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Special Report

Fundamentals of quality assessment of molecular amplification methods in clinical diagnostics

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The increasing interest in molecular biology diagnostics is a result of the tremendous gain of scientific knowledge in genetics, made possible especially since the introduction of amplification techniques. High expectations have been placed on genetic testing, and the number of laboratories now using the relevant technology is rapidly increasing—resulting in an obvious need for standardization and definition of laboratory organization. This communication is an effort towards that end. We address aspects that should be considered when structuring a new molecular diagnostic laboratory, and we discuss individual preanalytical and analytical procedures, from sampling to evaluation of assay results. In addition, different means of controlling contamination are discussed. Because the methodology is in constant change, no general standards can be defined. Accordingly, this publication is intended to serve as a recommendation for good laboratory practice and internal quality control and as a guide to troubleshooting, primarily in amplification techniques.

The following recommendations on quality assessment of molecular biology methods in clinical diagnostics refer to the preanalytical and analytical steps, particularly those of amplification techniques. In particular, the polymerase chain reaction (PCR) and methods based thereon are those primarily used for developing laboratory tests that have potential for future routine applications. We have not attempted to deal with individual applications, which are at present in constant change and evolution and for many of which the clinical significance has yet to be established.

Because of the special requirements associated with amplification techniques, the aspects of good laboratory practice dealt with in depth are those regarding preanalytical and analytical aspects of nucleic acids amplifications. Many of the recommendations are aimed at avoiding contaminations and facilitating timely recognition of contaminants, should they occur. Although every amplification assay is prone to contamination, the technical effort associated with different types of nucleic analyses varies widely. For example, genotyping patients usually does not require optimization of the amplification conditions for improved detection limits. On the other hand, for detection of minimal residual disease or for virus detection, a very low detection limit is prerequisite. Accordingly, contamination risk may be not a major problem in one application but obviously can be critical in another. Also, if different enzymes have to be used in subsequent steps of a given test, as in reverse transcriptase (RT) PCR, the handling of the additionally required material must be considered a potential contamination hazard.

The potential of contamination is especially serious if, within the scope of diagnosis, the same DNA sequence is amplified repeatedly, or if amplification products are subjected to additional rounds of amplification, as exemplified in so-called seminested or nested PCR procedures. In nested PCR, the specificity of amplification can be enhanced through use of a second set of internal primers. As in "one-step" PCR, an excessive number of amplification cycles will generate nonspecific signals. In addition, nested assays are particularly prone to contamination because the PCR products generated during the first round of amplification are usually pipetted into new reaction tubes before reamplification. Alternatively, execution of nested PCR in a single reaction vessel, where possible, is preferable to the usual nested PCR approach *[1, 2]*. In general, the gain in specificity by reamplification of the products of the first round should be weighed

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against the increased risk of contamination. At present, the use of nested PCR application cannot be generally recommended for clinical diagnostics but should be performed only by experienced laboratories.

Regardless of the amplification system being considered, the possibility of spreading the amplification products by aerosols into other reaction vessels as a source of contamination always has to be a general concern. Avoidance of contaminations requires careful planning of preanalytical and analytical steps. For the purpose of these fundamental recommendations, we presume that a molecular diagnostic laboratory using amplification methods will provide a test program with assays that differ in their detection limit requirements, e.g., HLA typing and virus detection in blood specimens. The precautions taken should always reflect the needs of the most critical assay and should aim at the highest quality with respect to the test program. The laboratory staff must be clearly aware of the consequences of inadequate performance and quality control, and appropriate training must be given within the laboratory to ensure the high skills needed for molecular analysis. Once a contamination of reagents or stock solutions has occurred, it is often very difficult and time-consuming to localize and eliminate its source; consequently, all of the reagents may have to be discarded and replaced. Therefore, the aspects of laboratory organization and work flow discussed here are intended to assist the staff in preventing contaminants and to eliminate potential sources thereof. However, because danger of contamination cannot be entirely disregarded, even in the best circumstances, appropriate internal quality control is absolutely essential.

The current recommendations aim to define good laboratory practice rules for molecular biological methods. For the reasons given above, we do not differentiate between the more error-prone methods and those that are less so. We particularly emphasize techniques that utilize PCR, the most widely used method in the emerging field of nucleic acids diagnostics. However, most of the aspects that apply to PCR are also valid for other target amplification techniques.

