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Genetically Characterized Positive Control Cell Lines Derived from Residual Clinical Blood Samples

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Background: **Positive control materials for clinical diagnostic molecular genetic testing are in critically short supply. High-quality DNA that closely resembles DNA isolated from patient specimens can be obtained from Epstein–Barr virus (EBV)–transformed peripheral blood lymphocyte cell lines. Here we report the development of a process to (*a*) recover residual blood samples with clinically important mutations detected during routine medical care, (*b*) select samples likely to provide viable lymphocytes for EBV transformation, (*c*) establish stable cell lines and confirm the reported mutation(s), and (*d*) validate the cell lines for use as positive controls in clinical molecular genetic testing applications.**

Methods: **A network of 32 genetic testing laboratories was established to obtain anonymous, residual clinical samples for transformation and to validate resulting cell lines for use as positive controls. Three panel meetings with experts in molecular genetic testing were held to evaluate results and formulate a process that could function in the context of current common practices in molecular diagnostic testing.**

Results: **Thirteen laboratories submitted a total of 113 residual clinical blood samples with mutations for 14 genetic disorders. Forty-one EBV-transformed cell lines were established. Thirty-five individual point and deletion mutations were shown to be stable after 20 popu-**

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lation doublings in culture. Thirty-three cell lines were characterized for specific mutations and validated for use as positive controls in clinical diagnostic applications.

Conclusions: **A process for producing and validating positive control cell lines from residual clinical blood samples has been developed. Sustainable implementation of the process could help alleviate the current shortage of positive control materials.**

The intense, ongoing effort to understand the functioning of the human genome has dramatically advanced the field of medical genetics, and this has been accompanied by a corresponding increase in clinical molecular genetic testing (MGT)[21] as a component of standard medical care. Clinical testing for >800 diseases with genetic involvement was available at this writing, and the number increases steadily *(1)*. MGT involves an array of complex scientific technologies and is performed in diverse settings, including academic research laboratories, healthcare facilities, and large commercial clinical testing sites. Consequently, clinical MGT laboratories face unique and increasing challenges in quality control, quality assurance/performance evaluation, and test validation. These activities require high-quality, readily available positive control materials; however, a deficiency of these materials was determined to be "the issue of utmost urgency in the field of MGT today" by a 1999 study sponsored by the CDC *(2, 3)*.

The impact of the deficiency of positive controls is strikingly illustrated by the situation with respect to cystic fibrosis testing. Cystic fibrosis is the most prevalent serious inherited disease of childhood, with a carrier frequency of ~1:25 in Caucasian populations *(4)*. In 2001, the American College of Obstetrics and Gynecology recommended cystic fibrosis screening for all individuals seeking preconception or prenatal care or counseling, and the American College of Medical Genetics (ACMG) recommended a core mutation panel of 25 cystic fibrosis mutations for use in carrier screening *(5)*. However, positive control materials for all of the recommended mutations were not available through public sources for more than 2 years after the release of the recommendations, limiting the ability of clinical laboratories to respond to the dramatic increase in demand for testing.

New demands for positive control materials are also created by scientific progress in medical genetics. For example, mutations in the connexin 26 gene (*GJB2*) were linked to hereditary deafness in 1997 and are now estimated to cause up to 50% of all nonsyndromic autosomal recessive hearing loss *(6, 7)*. Genetic testing for mutations in *GJB2* is now offered clinically, although a positive control for the most common mutation, 35delG, is not publicly available.

The limited sources of positive control materials currently include materials derived from positive patient samples and exchanged informally as well as proficiency-testing samples available through the College of American Pathologists/ACMG accreditation process [summarized in Ref. *(2)*]. Positive control materials may also be obtained from cell line collections, such as those offered by the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository *(8)* at Coriell Cell Repositories (Camden, NJ) *(9)*. Genetic materials derived from cell lines are widely used by current proficiency-testing programs; however, only a limited number of cell lines have been genetically characterized, and in general they have not been validated under clinical testing conditions. Their use may also be restricted; for example, in some cases, cell lines in public collections cannot be used in commercial products or distributed to second parties as may be required for a proficiency-testing program.

A centralized, comprehensive repository of transformed cell lines with clinically validated mutations of medical importance is urgently needed to make genetic controls widely and readily available. Transformed peripheral blood lymphocyte cell lines have the capacity to provide large quantities of high-quality positive control materials that are qualitatively similar to the clinical samples that are submitted for patient testing. Ideally, such a repository would provide genetic control materials to diverse types of organizations, including clinical laboratories, proficiency-testing organizations, and manufacturers of diagnostic products. The repository should also have the resources to identify emerging trends in MGT and establish new cell lines to meet the needs for new controls.

Previously, we demonstrated that residual clinical blood samples up to 14 days old are good sources of lymphocytes for Epstein–Barr virus (EBV) transformation *(10)*. Here we report the development of a process to obtain anonymous, residual clinical blood samples with mutations of interest, to use them to establish EBV-transformed cell lines, and to validate the cell lines for use as positive controls in clinical MGT applications. We show that a variety of mutations can be collected by this method and that most mutations are stable through 20 population doublings (PDLs) of the transformed cell line. We tested the cell lines at reference laboratories and then tested them further at additional clinical laboratories in a simulated performance evaluation (PE). During development of this process, 33 cell lines with mutations associated with 11 genetic disorders were validated. These cell lines are available through Coriell.

[21] Nonstandard abbreviations: MGT, molecular genetic testing; ACMG, American College of Medical Genetics; NIGMS, National Institute of General Medical Sciences; EBV, Epstein–Barr virus; PDL, population doubling; PE, performance evaluation; DUMC, Duke University Medical Center; IRB, Institutional Review Board; MTHFR, 5′,10′-methylenetetrahydrofolate reductase; FGFR, fibroblast growth factor receptor; Hb, hemoglobin; and FVL, factor V Leiden.

This project was a 3-year collaborative effort between Duke University Medical Center (DUMC), Coriell, the CDC Public Health Practices Program Office, and numerous genetic testing laboratories. During the development of the process, 3 expert panel meetings were convened to discuss the project and obtain input from members of the genetic testing community.

Implementation of this protocol in a sustainable manner would substantially alleviate the current critical shortage of positive control materials for clinical MGT and provide a means by which future and changing needs for positive controls can be met.

Materials and Methods

Initially, we evaluated a process that consisted of the following steps: laboratory recruitment, residual blood sample collection, EBV transformation of blood lymphocytes, mutation confirmation in the transformed cell lines, mutation stability testing, reference laboratory testing, and simulated PE testing. Twenty-one cell lines were validated with this initial method. In the interests of developing an expedient and economic method for use with future cell lines, we modified the process based on the results obtained and validated 12 additional cell lines by use of the modified method (see *Discussion*).

