of reference materials helps to assure the proper design and function of a clinical assay. To date, there are no commercially available reference materials for Rett syndrome genetic testing.

To address the need for characterized genomic DNA reference materials for Rett syndrome testing, the Centers for Disease Control and Prevention (CDC) based Genetic Testing Reference Material Coordination Program (GeT-RM), in collaboration with members of the genetic testing community and the National Institute of General Medical Sciences (NIGMS) Repository at the Coriell Cell Repositories, have characterized the *MECP2* mutations in 35 publicly available cell lines. Twenty-seven of the 35 cell lines were generated as part of this project, using blood collected with informed consent from Rett syndrome patients with variants not previously represented in cell lines at the Coriell Repository. The availability of a renewable source of characterized reference materials for Rett syndrome helps to assure the accuracy of these genetic tests and facilitate research and test development.

Materials and Methods

Selection of Pre-Existing Cell Lines

Based on preliminary information from the Coriell Cell Repository and data generated before this study, eight cell lines (Table 1) with common point mutations or exon deletions in the *MECP2* gene known to cause Rett syndrome were selected for further characterization.

Anonymous Blood Collection from Consenting Patients with Rett Syndrome

Collaboration was established with a researcher (A.K.P.) at the University of Alabama, Birmingham, to generate new cell lines with point mutations, duplications, and deletions not then available in the Coriell Repository collection. A protocol was developed for anonymous contact, informed consent, and blood collection from Rett syndrome patients and was approved by the University of Alabama Institutional Review Board. Rett syndrome patients previously enrolled in the NIH Office of Rare Diseases Research (ORDR) and National Institute of Child Health and Human Development (NICHD)-sponsored Rare Disease Clinical Research Center Natural History Study (RDCRC NHS) consortium for Angelman, Rett, and Prader-Willi Syndromes Natural History study with requisite Rett syndrome genotypes not represented in the Coriell Repository collection (common *MECP2* point mutations and *MECP2* deletions and duplications) were contacted and asked to participate by UAB staff associated with the RDCRC study. Recruitment letters describing the study were sent to eligible Rett syndrome patients, along with other

Table 1 Previously Established Cell Lines Tested at Seven Clinical Genetic Testing Laboratories (Round 1)

		Laboratory and analysis						
Coriell cell line[†]	Consensus genotype	1: Array CGH	2: DNA seq[‡]	3: DNA seq; PCR dosage	4: DNA seq; MLPA	5: DNA seq	6: DNA seq; MLPA	7: DNA seq
GM11299	c.316C>T; p.R106W het	ND	c.316C>T; p.R106W het	c.316C>T; p.R106W het	ND	c.316C>T; p.R106W het	ND	c.316C> T; p.R106W het
GM11310	c.502C>T; p.R168* het	ND	c.502C>T; p.R168* het	c.502C>T; p.R168* het	ND	c.502C>T; p.R168* het	c.502C>T; p.R168* het	c.502C>T; p.R168* het
GM16270	c.763C>T; p.R255* het	ND	c.763C>T; p.R255* het	c.763C>T; p.R255* het	ND	c.763C>T; p.R255* het	c.763C>T; p.R255* het	c.763C>T; p.R255* het
GM16271	c.473C>T; p.T158M het	ND	c.473C>T; p.T158M het	ND	ND	ND	c.473C>T; p.T158M het	c.473C>T; p.T158M het
GM16480	c.916C>T; p.R306C het	No del/dup identified	c.916C>T; p.R306C het	ND	c.916C>T; p.R306C het	ND	c.916C>T; p.R306C het	c.916C>T; p.R306C het
GM16546	c.880C>T; p.R294* het	ND	c.880C>T; p.R294* het	ND	c.880C>T; p.R294* het	ND	c.880C>T; p.R294* het	c.880C>T; p.R294* het
GM17540	c.401C>G p.S134C het	ND	c.401C>G p.S134C het	ND	c.401C>G p.S134C het	ND	c.401C>G p.S134C het	c.401C>G p.S134C het
GM11313	EX3_4del het	EX3_4del het	ND	EX3_4del het	ND	No seq var identified	EX3_4del het	ND

[†]All cell lines are female.

