Appendix 1

More detailed guidelines about laboratory methods, organisation and reporting are given here. These guidelines are based as much as possible on existing internationally available documents. Aspects which particularly pertain to CF have been highlighted or have been added.

Methods

There are three essential steps in mutation screening: the DNA extraction, DNA banking, and the mutation testing method.

DNA extraction A number of methods exist for the preparation of nucleic acid samples for molecular genetics analysis. Since this stage can have a significant impact on the quality of the final results, care should be exercised to ensure that a validated protocol is followed, independent of whether the extraction method was developed in-house, obtained from the literature, or purchased as a kit from a manufacturer. Protocols that have been either developed in-house or as a modification of a manufacturer's kit should be validated (see further). Protocols that follow exactly a validated published method should undergo thorough performance verification.[10,11] Written procedures of the methods used for DNA purification, including the sources of all components used, should be kept. Complete references should be included in standard operating procedure manuals. Changes in any of the procedures or source of components should be documented and approved by the laboratory, with date and initials recorded.[12]

The ideal method of DNA isolation in a molecular diagnostic laboratory must be simple, fast, safe and economical, but also precise and reliable. It must yield a high quality and quantity of high molecular weight DNA.

DNA banking Isolated material must be stored at 4°C or frozen. Excess DNA sample material should be stored at a temperature not higher than 0–5°C to ensure long-term stability. Concerning DNA stability and storage we refer to the literature.[13,14] An optimal storage procedure would involve aliquoting of the DNA solution in one primary stock solution frozen at –80°C, and multiple portions for subsequent analyses stored at 4°C and/or –20°C. This procedure avoids repeated freeze/thaw cycles and minimises the possibility of DNA contamination.

Cystic fibrosis mutation testing methods A wide range of mutation testing methods[15–24] is currently used in diagnostic laboratories.[25] The most frequently used mutation detection methods for cystic fibrosis are heteroduplex analysis, restriction enzyme analysis, reverse dot-blot, the commercial kits INNO-LiPA CF2 (Innogenetics nv, Gent, Belgium), Elucigene CF4 and CF12 (AstraZeneca Diagnostics, Abingdon, Oxfordshire, UK), and OLA Cystic Fibrosis Assay (PE Applied Biosystems, New Jersey, USA). Single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), two-dimensional DNA electrophoresis, and sequencing are mostly used as CF mutation screening methods.[26] The ideal method for mutation testing in a molecular diagnostic laboratory must be rapid and cheap, allow automisation, 100% efficiency and avoid the use of radioactivity and toxic reagents.

Whatever the methods used in a diagnostic laboratory, it is important that they are thoroughly validated (see test validation and characterisation). Written standard operating procedures, including the sources of all components, should be kept. Changes in any of the procedures or source of components should be documented and approved, with date and initials recorded.[12]

Laboratory organisation

Quality system A quality system for a molecular genetics laboratory should be directed at all fundamental aspects of its function. This means the setting-up of a quality system according to a molecular genetic translation of the criteria of good laboratory practices as developed by the OECD, the EN 45001 standard (the general criteria for the operation of testing laboratories) or ISO 17025, which are accepted in the European Union as the present standard. A quality system also includes the obligation to join external quality assessment schemes, and to make use of their results. Information about the requirements for a quality system is described in the literature.[10,27–29]

Because the methodology of molecular biological diagnostics is constantly changing, no general standard can at present be defined. We suggest the guidelines formulated by the National Committee for Clinical Laboratory Standards (NCCLS) and the European Concerted Action (BMH-CT93-1673) (Eucromic)[30] be followed. A guide to fundamentals of quality assessment of molecular amplification methods in clinical diagnostics has recently been published by M Neumaier.[31] These guidelines describe in detail reagent quality control, equipment calibration and maintenance, proficiency testing, training of technical staff, internal and external quality control.

Specimen types, specimen identification and access Collection and identification of the specimens are (often) carried out by non-laboratory personnel. Written procedures for proper collection, packing, shipment and handling of specimens are recommended by all the guidelines,[7,10,32,33] documents, and quality assessment authorities consulted.[12,34] Based on a review of these guidelines, we propose implementation of the following items.

(1) Specimen types
Since genetic analysis in cystic fibrosis is PCR-based, a minimal amount of DNA is required in the vast majority of cases. Unless extensive analysis is expected or planned, a mouthwash sample (or a buccal scrape sample in the

case of a baby) is sufficient. Otherwise a small volume of blood (2–5 ml) in EDTA will provide enough DNA for extensive analysis.

Prenatal diagnosis of cystic fibrosis is usually carried out on a chorionic villus sample taken during the first trimester of pregnancy. The samples should be checked by microscopic inspection and dissected immediately. Ideally they are independently prepared samples, which should all be tested. This minimises the potential problem of maternal cell contamination of the sample.