LABORATORY RECRUITMENT

Thirty-two laboratories participated in this project, recovering residual blood samples, serving as reference testing laboratories, and providing sites for simulated proficiency testing. A list of the participating laboratories and their roles in the project is provided as Table S1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue11. The laboratories were situated at private and public academic healthcare centers, community hospitals, not-for-profit healthcare organizations, commercial clinical testing facilities, and an industrial research and development site. Thirteen laboratories submitted anonymous, residual clinical blood samples, and many also served as reference laboratories and/or simulated proficiency-testing sites. To recruit additional clinical testing sites, a notice describing the project was sent to the members of the Molecular Section of the AACC and the Association for Molecular Pathology via their listserves. More than 30 responses were received. We also directly contacted laboratories listed at the GeneTests website *(1)*.

All sample-submitting laboratories obtained Institutional Review Board (IRB) approval. A model IRB protocol was developed by the expert panelists and submitted by the participating investigators to their own IRBs. Many institutions determined that the protocol was exempt from review because the samples were residual, completely anonymous materials and no patient contact was involved. In other cases, approval was granted under an expedited review.

BLOOD SAMPLE COLLECTION

Blood lymphocytes for EBV transformation were obtained from anonymous, residual blood samples that were found to contain mutations of interest during routine clinical testing or were considered likely to contain mutations based on clinical information and/or family history. In a preliminary study to determine transformability of residual clinical samples *(10)*, we had found that samples up to 14 days old, drawn with either acid-citrate-dextrose (ACD) or EDTA as the anticoagulant and stored at ambient temperature (0- to 7-day-old samples) or 4 °C (0- to 14-day-old samples), were reasonable candidates for transformation. A minimum sample volume of 1.0 mL was recommended for sample-handling reasons, and both previously opened and unopened tubes were acceptable. Microbial contamination was apparent in only one culture derived from a previously opened tube. The following guidelines were provided to submitting laboratories along with the necessary materials:

- Styrofoam-insulated shipping containers with sample tube holders, ziplock bags, and absorbent material per US Department of Transportation regulations for diagnostic specimens;
- Sample labels printed with unique project-specific identifiers;
- Packing and shipping instructions;
- Preprinted, prepaid Federal Express shipping labels;
- Submission forms for reporting nonidentifying patient and sample information; and
- A list of target mutations for which a critical clinical need for positive controls had been determined by genetic testing expert panelists.

When candidate specimens were identified, the laboratory contacted the study coordinator at DUMC to confirm the desirability of the specimen, and then removed all patient and institutional identifiers from the residual blood sample, affixed project identifier labels, and shipped the sample and the completed submission form (via Federal Express, priority overnight) to Coriell for transformation. If residual DNA was available, it was included with the blood sample. Because of the critical nature of some mutations and the time required for clinical testing, some samples that were considered likely to contain important mutations based on clinical information and/or family history were submitted before the results of genetic testing were reported. These samples were retested for the specified disease after submission, using either residual DNA or DNA extracted from the resulting cell line.

Patient confidentiality and complete anonymity of the samples were ensured. All patient and institutional identifiers were removed from both blood and DNA sample tubes. No records were maintained by the submitting laboratory regarding which samples had been contributed to the project, and the preprinted shipping labels identified DUMC as the shipper; therefore, both DUMC and

Coriell were blinded to the source of the samples. The sample submission forms and the residual DNA, if any, were forwarded to Duke by Coriell. Thus, no patient or institutional identifiers or links were retained. Mutations that were so unusual that they could by deduction be linked to a particular individual or family were excluded from the study. Decisions were made on a case-by-case basis, and one submission was excluded from the study on these grounds.

For each blood sample, the following information was requested, either from the submitting laboratory via the sample submission form or from Coriell after receipt of the sample: patient age, sex, and race/ethnicity; clinical phenotype and clinical diagnosis; sample mutation status; sample age; anticoagulant; tube status (unopened or previously opened); storage conditions; blood volume and tube type; volume and concentration of residual DNA, if any; and whether the sample had undergone hemolysis before setup for transformation. Coriell recorded the following culture information: evidence of contamination, both microbial and from other cell lines; success of transformation attempt; days in culture (days from transformation setup until freeze if successful, or until culture was discarded if unsuccessful); and periodic comments on culture appearance.

BLOOD LYMPHOCYTE TRANSFORMATION

Lymphocyte transformation, cell culture, and cell pellet preparation were performed at Coriell. Transformation of the residual peripheral blood samples was performed according to standard EBV transformation protocols as described previously *(10)*. Transformed cultures were grown to $\sim 1 \times 10^8$ cells and then cryopreserved. "Successful" transformations were free from contamination and were viable after cryopreservation, as evidenced by a doubling of the cell number within 4 days of recovery.

MUTATION CONFIRMATION

The mutation status of each submitted sample was verified by genetic testing of residual DNA from the original testing site, if available. For all successful transformations, the mutation was confirmed in each cell line after the culture had increased to $\sim 1 \times 10^8$ cells. In most cases, the confirmatory testing was performed with standard molecular genetic techniques at the Duke University Molecular Diagnostics Laboratory. Selected samples were tested for Huntington disease at Chapman Institute of Medical Genetics (Tulsa OK), and for particular cystic fibrosis mutations at EraGen Biosciences, Inc. (Madison, WI). The following mutations were included:

- Cystic fibrosis (*CFTR*): all mutations in the 2001 ACMG-recommended screening panel plus S1235R, IVS8 polyT status, and additional mutations included in the Roche CF-31 Linear Array (Roche Molecular Diagnostics) and the OLA CF Assay (Applied Biosystems). [Note: in the context of subsequent field testing, all cystic fibrosis cell lines were also tested for 394delTT, 3199del6, and all mutations included in the 87 CF panel offered by Genzyme Genetics.]
- 5′,10′-Methylenetetrahydrofolate reductase deficiency (*MTHFR*): 677C>T
- Hereditary hemochromatosis (*HFE*): C282Y, H63D, and S65C
- Fibroblast growth factor receptor 3 (*FGFR3*) gene: targeted analysis for 749C>G only (Meunke syndrome)
- Fragile X (*FMR1*): PCR and Southern blot analysis to detect both pre- and full mutations plus methylation status
- Connexin 26 (*GJB2*): targeted analysis for 35delG only
- α-Globin: deletion mutations
- Factor V: factor V Leiden (FVL; R506Q)
- Prothrombin: 20210G>A only
- Hemoglobin (Hb) B: targeted for Hb S and Hb C.

ANALYSIS OF MUTATION STABILITY IN CULTURE

The initial EBV-transformed culture was expanded to 1×10^8 cells (first passage), and the mutation(s) was confirmed by genetic analysis. The cell lines were then cultured through 4 additional 10-fold expansions, a total of ~20 PDLs, and retested (fifth-passage cultures) to confirm the stability of the mutation. The culture expansion also confirmed that each cell line was robust and suitable for producing large quantities of genetic material. Cell lines with unstable mutations or that failed to proliferate through 20 PDLs were not developed further as positive controls for this study; however, such cell lines may be interesting for future research.