[‡]Exons 2 to 4 only.

del, deletion; dup, duplication; EX3_4del, gross deletion of exons 3 and 4 in *MECP2*; het, heterozygous; ND, no data (sample not tested in this laboratory); seq, sequencing; var, variant.

informational materials, a consent form/waiver of assent form, and a contact information form. Interested participants returned completed documents and received a research code and a blood collection kit. Blood samples collected during routine health care visits were coded and were sent, without identifiers and using preaddressed, prepaid shipping containers, to the Coriell Cell Repository for lymphoblastoid cell line development. Through this process, 33 blood samples were received and 30 new cell lines were generated. For two of these lines, GM23675 and GM23734, the blood samples were from male patients with duplications of the *MECP2* gene.

Cell Line Generation

Epstein–Barr virus transformation of B lymphocytes was performed on whole-blood samples collected from consenting patients or their families, as described previously.[16,17]

DNA Preparation

Approximately 2 mg of DNA was prepared from cultures of each of the selected cell lines by the Coriell Cell Repositories using the Gentra Autopure method (Qiagen, Valencia, CA) according to the manufacturer's instructions or using methods described previously.[18]

Testing Laboratories

Eight College of American Pathologists–accredited clinical genetic testing laboratories were contacted and volunteered to test the DNA samples using their current *MECP2* assay methods. Laboratories 1 to 7 participated in Round 1 of testing (characterization of eight previously available cell lines) and Laboratories 1 to 5 and 8 participated in Round 2 (testing of the newly generated cell lines). Each sample was tested at three to five laboratories in Round 1 and at two or three laboratories in Round 2 (Tables 1 and 2).

Protocol

The volunteer clinical laboratories received a 10-μg aliquot of DNA from each of the cell lines for *MECP2* testing. The expected mutation in each of the samples was not revealed to the laboratories in advance. Each laboratory genotyped the samples using their standard *MECP2* DNA sequencing and/or deletion–duplication assay. The results were sent to the study coordinator (L.V.K.), who examined the data for discrepancies. Testing was performed in two rounds. In the first round, aliquots of DNA from each of the eight existing Coriell cell lines (Table 1) were sent to seven laboratories. In Round 2 testing, DNA samples from 27 of the 30 cell lines generated from Rett syndrome patients for this project (Table 2) were sent for analysis to six laboratories (including one newly participating laboratory). DNA from the remaining three newly generated cell lines was not used, either because it was not available when the study began or because the expected mutation could not be confirmed during initial analysis before the study.

Assays Used in Rounds 1 and 2

DNA sequence analysis, array CGH, MLPA, and PCR dosage analysis were used to analyze the samples (Tables 1 and 2).

Table 2 Newly Established Cell Lines Tested at Six Clinical Genetic Testing Laboratories (Round 2)