Amniocytes can also be used for molecular genetic analysis – either directly spun down from amniocentesis sample or after 10–14 days culture. Direct analysis should be carried out with caution, as the foetal cells are invariably contaminated with maternal cells. Tissue culture of the cells can either remove/reduce maternal complication or result in overgrowth of maternal fibroblasts. The analysis of a few polymorphic markers (CA repeats or STR) can identify the presence of contamination when compared with maternal DNA.

(2) Specimen identification
The specimen container should be clearly marked with a unique patient identifier, such as a hospital patient identification number. In most situations, the patient's name is not sufficient, although the combination of patient name and birth date is generally enough to prevent identification errors. The container should also be labelled with the date and time that the specimen was acquired.

(3) Request forms
All specimens should be accompanied by a request form which contains as much of the following information as possible

- Patient's name
- Date of birth
- Date of collection
- Gender
- Ethnicity (if applicable)
- Place of birth of patient, parents and grandparents
- Unique identifier found on the specimen container
- Specimen type (blood, amniotic fluid, etc)
- Reason for requesting the test, based on clinical information
- Relevant clinical or laboratory information, including sweat test results
- Pedigree (recommended for all cases)
- Referring physician or health professional
- Weeks of gestation (for prenatal diagnosis)
- Biling information (if applicable)

(4) Criteria for rejecting specimens[10]
It is recommended that each laboratory has written criteria for acceptance or rejection of specimens.

Rejection of specimens is strongly recommended if either the specimen or the request form lacks sufficient information for the laboratory or clinician to uniquely identify the specimen, or lacks other information necessary to determine if the specimen or test requested is appropriate for answering the clinical question. It is also strongly recommended that improperly handled or transported specimens should be rejected.

Other conditions for accepting or rejecting specimens are left to the discretion of the laboratory. Given the power of gene amplification techniques, it is difficult to set an arbitrary minimum cellular content or volume for specimen acceptance, but it is recommended that each laboratory develops its own standards. It is recommended that prenatal specimens, especially chorionic villus samples, be assessed for maternal cell contamination; this is done morphologically and by PCR-based DNA analysis.

(5) Accessing specimens
Each specimen should be assigned a unique laboratory identifier when accepted for testing. This identifier should be linked with the unique patient/family identifier and with other identifiers, such as those for individual electrophoretic gels or blots that may be used in the laboratory. The unique laboratory identifier should differentiate between specimens from different patients, between different specimens submitted from the same patient, and between specimens, from different patients of the same family.

(6) Specimen transport and storage
Each laboratory should establish criteria based on its own experience in successful extraction of analysable DNA from the various sample types. In general, frozen tissue should be transported on dry ice, fresh tissue on wet ice, and fixed or dried tissue at room temperature. Amniotic fluids should be transported at room temperature which allow the establishment of cell cultures. DNA can be extracted from whole blood stored at room temperature for a week or more.[14] Frozen tissue can be stored at −70°C indefinitely; fixed or dried samples can be stored at room temperature for variable periods depending on the laboratory's experience.

(7) Specimen retention
A primary issue regarding specimen retention involves ethical and legal considerations such as specimen ownership, confidentiality, and informed consent.

Until universal recommendations are adopted or regulations are implemented, each laboratory should establish its own policy regarding specimen retention and the use of archived specimens or stored DNA (anonymisation

when used in research etc). Such policies will need to be in compliance with institutional regulations that may exist with federal and/or state regulations as they become implemented.

Controlling false-positive nucleic acid and target amplification reactions A significant challenge facing the diagnostic amplification of nucleic acids is the occurrence of false-positive results due to contaminating nucleic acids. The ability of amplification techniques to produce large numbers of copies of a sequence from minute quantities of nucleic acid necessitates that extreme care should be taken to avoid false-positive results due to transfer of DNA between samples.

(1) Laboratory design
Ideally, three physically separate areas of the laboratory should be available for reagent preparation, specimen preparation, and amplification and product detection. The reagent preparation area, for those laboratories using only commercially available kits, is considered to be at the site of manufacture. In laboratories where enzymatic or chemical means of inactivating amplified products are used, the demands for physical separation of pre- and postamplification procedures may be somewhat reduced, but good laboratory practice should still be diligently exercised.

(a) Workflow
Specimens should be processed in an area of the laboratory that is isolated from amplification and detection areas. Ideally, the specimen preparation area should be under positive pressure to other areas of the laboratory. If the specimen preparation area cannot be maintained at a positive pressure to other areas of the laboratory, specimen preparation should be performed in a class II biological safety cabinet to prevent contamination. The pre- and postamplification laboratories should be served by separate ventilation systems. Also, the postamplification area should be under negative pressure. Traffic of personnel should be from the specimen preparation area, with a change of laboratory coats, to the pre- and postamplification areas. Laboratory coats should be dedicated to specific areas and changed when going in and out of each area. With the introduction of commercially licensed tests and new methods, some of these requirements may be reduced. However, if contamination becomes a problem, then separate areas of the laboratory should be devised to accommodate these different processing stages.