REFERENCE LABORATORY TESTING

Cell pellets with at least 1.0×10^7 cells were prepared from fifth-passage cultures and shipped to reference testing laboratories on dry ice, accompanied by forms for reporting the results. Each cell line was analyzed by at least 5 reputable genetic testing facilities and by at least 2 common molecular methods. The laboratories were informed of the disease and in some cases the particular mutation or mutated region of the gene, as was necessary for some sequence-based assays and for mutations not included in standard testing. The results-reporting forms were custom designed for this project and requested information on the DNA isolation method, DNA quality, genetic testing method(s), and genetic results. Results were returned by fax to DUMC.

SIMULATED PE

After analysis by the reference laboratories, cell lines with confirmed mutations were sent to at least 5 genetic testing facilities for simulated PE. The laboratories were instructed to include the samples in regular patient runs and treat them as much as possible like routine clinical samples. For the simulated testing, pellets from the project cell lines were prepared as above and were accompanied by pellets from 2 additional cell lines that previ-

ously had been shown to be negative for all mutations carried by the project cell lines [GM03469 and GM00130; NIGMS Human Genetic Cell Repository; characterization of these cell lines is described in the accompanying Technical Brief *(11)*]. The same 2 cell lines were used for all 11 diseases; however, for each disease, separate pellets were prepared and unique project identifier numbers were assigned. New identifiers were also assigned to the project cell lines so that laboratories that had performed reference testing would not be able to identify the simulated proficiency samples, although whenever possible, laboratories selected for the simulated PE testing were different from those that performed reference laboratory testing. The mutations in the samples were blinded, and it was not revealed that some were negative. Only the disease was indicated. The pellets were shipped to laboratories that included the known mutations in their standard clinical testing. It was occasionally necessary to request special testing for less common mutations. In these cases, the testing was performed on all samples in a given disease set, not just the sample known to carry the mutation.

Results

SAMPLE SUBMISSION AND TRANSFORMATION SUCCESS

Participating clinical genetic testing laboratories recovered 113 anonymous residual clinical blood samples and shipped them to Coriell for EBV transformation. These blood samples carried mutations for 14 different diseases and had 44 distinct genotypes (Table 1). Eighty-eight of the submitted samples fit the previously established recommendations for sample age and storage conditions *(10)*, and a total of 41 cell lines were established, for a success rate of 47% (only samples that fit the guidelines were successful). The 88 conforming samples were submitted a mean (SE) of 9.2 (0.4) days after collection. In the previous analysis of 34 samples, sample age was the most significant predictor of transformation success, and no cell lines could be derived from samples collected more than 14 days earlier *(10)*. Results from the set of 113 samples confirmed the previous observations on the importance of sample age, and no samples more than 14 days old transformed successfully although the set included 8 samples that were 15–17 days old. The decrease in transformation success with increasing sample age is illustrated in Fig. 1, and the results suggest a linear relationship between these 2 variables.

Table 1. Diseases represented among the submitted residual blood samples.

Disease name (gene symbol)	No. of samples submitted	Distinct genotypes
Achondroplasia (*FGFR3*)	1	1
α-Thalassemia (*HBA1*; *HBA2*)	10	4
Cystic fibrosis (*CFTR*)	28	13
Nonsyndromic hearing loss (*GJB2*)	1	1
Muenke syndrome craniosynostosis (*FGFR3*)	2	1
Fragile X syndrome (*FMR1*)	11	10
Hereditary hemochromatosis (*HFE*)	14	5
Huntington disease (*HD*)	2	2
MTHFR deficiency (*MTHFR*)	4	2
Prothrombin thrombophilia (*F2*)	1	1
Kennedy disease (*SBMA*)	1	1
Myotonic dystrophy (*DMPK*)	1	1
Saethre–Chotzen craniosynostosis (*TWIST*)	1	1
Spinal muscular atrophy (*SMN1*)	1	1
Total (n = 14 diseases)	78	44

STABILITY OF MUTATIONS

In the initial protocol, 24 viable cell lines were expanded in culture to determine the stability of the selected mutations. Eighteen of the cell lines were derived from residual blood samples as described, and 6 were selected from the NIGMS Human Genetic Cell Repository. The NIGMS cell lines had been previously determined to carry mutations of particular significance for genetic testing purposes, and most carried multiple mutations, often in several different disease genes. Although only 1 disease per cell line was selected for validation as a positive control, several of the additional mutations in the NIGMS cell lines were tested for stability. The results are shown in Table S2 in the online Data Supplement. The cell lines derived from residual clinical samples were tested only for the disease for which they were originally submitted (required by the IRB protocol). For the purposes of this study, the cell lines obtained from NIGMS were considered first passage when received, although they may have been in culture longer than those derived from residual blood samples. In all other respects, all 24 lines were handled similarly.

Twenty-two of the 24 cell lines successfully completed five 10-fold expansions, ~20 PDLs. Two cell lines [DUK15765; α-thalassemia type 2 ($\alpha^{-3.7}/\alpha^{-3.7}$) and

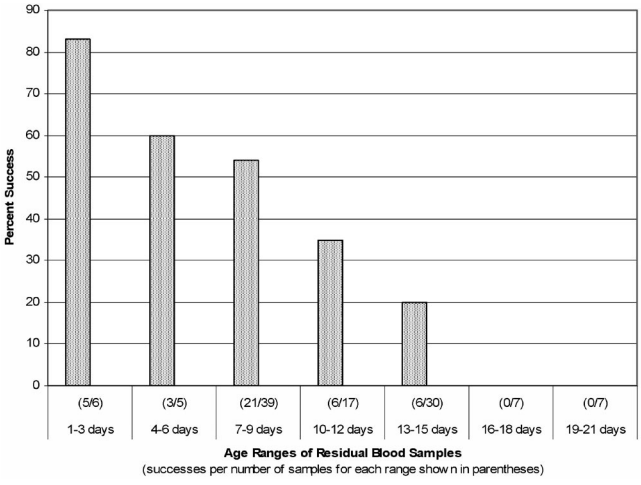

Fig. 1. Percentage of residual blood samples that transformed successfully vs age of the samples.

The number of successes and the total number of submitted samples in each 3-day age range are shown in parentheses *below* each *column*.

DUK11538; *HFE* H63D/WT), both derived from residual blood] ceased proliferation at approximately the fourth 10-fold expansion. These cell lines were not considered to be optimum candidates for positive controls, and no further testing was performed. For the remaining cell lines, the fifth-passage cultures were analyzed for the previously characterized mutations.

As shown in Table S2 in the online Data Supplement, all point mutations and all deletion mutations were found to be stable. A total of 32 individual point mutations, one 1-bp deletion, one 3-bp deletion (*CFTR* ΔF508), and one larger deletion (α-globin southeast Asian 20.5-kb deletion; $-^{SEA}$) were assessed. The *CFTR* IVS8 polyT tracts were stable in all cell lines tested.