Coriell catalog no.	Sex	Consensus genotype	Laboratory and analysis					
			1: DNA seq; MLPA	2: DNA seq; MLPA	3: DNA seq; PCR dosage	4: DNA seq; MLPA	5: DNA seq; MLPA	8: DNA seq; MLPA
GM23459	F	del 5′ exon 4 het	ND[†]	ND	c. 1226_1337del 5′ exon 4 het[‡]	ND	ND	del 5′ exon 4 het
GM23461	F	c.455C>G; p.P152R het	ND	c.455C>G; p.P152R het, MLPA neg	ND	ND	ND	c.455C>G; p.P152R het
GM23462	F	c.808C>T; p.R270* het	ND	ND	ND	c.808C>T; p.R270* het	ND	c.808C>T; p.R270* het
GM23463	F	c.1173_1207del p.Glu392* het	ND	c.1173_1207del, p.Glu392*, MLPA neg	c.1173_1207del p.Glu392* het	ND	ND	ND
GM23503	F	c.806delG; p.Gly269Alafs*20 het	ND	c.806delG; p.Gly269Alafs*20 het, MLPA neg	ND	ND	ND	c.806delG; p.Gly269Alafs*20 het
GM23598	F	c.808C>T; p.R270* het	ND	c.808C>T; p.R270* het, MLPA neg	ND	ND	ND	c.808C>T; p.R270* het
GM23599	F	del exon 3—4 het	ND	del exon 3—4 het, seq neg	ND	ND	ND	del exon 3—4 het
GM23605	F	c.421C>G; p.Y141* het	c.421C>G; p.Y141* het	ND	ND	ND	ND	c.421C>G; p.Y141* het
GM23606	F	c.27-8C>G (intron 2 IVS8C>G) het	ND	c.27-8C>G (intron 2 IVS8C>G) het, MLPA neg	ND	ND	ND	c.27-8C>G (intron 2 IVS8C>G) het
GM23607	F	c.1157_1200del44; p.L386Gfs*4 het	ND	c.1157_1200del44; p.L386Gfs*4 het, MLPA neg	ND	ND	c.1157_1200del44; p.L386Gfs*4 het	c.1157_1200del44; p.L386Gfs*4 het
GM23624	F	c.398G>C; p.R133P het	ND	c.398G>C; p.R133P het, MLPA neg	ND	ND	ND	c.398G>C; p.R133P het
GM23625	F	c.808C>T; p.R270* het	ND	c.808C>T; p.R270* het, MLPA neg	ND	ND	ND	c.808C>T; p.R270* het
GM23626	F	c.455C>G; p.P152R het	ND	ND	ND	ND	c.455C>G; p.P152R het	c.455C>G; p.P152R het
GM23634	F	c.397C>T; p.R133C het	ND	c.397C>T; p.R133C het, MLPA neg	ND	ND	ND	c.397C>T; p.R133C het
GM23635	F	del exon 3 and part of exon 4, het	ND	ND	ND	del exon 3 and part of exon 4, het	ND	del exon 3 and part of exon 4, het
GM23647	F	c.378-3C>G (IVS3-3) het	c.378-3C>G (IVS3-3) het	ND	ND	ND	ND	c.378-3C>G (IVS3-3) het
GM23648	F	del exon 4 into *IRAK1*, het	ND	ND	del exon 4 into *IRAK1*, het	ND	ND	del exon 4 into *IRAK1*, het
GM23654	F	del exon 3 and part of exon 4, het	ND	ND	del exon 3 and part of exon 4, het; dupl 3′ end of exon 4[‡]	ND	ND	del exon 3 and part of exon 4, het
GM23659	F	c.397C>T; p.R133C het	c.397C>T; p.R133C het	ND	ND	ND	ND	c.397C>T; p.R133C het
GM23663	F	c.397C>T; p.R133C het	ND	ND	ND	c.397C>T; p.R133C het	ND	c.397C>T; p.R133C het

(*table continues*)

Table 2 (*continued*)

Coriell catalog no.	Sex	Consensus genotype	Laboratory and analysis					
			1: DNA seq; MLPA	2: DNA seq; MLPA	3: DNA seq; PCR dosage	4: DNA seq; MLPA	5: DNA seq; MLPA	8: DNA seq; MLPA
GM23675	M	Complete *MECP2* dupl + dupl of flanking *IRAK1* (exons 3, 4)	ND	Complete *MECP2* dupl + dupl of flanking *IRAK1* (exons 3, 4)	ND	ND	ND	Complete *MECP2* dupl + dupl of flanking *IRAK1* (exons 3, 4)
GM23676	F	Complete *MECP2* dupl + dupl of flanking *IRAK1*, het	ND	ND	ND	ND	Complete *MECP2* dupl + dupl of flanking *IRAK1*, het	Complete *MECP2* dupl + dupl of flanking *IRAK1*, het
GM23733	F	Complete *MECP2* dupl + dupl of flanking *FLNA-MECP2-IRAK1-L1CAM*, het	ND	ND	*MECP2* dupl exons 2–4 (bp 83,700–21,600)	ND	ND	Complete *MECP2* dupl + dupl of flanking *FLNA-MECP2-IRAK1-L1CAM*, het
GM23734	M	Complete *MECP2* dupl + dupl of flanking *FLNA-MECP2-IRAK1-L1CAM*	ND	*MECP2* dupl; *FLNA* (exon 29) dupl; *IRAK1* (exon 3, 4) dupl; *L1CAM* (exon 23) dupl	ND	ND	ND	Complete *MECP2* dupl + dupl of flanking *FLNA-MECP2-IRAK1-L1CAM*
GM23747	F	c.806delG; p.Gly269Alafs*20 het	ND	ND	ND	ND	c.806delG; p.Gly269Alafs*20 het	c.806delG; p.Gly269Alafs*20 het
GM23748	F	c.806delG; p.Gly269Alafs*20 het	ND	c.806delG; p.Gly269Alafs*20 het, MLPA neg	ND	ND	ND	c.806delG; p.Gly269Alafs*20 het
GM23846	F	c.1163_1188del26; p.Pro388Argfs*7 het	c.1163_1188del26; p.Pro388Argfs*7 het	ND	ND	ND	ND	c.1163_1188del26; p.Pro388Argfs*7 het