(b) Containment devices
In the event of separate laboratory space not being available to segregate pre- and postamplification activities, a class II biological safety cabinet should be used as a containment device for specimen preparation. Class I safety cabinets do not provide protection for material contained within them. Dead-air boxes with ultraviolet light attachments can provide a clean bench area for specimen preparation in a dedicated specimen preparation laboratory; UV lamps lose energy efficiency over time.

(2) Laboratory practice
Specific laboratory practice should be implemented to minimise the occurrence of false-positive results. These include the preparation of reagents and solutions, the correct use of pipettes, the use of laboratory coats and gloves and procedures for manipulation of reaction tubes. In addition, special care should be taken in the use of appropriate controls.[10,35–37]

(3) Selection and preparation of controls
A *positive control* that amplifies weakly but consistently should be selected. The use of dilute positive controls prevents the unnecessary generation of large amounts of amplified product that can result in contamination.

Applicable *reagent controls* should be included with each amplification batch run. These controls contain all necessary components of the reaction without the addition of template nucleic acid or human DNA.

Negative controls should be dispensed last so that they reflect the state of the reagents added.

Assays based on presence or absence of PCR products must include known control primers yielding a positive result to check for proper amplification and sizing of the PCR products and to ensure that a negative result is accurate. This should include a positive result with control primers detecting a spiked additive or a constitutive component.

When specimens are analysed for *sequence variation*, controls containing all alleles to be detected must be included.

Assays in which the result is based on *fragment size* must include size markers (sequencing ladders, etc) covering the range of expected results during gel electrophoresis.

Assays based on changing of electrophoretic mobility must include appropriate controls to ensure correct interpretation. New samples should be confirmed by alternative methods. Any unexpected result requires a repeat of the assay. Procedures for the analysis of possible new mutations should be available.

Test validation and characterisation For most tests performed in general clinical laboratories the process of test validation is fairly straightforward and can be done according to well established guidelines.[38] However, the unique aspects

of genetic testing make the validation of genetic tests a challenge in many instances. Validation of a clinical laboratory test should be on both analytical and clinical levels. Analytical validation involves determination of the various parameters themselves, such as accuracy, precision, analytical range, sensitivity, specificity, detection limit, interferences, and recovery.[38] Clinical validation refers to determination of the predictive value of the test, or the probability that a person with a positive test result will have or will develop the disease.[39]

Test validation should be conducted before a new test is introduced for clinical use. The test should be subjected to literature review and to analytical and laboratory/clinical correlation studies. This means characterisation of the detected mutations, establishing the performance properties of the test to ensure the test's ability to provide consistent and reliable results, establishing the clinical utility of the test, defining aspects of the procedure which should be carefully regulated to maintain test performance, and defining the limitations of the test.

Such validation is necessary to assure the safe and effective application of a genetic test for its intended use. Each laboratory should develop its own validation protocol. We recommend the recently published practical guide for the validation of genetic tests by E Prence[11] be followed and used.

Safety Training and use of safe laboratory practices are essential for the protection of all personnel. *Good Laboratory Practices*[12] and the Commission on Laboratory Accreditation of the College of American Pathologist[34] recommend a general safety file in each laboratory. This document should describe safety measures concerning infective samples, dangerous chemicals, radiation and electronic danger, and instructions for proper cleaning of the laboratory.

Reports

Reports of the results Genetic test results should be communicated to the referring physician or genetic professional and to any physician designated by the patient. Reports of test results should be issued in a standardised form, clearly intelligible to the non-specialist. In general, the laboratory should not directly report on the results to the patient: the laboratory should ensure that the clinician reporting to the patient has a full understanding of the results and the underlying clinical meaning of the result. The laboratory report should include:

- collecting date
- nature of the sample
- name of the individual
- date of birth/place of birth
- laboratory identification number of patient and sample
- date of report
- reason for testing
- the genotype and/or haplotype established for the individual
- interpretation of the data (should relate to the reason for testing prenatal diagnosis, carrier testing, the sensitivity of the test, etc)
- the signature of the laboratory director or other authorised individual and his/her name

It is recommended that along with this information, the following be included: the family number (if it has been assigned), and a pedigree with the genotype information indicated if applicable (eg linkage study).[10,33]

Nomenclature for designation of mutations A systematic common nomenclature is essential for the deposition of mutations into computerised databases and their subsequent accessibility to the research and clinical community. Databases of mutations in genes are required for efficient access by clinicians and researchers. Clinical geneticists can identify and study patients with the same mutations and perhaps provide prognostic information. Researchers can readily determine whether a specific mutation has been described.[15]

It is obvious that the most unambiguous nomenclature system is based on genomic DNA. However, length polymorphisms may create a problem in the numbering of nucleotides. Therefore a reference sequence standard needs to be established. We recommend the nomenclature system suggested by the Ad Hoc Committee on Mutation Nomenclature[40] and Antonarakis *et al.*[41]

Thanks to the electronically available database set up by L-C Tsui,[8] the nomenclature of cystic fibrosis mutations is universally accepted. Although the nomenclature for CF is simple, results of the European quality control trials (Dequeker and Cassiman[25]) demonstrated that a significant number of laboratories violated the nomenclature rules. Based on earlier published documents,[7,10,32,41–44] we describe in the following the major issues of the CF mutation nomenclature system.