Three cell lines contained trinucleotide repeat mutations, which can be unstable in vivo *(12)*. Two of the mutations, a 31-CAG repeat in the *HD* gene and a 56-CGG repeat fragile X (*FMR1*) mutation (male patient), appeared stable during cell culture expansion. However, a fragile X premutation in a cell line derived from a female patient (DUK35698) showed instability in methylation status and repeat number, both during the EBV transformation process and during subsequent expansion of the cell line. This cell line was not considered to be a good candidate for use as a positive control.

reference laboratory testing

Fifteen reference laboratories participated (see Table S1 in the online Data Supplement), and in almost all cases, the mutations were correctly identified. The DNA isolated by one laboratory (2 samples) was too severely degraded for testing; however, a second set of samples was shipped, and DNA was successfully isolated and analyzed. Two of 6 laboratories were unable to obtain a clear result for the fragile X cell line DUK13521, which carried a premutation of ~56 CGG repeats (PCR analysis results for 4 reference laboratories were 54–57 repeats). One laboratory was unable to obtain a strong enough signal on a Southern blot. Another laboratory reported that they obtained a uniform size of 55 repeats by 2 methods [PCR and double restriction digest (*Eco*RI and *Bss*HII) followed by Southern blot] and that a third method (single restriction digest with *Pst*I followed by Southern blot) reproducibly gave both a 55-repeat band and a full mutation band of ~400 repeats. The cause of this finding is unknown; however, 2 different laboratories subsequently tested this cell line using the *Pst*I method, and only the premutation band was detected. The apparent full-mutation band was therefore likely a laboratory artifact rather than a genetic characteristic of the sample or cell line. DUK13521 was successfully analyzed by all 5 laboratories in the simulated PE testing. The repeat size range reported for DUK13521 was within clinical testing limits established by the ACMG *(13)*. To obtain a more precise number, however, we sent a total of 5 DNA samples, including residual DNA from the laboratory that originally identified the blood sample, DNA isolated by Coriell from residual patient blood submitted for transformation, and DNA from early and late passages of the cell, to the NIST, which has developed a combination of PCR and DNA sequencing techniques specially tailored to the challenges of analyzing trinucleotide repeat mutations in *FMR1*. This method has been demonstrated by NIST to reliably give readings within ± 1 repeat for premutations in this size range *(14)*. All samples of DUK13521 were reported to contain 56 repeats. A standard reference material for fragile X repeat sizing is now available from NIST.

simulated pe testing

In a simulated PE test, a total of 263 samples were shipped to 25 clinical testing laboratories, including samples for 21 cell lines undergoing validation plus 2 negative control samples for each of 11 diseases. Replicate samples were sent to at least 5 laboratories. With few exceptions, the genotypes of the samples were correctly identified, and the small number of errors observed appeared random with respect to the individual cell lines. Only 3 discordant results were returned. Problems in sample preparation (such as degraded DNA) and failed PCR reactions were the most commonly reported problems. For 12 samples (4 different laboratories; all 12 samples were from different cell lines), no results were returned because of these problems. The simulated PE testing results indicate that all cell lines are excellent positive controls. The details are revealing from a laboratory proficiency standpoint and will be analyzed in a separate report.

revised validation protocol

On the basis of the above results, the initial protocol was abbreviated (see *Discussion*), and 12 additional cell lines were developed and validated for use as positive controls.

genetic description of clinically validated cell lines

The 33 validated cell lines include mutations associated with 11 diseases. Twenty-three different mutations are represented in 26 unique allelic variant combinations (Table 2).

Discussion

Clinically validated positive control materials are in critically short supply for MGT applications. We developed a process to generate EBV-transformed blood lymphocyte cell lines from anonymous, residual clinical blood samples with characterized mutations and to validate the cell lines for use as standard positive control materials for MGT applications. To ensure that the process would be practical and effective in meeting current clinical MGT needs, we convened 3 expert panel meetings and also sought input from members of the genetic testing community throughout process development.

In the initial process, we (*a*) collected residual clinical blood samples with important mutations, together with residual DNA from the samples if available; (*b*) EBV-

Table 2. Validated positive control mutations in EBV-transformed blood lymphocyte cell lines.

Cell line	Disease (gene)	Identified mutation(s)
DUK40878[b]	Cystic fibrosis (*CFTR*)	S1235R; 7T/7T
DUK58698[c]	Cystic fibrosis (*CFTR*)	1898 + 1G>A; 7T/7T
DUK10464[c]	Cystic fibrosis (*CFTR*)	1898 + 1G>A; 7T/9T
DUK99211[c]	Cystic fibrosis (*CFTR*)	1898 + 1G>A; 7T/7T
DUK64169[c]	Cystic fibrosis (*CFTR*)	1898 + 1G>A; 7T/7T
DUK54361[c]	Cystic fibrosis (*CFTR*)	2184delA; 7T/9T
DUK15576[c]	Cystic fibrosis (*CFTR*)	394delTT; 7T/9T
DUK82747[c]	Cystic fibrosis (*CFTR*)	I148T; 7T/9T
DUK62150[c]	Cystic fibrosis (*CFTR*)	I148T; 9T/9T
DUK65584[c]	Cystic fibrosis (*CFTR*)	1078delT; 7T/7T
DUK54732[c]	Cystic fibrosis (*CFTR*)	I148T; 7T/9T
GM07441[b]	Cystic fibrosis (*CFTR*)	3120 + 1G>A; 621 + 1G>T;[d] 7T/9T
GM13591[b]	Cystic fibrosis (*CFTR*)	ΔF508; R117H; M470V;[d,e] 5T/9T
DUK21185[b]	MTHFR deficiency (*MTHFR*)	677C>T homozygous
DUK61832[b]	MTHFR deficiency (*MTHFR*)	677C>T homozygous
DUK84629[c]	MTHFR deficiency (*MTHFR*)	677C>T homozygous
GM16028[b]	MTHFR deficiency (*MTHFR*)	677C>T
DUK53834[b]	Hereditary hemochromatosis (*HFE*)	H63D
DUK29765[b]	Hereditary hemochromatosis (*HFE*)	C282Y
DUK32053[b]	Hereditary hemochromatosis (*HFE*)	H63D homozygous
DUK87691[b]	Hereditary hemochromatosis (*HFE*)	S65C
DUK22472[b]	Hereditary hemochromatosis (*HFE*)	S65C
DUK24771[b]	Hereditary hemochromatosis (*HFE*)	C282Y
DUK34385[b]	Hereditary hemochromatosis (*HFE*)	H63D; S65C
GM14641[b]	Factor V Leiden thrombophilia (*F5*)	R506Q
GM16000[b]	Prothrombin thrombophilia (*F2*)	20210G>A homozygous
DUK69915[b]	Huntington disease (*HD*)	31/18 CAG repeats
DUK13521[b]	Fragile X syndrome (*FMR1*)	56 CGG repeats (male)
DUK60302[b]	Craniosynostosis/Muenke syndrome (*FGFR3*)	749C>G
DUK19946[b]	Nonsyndromic hearing loss/connexin 26 (*GJB2*)	35delG
DUK71006[b]	α-Thalassemia (*HBA1*; *HBA2*)	Type 1 ($--^{SEA}/\alpha\alpha$)
DUK66652[c]	α-Thalassemia (*HBA1*; *HBA2*)	Type 1 ($--^{SEA}/\alpha\alpha$)
GM16266[b]	Sickle cell/Hb C disease (*HBB*)	Hb S; Hb C

[a] Cell lines with the prefix DUK were derived from anonymous, residual clinical blood samples recovered for this study. Cell lines with the prefix GM were obtained from the NIGMS collection at Coriell Cell Repositories. Mutations are heterozygous unless otherwise indicated.