Laboratories 6 and 7 did not participate in Round 2.
[†]Deletion 23,700 to 22,200; duplication 21,900 to 21,600.
[‡]Sequencing and PCR dosage.
F, female; M, male; dupl, duplication; het, heterozygous; ND, no data (sample not tested in this laboratory); neg, negative; seq, sequencing.

DNA Sequence Analysis

The sequence of exons 1 to 4 of the *MECP2* gene was determined using bidirectional Sanger sequencing with either fluorescently tagged primers or dideoxy terminators. The reactions were analyzed using capillary electrophoresis.

Deletion–Duplication Analysis

With the exception of Laboratories 1 and 3 (described below), gross deletion–duplication analysis was performed using an MLPA kit (P015-D2; MRC Holland, Amsterdam, The Netherlands).

Array CGH and PCR Dosage Analysis

At Laboratory 1 (Round 1 only), genomic DNA from these cell lines was examined by array-based CGH, using the current version of ExonArrayDx (Agilent Technologies, Santa Clara, CA). This custom-designed array contains DNA oligonucleotide probes that cover all exons and introns of the *MECP2* gene and was designed to detect most single-exon deletions and duplications.

At Laboratory 3 (Rounds 1 and 2), dosage analysis was performed, in addition to DNA sequencing. Fragments sampling the four exons and proximal promoter of the *MECP2* gene, amelogenin and two autosomal gene fragments, were amplified in a single multiplex polymerase chain reaction (PCR) in the presence of fluorescently-labeled primers. Products of the amplification reactions were analyzed on an Applied Biosystems automated capillary DNA sequencer (Life Technologies, Carlsbad, CA). Dosage analysis was performed using SeqPilot software version 3.1.0.2 (JSI medical systems, Kippenheim, Germany).

Results

The goal of this study was to generate a comprehensive panel of publicly available characterized genomic DNA reference materials for Rett syndrome genetic testing. We sought to include the eight most common *MECP2* point mutations, p.R106W, p.R168X, p.R255X, p.T158M, p.R306C, p.R294X, p.R270X,

and p.R133C (referenced at NM_004992.3 and NM_001110792.1),[6] which affect approximately 60% of patients with Rett syndrome. In addition, we generated cell lines with other common point mutations, deletions, and duplications known to cause this disorder. Although Rett syndrome is most common in females, we included two samples from males with *MECP2* mutations.

We first selected eight cell lines derived from patients with Rett syndrome from the NIGMS Repository at the Coriell Cell Repositories. These lines, which contain six of the eight most common mutations that cause Rett syndrome (as well as one additional point mutation, p.S134C, and one deletion of *MECP2* exons 3 and 4), were tested by DNA sequence analysis and deletion−duplication analysis at seven laboratories (Round 1) (Table 1). The mutation previously identified in each cell line was confirmed, and results were concordant among laboratories and assay platforms (Table 1).

In Round 2, DNA from 27 newly generated cell lines was sent to six volunteer clinical laboratories for analysis (Round 2). DNA from 3 of the original 30 cell lines was not used, either because it was not available when the study began or because the expected mutation could not be confirmed during initial analysis before the study. The DNA samples in Round 2 were each tested by two or three laboratories using DNA sequence analysis and an assay to determine gene dosage (MLPA or dosage PCR) (Table 2). The results confirmed the expected genotype (based on information from the submitter) of the sample and were concordant among all laboratories and assay platforms (Table 2).