(1) General recommendations
A single letter code is used for designating amino acids. Nucleotides are designated as DNA bases, not as RNA bases. Nucleotides are designated in the sense strand (eg ATG for a methionine codon). With this nomenclature system, mutation designations starting with a letter refer to an amino acid and the number refers to a codon position. In contrast, mutation designations starting with a number refer to a nucleotide position in the coding sense strand, and subsequent letters refer to DNA bases. Nucleotide changes are indicated by arrows. Superscripts and subscripts should not be used, and there should be

no spaces between the numbers and letters in a mutation designation.

(2) Missense and nonsense mutations

Missense and nonsense mutations are described in terms of the change in the gene product. A missense mutation is designated by the number of the amino acid position and the single-letter abbreviations of the amino acids involved. The abbreviation for the normal amino acid precedes the number, and the mutant amino acid follows the number, with no spaces between. For example, G551D indicates that the glycine residue at position 551 in the protein has been replaced by an aspartic acid residue. If different nucleotide substitutions lead to the same amino acid substitution, such as that occurring at S549 in the *CFTR* gene, the mutation designation should include the nucleotide change, within parenthesis, immediately following the designation for the amino acid substitution. Using the cystic fibrosis gene example, this would be S549R(1777 A/C), or S549R(1778 T/G).

Nonsense mutations are designated similarly, except that X represents a termination codon. For example, G542X indicates that the glycine residue at amino acid position 542 has been replaced by a termination codon.

(3) Insertions and deletions

Insertion or deletion mutations are designated by a nucleotide number of the sense strand, followed by ins (for insertion) or del (for deletion). The nucleotide position is the one preceding an insertion, or the first that is deleted. The exact nucleotides are specified if only one or two are involved. For example, 441delA indicates the deletion of deoxyadenylic acid at nucleotide position 441. The 'name' 241delAT indicates the deletion of deoxyadenylic acid at nucleotide position 241, and the deletion of deoxythymidylic acid from nucleotide position 242. Mutations involving both substitution and a small insertion or deletion can be designated by the first altered base, followed by the nucleotide change. For example, 2183AA–G indicates replacement of AA at nucleotide positions 2183 and 2184 in the normal sequence by G in the mutant allele.

(4) In-frame deletions

Deletions of single amino acids result from deletions of three bases and are represented by a Δ followed by the single-letter code of the amino acid and its position, eg ΔF508 is the deletion of phenylalanine (F) at position 508. An acceptable alternative is 'Delta', 'delta' or 'del'. It has been proposed to write amino acid deletions in the future as amino acid codon del e.g. F508 del.[41]

(5) Complex deletions/insertions

There are several mutations which involve the deletion or insertion of four or more bases and these are usually named as the number of bases which are deleted or inserted, eg 1461ins4 which is the insertion of four bases (in this case AGAT) after base 1461, and 1949del84 which is the deletion of 84 bases from base 1949. There are some much larger deletions which remove one or more complete exons, eg *CFTR*del2 which is the deletion of exon 2.

(6) Splicing mutations

These are (usually) the substitution of a base in the splice acceptor site (an AG dinucleotide at the 3' end of the intron) or the splice donor site (a GT dinucleotide at the 5' end of the intron), both of which are highly conserved in human genomic DNA. The position of the mutated base is numbered from the first or last base in the exon, as intronic bases are not themselves numbered. Thus the mutation 621 + 1G > T is the substitution of a guanine by a thymidine at the first base in intron 4 (the last base of exon 4 being numbered 621 and the first base of intron 4 being 621 + 1). Similarly, 621 + 2T > C is a substitution at the second base in intron 4. Numbering of the acceptor site bases is from the 5' end of the exon, eg 1717–1G > A is the substitution of the last base of intron 10 (a guanine) by an adenine. Some splicing mutations are quite distant form intron/exon boundaries, eg 3849 + 10 kbC > T.

(7) Other nomenclature rules

Nomenclature rules suggested for larger deletions and insertions, splicing mutations, mutations in the noncoding sequence and the more complex mutations are addressed by the Ad Hoc Committee on Mutation Nomenclature.[40]