[b] Validated by initial protocol.

[c] Validated by revised protocol.

[d] Mutation not previously described (NIGMS cell lines).

[e] M470V was detected by 2 laboratories during simulated PE testing. It was not validated as a positive control in this study.

transformed the blood lymphocytes and confirmed the reported mutations with residual DNA, if available; (*c*) verified the mutation status of the transformed cell lines; (*d*) assessed mutational stability and cell line vigor through five 10-fold culture expansions (20 PDLs); (*e*) reference-tested the cell lines (frozen cell pellets) for the mutations of interest, using at least 5 different laboratories; and (*f*) performed simulated PE testing of the cell lines (again as frozen cell pellets) alongside patient samples in at least 5 clinical genetic testing laboratories.

We previously reported that residual clinical samples were good sources of viable lymphocytes for EBV transformation *(10)*. The use of residual clinical samples has several benefits. It is convenient and economical because recontacting patients and drawing additional blood is not necessary. In addition, by enlisting several clinical testing laboratories, including high-volume commercial laboratories and specialty clinics, it is possible to obtain a wide variety of mutations. In this study, a total of 113 residual samples with mutations for 14 genetic diseases were contributed by a network of 13 laboratories, which included both large commercial processing facilities and smaller genetics research centers.

A potential drawback of residual samples is the time limitation on viability of the blood lymphocytes for transformation. The current study confirmed our previous observations: no samples older than 14 days transformed successfully, and the transformation rate was directly related to the age of the sample, decreasing from 83% for samples 1–3 days old to 20% for samples 13–15 days old. The time required for initial clinical testing varies depending on the laboratory and methods, and a turnaround

time of 3 weeks is not uncommon. Even when the testing is completed in a few days, it may be more than a week before the results are interpreted by qualified personnel and positive samples are identified. In this study, we overcame this difficulty by enlisting several laboratories with rapid turnaround times and enthusiastic personnel to contribute samples. We obtained samples with a mean age of ~9 days and achieved an overall transformation success rate of 47%. Although we developed numerous cell lines from these residual samples, transformation success approaches 100% for freshly drawn specimens *(15)*. The possibility of using targeted, consented blood donations should be considered for some diseases and for specific, urgently needed mutations.

Cell lines used to provide control materials should be genetically stable with respect to the characterized mutation(s). In this study, the mutation stability in the transformed cell lines was found in general to mimic the stability in vivo. Nineteen cell lines carried mutations of types generally known to be stable in human populations, specifically, various point mutations, a 3-bp cystic fibrosis deletion mutation, and a large α-thalassemia deletion. All remained stable in the cell lines after five 10-fold expansions in culture, ~20 PDLs. Three cell lines carried trinucleotide repeat mutations, which are frequently unstable from generation to generation and can vary in length within an individual [reviewed in Ref. *(12)*]. Repeat length was maintained in 2 cell lines: one carrying an expansion mutation in *FMR1* (male patient) and the other a Huntington disease expansion mutation. Both were in the premutation size range, in which the patient is unlikely to show symptoms of the disease but a disease-causing further expansion mutation could be transmitted to offspring. Repeat length was not maintained in a third cell line, derived from a female individual with an *FMR1* expansion in the premutation range. Both the number of repeats and the methylation state of the repeat region changed during the EBV transformation process and as the transformed cells proliferated in culture. Specific determinants of whether a trinucleotide repeat mutation will be unstable in a cultured cell line are not known. We recommend that mutation status be closely monitored in cell lines used to produce positive control genetic material for trinucleotide repeat mutations.

EBV-transformed lymphoblast cell lines are generally robust and are known to be extremely long lived in culture. They typically are not truly immortal, however, and most EBV cell lines cease growth by 160 PDLs *(16, 17)*. Even with this limitation, appropriate early-passage stock management should assure a practically unlimited supply of genetic material from most EBV-transformed cell lines. In this study, we routinely obtained 10 μg of DNA per 10^6 cells, using the Gentra Puregene® DNA Purification Kit (Gentra Systems). Approximately 100 000 aliquots containing 100 μg of DNA each could theoretically be prepared if an entire culture were expanded through 20 PDLs. Twenty-two of the 24 project cell lines that we expanded in culture grew well through 20 PDLs and continued to grow vigorously beyond that point to produce sufficient cells for the reference laboratory and simulated PE testing. Two cell lines ceased proliferation at ~16 PDLs, although no reason was readily apparent, because neither cell line was derived from an elderly patient and neither carried a mutation known to affect lymphocyte growth. Although these cell lines were not clinically validated in this study, theoretically, 16 PDLs could still produce a substantial quantity of genetic material. If the cell lines carried particularly valuable mutations, they could be used to produce genetic control material, although special care should be taken with early passage culture and storage management.

All cell lines performed well in reference laboratory testing for the mutation(s) of interest. The few problems encountered were generally related to the difficulty in isolating high-quality DNA from the cell pellets, which were shipped frozen on dry ice.

Simulated PE testing of the cell lines indicated how they were likely to perform as positive samples in clinical analysis. Overall the samples performed very well, and no systematic errors or difficulties were encountered with any particular cell line.

On the basis of the results of the initial protocol, and with input from expert panelists and other members of the genetic testing community, we revised and streamlined the cell-line development and validation process. The abbreviated process likely will be sufficient for all mutations except trinucleotide repeat expansion mutations, which would require special handling as discussed above.

In the abbreviated process, the blood sample submission protocol remained unchanged, including recommendations for selection of samples likely to transform. However, we determined that submission and subsequent retesting of residual clinical DNA did not provide sufficient benefit to justify the effort. We had solicited residual DNA with the intent of verifying the presence of the reported mutation before a substantial amount of effort had been expended on cell culture. We found that the genetic results reported by the submitting laboratories were reliable and that, therefore, analysis of DNA derived from the established cell line was sufficient to confirm the presence of the mutation.

We also found that assessing stability of the mutation may not be required for point and deletion mutations that are typically stable in vivo. In this study, all mutations of this type were found to be stable in EBV-transformed cell lines cultured through ~20 PDLs. The culture expansion process requires ~10–12 weeks; thus, eliminating this assessment dramatically reduces the time and expense involved, although information on potential longevity of individual cell lines would also be forfeited.