Discussion

DNA samples from 8 Rett syndrome cell lines in the Coriell Cell Repository and 27 newly generated cell lines were tested at from two to five separate clinical genetic testing laboratories for *MECP2* mutations using DNA sequence analysis and methods to assess copy number (MLPA, semiquantitative PCR, or array CGH). We identified samples with all eight of the common point mutations (listed under *Results*) known to cause approximately 60% of Rett syndrome cases, as well as a number of other *MECP2* variants, including deletions, duplications, frame shifts, and splice site mutations. Two of the samples tested were from males with duplications in *MECP2* gene. The results were concordant among laboratories and assay platforms.

Molecular diagnosis is critical for diagnosis of patients with *MECP2*-related disorders. The Rett syndrome phenotype usually becomes apparent at about 18 months of age, but the timing varies among individuals. Physicians use the particular combination of symptoms to classify patients into classic Rett syndrome or variant Rett syndrome.[6] Some patients with variant Rett syndrome may have mutations in *CDKL5*,[19] and patients with mutations in *FOXG1* have a congenital form of Rett syndrome.[1] Genetic testing can help to confirm or establish the diagnosis in many cases, especially when the patients are young and the phenotype may not be completely apparent.[4,5] Testing may also be important for at-risk relatives and for prenatal diagnosis or preimplantation genetic diagnosis.

To date, there are no commercially available reference materials or assays for Rett syndrome genetic testing. In the absence of FDA-approved Rett syndrome assays, clinical laboratories that test for this disorder must develop and validate their own assays. This involves not only the design of the assay, but also the establishment of performance characteristics such as accuracy, precision, analytical sensitivity, and analytical specificity. Establishment of these metrics requires the use of well-characterized reference materials encompassing the wide variety of variant types that can be expected in the patient population. In addition, clinical laboratories must also perform quality-control measures and participate in proficiency testing or other alternative assessment activities, as mandated by regulatory requirements and professional guidelines (College of American Pathologists *http://www.cap.org/apps/cap.portal*, last accessed January 11, 2013; Washington State Legislature, *http://www.doh.wa.gov/hsqa/fsl/lqa_home.htm*, last accessed January 11, 2013; New York State Clinical Laboratory Evaluation Program, *http://www.wadsworth.org/clep*, last accessed January 11, 2013).[9,10] All of these assay development, validation, and quality-assurance activities require the use of characterized reference materials. The reference materials for Rett syndrome should contain, in both male and female genomes, the variants that the assays are designed to detect (eg, point mutations, deletions, and duplications). This allows laboratories to make sure that their assays are robust and have sufficient sensitivity to detect relevant variants in patients with Rett syndrome.

Clinical laboratories and proficiency test providers often use genomic DNA from residual patient specimens and available cell lines, because publicly available reference materials for Rett syndrome testing are lacking. This approach, although necessary at present, is not ideal. Residual patient specimens are usually nonrenewable and of limited supply, and both residual DNA and cell lines may not be completely characterized. For example, several of the cell lines initially evaluated for this project did not contain the expected *MECP2* mutation based on information provided by the submitter. Laboratories may also have difficulty obtaining the variety of residual specimens necessary to adequately represent the spectrum of common point mutations and deletions and duplications that occur in Rett syndrome patients.

Cell lines previously available from the Coriell Cell Repositories represented only limited Rett syndrome genotypes. The panel of 35 publicly available genomic DNA samples developed and characterized as part of this study contains a wide variety of deletions and duplications in both male and female samples that can be used by clinical laboratories to assure the quality of Rett syndrome testing. In addition, the two samples from male patients allow laboratories to assess their ability to detect mutations when present in the haploid

state. These and other characterized genomic DNA samples are publicly available from the NIGMS Repository at the Coriell Cell Repositories (Camden, NJ). More information about the CDC GeT-RM program and other genomic DNA reference materials are available at the GeT-RM website (*http://wwwn.cdc.gov/dls/genetics/RMMaterials*, last accessed January 8, 2014).

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