In the initial process, frozen cell pellets from each cell line were sent to 5 reference laboratories for testing, and

subsequently to 5 more laboratories accompanied by blinded negative controls for simulated PE testing alongside patient samples. In all cases, results of the reference laboratory testing confirmed the presence of the targeted mutation(s), and the simulated PE testing showed that the reference laboratory testing provided an excellent indication of how the samples would perform in a clinical run. Because there were no substantial disagreements between results from 5 reference laboratories, testing by 2 reference laboratories with 2 different molecular methods may be adequate for validation of the mutation(s) in each cell line. If results do not agree, additional analysis could be performed.

Initially, frozen cell pellets were provided to the testing laboratories, with the rationale that isolating DNA from frozen lymphocytes would closely approximate the isolation of DNA from patient samples. However, we found that preparing and shipping frozen cell pellets was time-consuming and expensive, and many laboratories were not accustomed to handling and validating frozen samples. Isolation of high-quality DNA was the most common difficulty encountered in our analyses of the cell lines by the initial protocol. Although DNA isolation is an important element of the clinical testing process, it does not necessarily relate to molecular analysis of specific mutations. Providing the testing laboratories with high-quality, purified DNA instead of frozen cells could simplify the validation process.

The final, abbreviated protocol for collection and validation of additional cell lines can be summarized as follows: Residual, clinical blood specimens with targeted mutations were recovered by participating laboratories and submitted anonymously, along with nonidentifying patient and sample information, to a central facility for EBV transformation. After a blood lymphoblast cell line was successfully established, the presence of the reported mutation was confirmed by genetic analysis of the transformed cells. After confirmation of the mutation, DNA samples from the cell line were sent to 2 reference testing facilities for analysis with at least 2 different molecular techniques. If the results were not concordant, additional analysis was performed. Cell lines thus validated could be maintained at a cell-banking facility with the capability of producing and distributing genetic material for MGT and research purposes.

This protocol for collecting residual clinical blood samples and using them to establish cell lines holds promise as a means to alleviate the critical shortage of high-quality positive genetic control materials. An essential element in the successful implementation of this protocol as an ongoing effort would be the recruitment of clinical testing laboratories with rapid sample turnaround times to routinely recover and submit residual samples with mutations of interest. Logistic and financial support are needed to sustain the enterprise. Because of time limitations on sample age and the unpredictability of the types of mutations identified in residual patient samples, targeted collection of blood samples through specialty clinics and patient/family support organizations with direct patient contact should also be considered as an option for obtaining source material for specific mutations.

VALIDATED CELL LINES

Thirty-three cell lines with medically important mutations were validated for use as positive controls in clinical applications. These cell lines are available through the CDC Cell and DNA Repository or through the NIGMS Human Genetic Cell Repository, both of which are maintained by Coriell Cell Repositories (http://locus.umdnj.edu/ccr/). Twenty-one cell lines were validated during the initial development of the process, and the other 12 were validated with a revised, streamlined protocol. The mutations and diseases targeted for sample collection and cell-line development for this project had been identified by members of the genetic testing community as being urgently needed and/or in short supply from existing resources. Twenty-seven of the cell lines were derived from residual clinical blood samples, and 6 were selected from the NIGMS Human Genetic Cell Repository. The cell lines carry mutations associated with 11 genetic diseases, and the allelic variants of 21 of the newly derived cell lines were not, to the best of our knowledge, previously available from public sources.

CYSTIC FIBROSIS

Thirteen of the validated cell lines carry cystic fibrosis mutations. Cystic fibrosis is the most prevalent serious inherited disease of childhood, and more than 1000 mutations of the *CFTR* gene are listed in the Cystic Fibrosis Mutation Data Base *(18)*. Two cell lines (GM07441 and GM13591), obtained from the NIGMS collection, are compound heterozygotes for mutations included in the screening panel and are therefore particularly valuable as positive controls. GM13591 was also shown by 2 testing laboratories to be heterozygous for the variant M470V, which may be associated with disease in individuals with particular haplotype backgrounds *(19, 20)*. Nine cell lines derived from residual clinical samples carry 4 mutations included the 2001 ACMG panel that were not available from public sources at the time this study was initiated: 1898 + 1G>A is carried by 4 cell lines, I148T by 3, and 2184delA and 1078delT by 1 each. Another cell line carries 394delTT, and although this mutation is not included in the screening panel, it is common in certain populations *(21, 22)*, and screening may be recommended by regional health authorities. The mutation S1235R, carried by DUK40878, has been detected at a relative frequency of 7.1% in some populations *(23)*. Although it is not currently included in the screening panel, there is evidence that this mutation may be associated with disease, and clinical testing is available. All combinations of the IVS8 polyT tract variant alleles 5T, 7T, and 9T are represented among the cystic fibrosis cell lines except for 5T/5T and

5T/7T. The T-allele variants influence splicing efficiency of the *CFTR* transcript *(24)*, can influence penetrance of some disease mutations, and may be associated with disease in some haplotype backgrounds *(19, 20)*.

Note: Since completion of this study, cell lines carrying 1898 + 1G>A and 2184delA have been submitted independently to NIGMS, although they have not been validated by the process described here. In addition, ACMG has released updated recommendations for *CFTR* population carrier screening *(25)*: I148T is no longer included because it does not appear to cause disease, and 1078delT is no longer included because it has a prevalence in the general population of <0.1%. However, these considerations aside, the development of cell lines containing these mutations illustrates the potential of the method described here to meet existing clinical needs.

MTHFR DEFICIENCY

MTHFR is important in folate metabolism, and mutations in this gene may lead to increased concentrations of homocysteine and a variety of symptoms, including severe neurologic impairment *(26)*. A thermolabile variant, 677C>T, does not appear to be associated with neurologic symptoms, but may be associated with an increased risk for vascular disease *(27)*. The 677C>T mutation is common, with an estimated frequency of up to 24% depending on the population *(28)*, and clinical testing for *MTHFR* 677C>T is widespread as part of a cardiovascular risk panel. The cell line GM16028 is 1 of 4 heterozygotes that have been identified with this mutation in the NIGMS collection. Despite the high frequency of the mutation, no homozygous cell lines were previously known to be available. The 3 homozygous cell lines developed in this study will provide useful control material.

HEREDITARY HEMOCHROMATOSIS

Mutations in the *HFE* gene can lead to hereditary hemochromatosis *(29)*, and the mutation frequency may be in excess of 10% in the Caucasian population. Testing for mutations in *HFE* is common. The NIGMS collection contains numerous cell lines homozygous for the C282Y mutation and 5 cell lines with the C282Y/H63D genotype. However, only 1 NIGMS cell line is homozygous for H63D (GM13591; validated for cystic fibrosis in this study), and 2 cell lines each have a single C282Y or H63D mutation. Unlike the validated cell lines in this study, the NIGMS cell lines have not been tested for the recently described mutation S65C *(30, 31)*. The *HFE* cell lines derived from clinical samples for this project supplement the available genotypes, adding 2 C282Y and 1 H63D heterozygote. In addition, 2 cell lines heterozygous for S65C and 1 compound heterozygote, H63D/S65C, were developed. No cell lines with the S65C mutation have been previously characterized. Because of the proximity of S65C to H63D, incorrect identification of the genotype could occur with some diagnostic assays. The S65C cell lines, particularly the H63D/S65C cell line, are therefore important controls for the validation of clinical assays.

THROMBOPHILIA

Genes for coagulation factor V (*F5*) and prothrombin (*F2*) are commonly analyzed to estimate patient risk for thrombosis. In this study, GM14641 and GM16000 were validated as positive controls for FVL (R506Q; heterozygote) and the prothrombin polymorphism 20210G>A (homozygote), respectively. Both of these cell lines also contain other common mutations (not validated in this study) and therefore have added value as positive controls. GM14641 is 1 of 4 identified FVL heterozygotes in the NIGMS collection, and 1 FVL homozygote is also available. GM16000 is the only 20210G>A homozygote available through NIGMS. One 20210G>A heterozygote is also available. Although these mutations are both relatively common, few cell lines have been characterized, illustrating the importance of a focused effort to develop and validate positive controls for mutations of interest.

HUNTINGTON DISEASE

Huntington disease is a severe neurologic disorder resulting from an increased number of CAG repeats in the huntingtin (*HD*) gene *(32, 33)*. Many cell lines with *HD* alleles in the abnormal range (>36 CAG repeats) are available; however, none of those in the NIGMS collection have fewer than 44 repeats. DUK69915 is an important addition because, with 31 CAG repeats, it falls in the intermediate (27–35 repeats) range for this disorder. Individuals carrying alleles with repeats in this range do not display symptoms of Huntington disease. These alleles are unstable, however, and such individuals are at risk of transmitting disease-causing alleles to their offspring. Accurate measurement of the repeat number is therefore critical for genetic counseling purposes.

FRAGILE X SYNDROME

Fragile X syndrome is the most common inherited form of mental retardation. It is caused by expansion of the CGG-repeat region and abnormalities in the methylation pattern of the *FMR1* gene *(34, 35)*. The normal repeat number is from 6 to ~55. Repeats in the range of ~55 to 200–230 are considered premutation or intermediate range, and repeats in excess of 200–230 are in the full mutation, disease-causing, range. Although individuals with repeats in the intermediate range may be asymptomatic, they are at risk of transmitting a full-expansion mutation allele to their offspring. The NIGMS collection contains several fragile X cell lines, including some in the premutation range. None of these cell lines, however, is in the upper normal or lower intermediate range (~45–60 repeats; gray zone). Gray zone alleles may also be unstable but are highly unlikely to expand to a full mutation in a single generation. DUK13521, with 56 repeats, represents an important addition to the available controls for the detection of intermediate-range alleles.

MUENKE SYNDROME

Mutations in *FGFR* genes commonly lead to abnormal skeletal development. DUK60302 carries a point mutation (749G>C) in the *FGFR3* gene that leads to Meunke syndrome *(36)*, a nonsyndromic craniosynostosis. Although the Meunke syndrome mutation is uncommon, clinical testing for this disorder is available. DUK60302 could provide important positive control material.

CONNEXIN 26

Defects in the connexin 26 gene (*GJB2*) are thought to be responsible for ~50% of all nonsyndromic autosomal recessive deafness, and ~70% of the currently identified connexin 26 mutations are of the type carried by DUK19946 *(6, 7)*. Since the link between connexin 26 and deafness was established in the 1990s, a demand for clinical testing for mutations in the connexin 26 gene has developed. Although clinical testing is now widely available, to the best of our knowledge, no cell lines carrying mutations in this gene were previously available. DUK19946 therefore represents an important positive control.

α-THALASSEMIA

Deletions in the α-globin gene cluster are common in certain populations and cause α-thalassemia with various degrees of severity, depending on the type of deletion *(37)*. Although several different deletions have been identified in the α-globin gene cluster, only 3 α-thalassemia lymphoblast cell lines are currently described in the NIGMS collection. Two of these are type 1 deletions (both α-globin genes deleted; 1 heterozygous SEA deletion and 1 heterozygous FIL deletion), and the third is a type 2 deletion (1 gene deleted; heterozygous). DUK71006 and DUK66652 represent additional cell lines with heterozygous SEA deletions.

Hb SC

GM16266 was identified in the NIGMS collection as a cell line with unusual potential as a positive control. It carries 2 separate point mutations in the β-hemoglobin (*HBB*) gene: the Hb S mutation, which is responsible for sickle cell disease, and the Hb C mutation, which is associated with chronic hemolytic anemia. The Hb S and Hb C mutations occur in the same codon. Hb S leads to the substitution of valine for glutamic acid, whereas Hb C leads to the substitution of lysine. The 2 mutations in a compound heterozygous state cause Hb SC disease, which has characteristics of both sickle cell and Hb C disease. Because the 2 mutations occur in such close proximity to one another, they are often tested for simultaneously. This cell line provides a double-positive control for these assays.

In conclusion, EBV-transformed cell lines should continue to provide sources of high-quality, biologically relevant genetic material. For residual clinical blood samples, additional research into methods for selecting samples likely to transform could increase the success rate. Studies of trinucleotide repeat instability could lead to the development of cell lines in which these repeats are stable. The combined efforts of the genetic testing community and healthcare organizations can ensure that clinical MGT practices keep pace with advances in medical genetics.

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References

1. GeneTests: medical genetics information resource [Online Database]. Seattle, WA: University of Washington, 1993–2005, updated weekly. http://www.geneclinics.org/ (accessed February 2005).
2. Williams LO, Cole EC. General recommendations for quality assurance programs for laboratory molecular genetic tests, final monograph. Atlanta, GA: Centers for Disease Control and Prevention, Public Health Practice Program Office, Division of Laboratory Services, 1999. www.phppo.cdc.gov/dls/pdf/genetics/dyncor.pdf (accessed August 2005).
3. Williams LO, Cole EC, Lubin IM, Iglesias NI, Jordan RL, Elliott LE. Quality assurance in human molecular genetics testing: status and recommendations. Arch Pathol Lab Med 2003;127:1353–8.
4. Richards CS, Bradley LA, Amos J, Allitto B, Grody WW, Maddalena A, et al. Standards and guidelines for CFTR mutation testing [erratum in Genet Med 2002;4:471.]. Genet Med 2002;4:379–91.
5. Grody WW, Cutting GR, Klinger KW, Richards CS, Watson MS, Desnick RJ; Subcommittee on Cystic Fibrosis Screening, Accreditation of Genetic Services Committee, American College of Medical Genetics. Laboratory standards and guidelines for population-based cystic fibrosis carrier screening. Genet Med 2001;3:149–54.
6. Denoyelle F, Weil D, Maw MA, Wilcox SA, Lench NJ, Allen-Powell DR, et al. Prelingual deafness: high prevalence of a 30delG mutation in the connexin 26 gene. Hum Mol Genet 1997;6:2173–7.

7. Scott DA, Kraft ML, Carmi R, Ramesh A, Elbedour K, Yairi Y, et al. Identification of mutations in the connexin 26 gene that cause autosomal recessive nonsyndromic hearing loss. Hum Mutat 1998;11:387–94.
8. National Institute of General Medical Sciences (NIGMS) human genetic cell repository. http://locus.umdnj.edu/nigms/ (accessed August 2005).
9. Coriell Institute for Medical Research. Coriell cell repositories. http://locus.umdnj.edu/ccr/ (accessed August 2005).
10. Bernacki SH, Stankovic AK, Williams LO, Beck JC, Herndon JE, Snow-Bailey K, et al. Establishment of stably EBV-transformed cell lines from residual clinical blood samples for use in performance evaluation and quality assurance in molecular genetic testing. J Mol Diagn 2003;5:227–30.
11. Bernacki SH, Beck JC, Muralidharan K, Schaefer FV, Shrimpton AE, Richie KL, et al. Characterization of publicly available lymphoblastoid cell lines for disease-associated mutations in 11 genes. Clin Chem 2005;51:2156–9.
12. Bowater RP, Wells RD. The intrinsically unstable life of DNA triplet repeats associated with human hereditary disorders. Prog Nucleic Acid Res Mol Biol 2001;66:159–202.
13. Maddalena A, Richards CS, McGinniss MJ, Brothman A, Desnick RJ, Grier RE, et al. Technical standards and guidelines for fragile X: the first of a series of disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American College of Medical Genetics. Quality Assurance Subcommittee of the Laboratory Practice Committee. Genet Med 2001;3:200–5.
14. O'Connell CD, Atha DH, Jakupciak JP, Amos JA, Richie K. Standardization of PCR amplification for fragile X trinucleotide repeat measurements. Clin Genet 2002;61:13–20.
15. Beck JC, Beiswanger CM, John EM, Satariano E, West D. Successful transformation of cryopreserved lymphocytes: a resource for epidemiological studies. Cancer Epidemiol Biomarkers Prev 2001;10:551–4.
16. Sugimoto M, Furuichi Y, Ide T, Goto M. Incorrect use of "immortalization" for B-lymphoblastoid cell lines transformed by Epstein-Barr virus. J Virol 1999;73:9690–1.
17. Sugimoto M, Ide T, Goto M, Furuichi Y. Reconsideration of senescence, immortalization, and telomere maintenance of Epstein-Barr virus-transformed human B-lymphoblastoid cell lines. Mech Ageing Dev 1999;107:51–60.
18. Cystic Fibrosis Genetic Analysis Consortium. Cystic fibrosis mutation database. http://www.genet.sickkids.on.ca/cftr/ (accessed February 2005).
19. Lee JH, Choi JH, Namkung W, Hanrahan JW, Chang J, Song SY, et al. A haplotype-based molecular analysis of CFTR mutations associated with respiratory and pancreatic diseases. Hum Mol Genet 2003;12:2321–32.
20. Noone PG, Pue CA, Zhou Z, Friedman KJ, Wakeling EL, Ganeshananthan M, et al. Lung disease associated with the IVS8 5T allele of the CFTR gene. Am J Respir Crit Care Med 2000;162:1919–24.
21. Kere J, Estivill X, Chillon M, Morral N, Nunes V, Norio R, et al. Cystic fibrosis in a low-incidence population: two major mutations in Finland. Hum Genet 1994;93:162–6.
22. Schwartz M, Anvret M, Claustres M, Eiken HG, Eiklid K, Schaedel C, et al. 394delTT: a Nordic cystic fibrosis mutation. Hum Genet 1994;93:157–61.
23. Monaghan KG, Feldman GL, Barbarotto GM, Manji S, Desai TK, Snow K. Frequency and clinical significance of the S1235R mutation in the cystic fibrosis transmembrane conductance regulator gene: results from a collaborative study. Am J Med Genet 2000;95:361–5.
24. Niksic M, Romano M, Buratti E, Pagani F, Baralle FE. Functional analysis of *cis*-acting elements regulating the alternative splicing of human CFTR exon 9. Hum Mol Genet 1999;8:2339–49.
25. Watson MS, Cutting GR, Desnick RJ, Driscoll DA, Klinger K, Mennuti M, et al. Cystic fibrosis population carrier screening: 2004 revision of American College of Medical Genetics mutation panel. Genet Med 2004;6:387–91.
26. Rosenblatt DS, Lue-Shing H, Arzoumanian A, Low-Nang L, Matiaszuk N. Methylenetetrahydrofolate reductase (MR) deficiency: thermolability of residual MR activity, methionine synthase activity, and methylcobalamin levels in cultured fibroblasts. Biochem Med Metab Biol 1992;47:221–5.
27. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.
28. Franco RF, Araujo AG, Guerreiro JF, Elion J, Zago MA. Analysis of the 677 C→T mutation of the methylenetetrahydrofolate reductase gene in different ethnic groups. Thromb Haemost 1998;79:119–21.
29. Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, Basava A, et al. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. Nat Genet 1996;13:399–408.
30. Mura C, Raguenes O, Ferec C. HFE mutations analysis in 711 hemochromatosis probands: evidence for S65C implication in mild form of hemochromatosis. Blood 1999;93:2502–5.
31. Barton JC, Sawada-Hirai R, Rothenberg BE, Acton RT. Two novel missense mutations of the HFE gene (I105T and G93R) and identification of the S65C mutation in Alabama hemochromatosis probands. Blood Cells Mol Dis 1999;25:147–55.
32. Ambrose CM, Duyao MP, Barnes G, Bates GP, Lin CS, Srinidhi J, et al. Structure and expression of the Huntington's disease gene: evidence against simple inactivation due to an expanded CAG repeat. Somat Cell Mol Genet 1994;20:27–38.
33. Andrew SE, Goldberg YP, Kremer B, Telenius H, Theilmann J, Adam S, et al. The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease [Comment]. Nat Genet 1993;4:398–403.
34. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 1991;65:905–14.
35. Eichler EE, Richards S, Gibbs RA, Nelson DL. Fine structure of the human FMR1 gene [erratum in Hum Mol Genet 1994;3:684–5]. Hum Mol Genet 1993;2:1147–53.
36. Muenke M, Gripp KW, McDonald-McGinn DM, Gaudenz K, Whitaker LA, Bartlett SP, et al. A unique point mutation in the fibroblast growth factor receptor 3 gene (FGFR3) defines a new craniosynostosis syndrome. Am J Hum Genet 1997;60:555–64.
37. Weatherall DJ. The thalassaemias [Review]. BMJ 1997;314:1675–8.