1. Laboratory Organization and Laboratory Equipment

The problems associated with the avoidance of contamination in PCR necessitate a decisive and strictly-adhered-to laboratory organization, including room and space planning. Ideally, a PCR laboratory should be divided into four separate work areas, each having dedicated special equipment for: (*a*) reagent storage and set-up, (*b*) sample preparation, (*c*) PCR reaction mix assembly and amplification, and (*d*) PCR product analysis. The following guidelines refer to these specific work areas.

The respective rooms must be marked as related to the specific areas; on no account may equipment such as pipettors or reagents be removed from their respective work area or exchanged between work areas. Access to the individual work areas should follow a strict sequence, i.e., proceeding in only one direction, from the reagent storage and set-up area to the PCR product analysis area (access sequence). Previous experience has proven that ignorance, thoughtlessness, or carelessness in adhering to the said sequence can lead to the spreading of severe carryover contamination. Consequently, use of different laboratory coats (e.g., of different colors) is recommended in the individual work areas to identify directly a potential source of contamination. In addition, the space-specific coats must be left behind when workers leave the respective work area.

The procedures and the access sequence must be adhered to by all staff accessing the laboratory area, including the cleaning personnel, for improper cleaning procedures can be a major source of contaminants. In principle, the laboratory is cleaned from reagent storage and set-up in the direction of the PCR product analysis area. Likewise, separate cleaning utensils should be available for each work area to prevent cross-contamination.

1.1. Reagent Storage and Set-up

1.1.1. Operations. The following operations are done in the reagent storage and set-up area: preparation of stock solutions, preparation of aliquoted solutions, and preparation of master mix solutions. Cleaning of the workplace has to be performed immediately after the termination of the work. Also, specific work areas must not be accessed if work was performed earlier in any of the other working areas, particularly in the PCR product analysis area.

1.1.2. Job description, work flow. Delivery of stock reagents and material for sample preparation is best done directly to the reagent storage and set-up area. Delivery to the PCR analysis area should be avoided (see below). Vessels containing reaction mixtures should always be centrifuged briefly before opening. The required reagents are stored exclusively in this area and processed here into the stock solutions needed.

After the stock solutions have been checked for suitability, they should be divided into aliquots for storage and further use, to reduce the danger of contamination through frequent opening of reaction vessels and pipetting.

Vessels containing reaction mixtures should always be centrifuged briefly before freezing. In general, most solutions used for PCR are stored frozen. Frequent use of freeze/thaw cycles of master stock solutions to remove aliquots for individual reactions must be avoided; instead, freeze stock solutions in small aliquots. Because the required volume for these stock solutions is determined by the number of PCR reactions usually carried out in one assay run in the laboratory, no specific volume recommendations are given here. Aliquot stocks of suitable sizes are predispensed into microreaction vessels and are subsequently frozen for storage. Plastic containers such as

Tupperware™ are ideal for keeping batches of frozen stocks.

In addition, the reagent storage and set-up working area can serve for setting up the master mix solutions, i.e., preparations containing all reaction components except the nucleic acids to be tested. The objective is to prepare aliquots of the master mix solutions into the appropriate reaction format for a given assay and to store them until required for use. The suitability and stability of reaction components, especially polymerase enzymes, are to be checked (e.g.) by pilot reactions, and the results of the evaluations have to be documented. For "hot-start" techniques (enzyme addition after a first denaturation step at high temperature), the polymerases are also omitted from the master mix .

1.1.3. Clothing. Protective clothing should preferably feature close-fitting sleevebands and should close tightly at the front. Surgical gowns are ideally suited for this purpose and are usually available, at least in a hospital laboratory. Throughout the entire work, workers must wear gloves and should change them frequently. When leaving the area, workers must leave their protective clothing behind.

An additional safeguard against contamination of specimens is the use of disposable operating caps. These are worn only rarely in routine practice, but their use should be strongly considered, particularly in this work area, where a contamination may ruin whole sets of stock reagents and master mixes.

1.1.4. Equipment. Pipetting by mouth is strongly prohibited. Pipettes and pipetting aids must be autoclavable. Electric microdispensers for aliquoting increase the pipetting precision and thus limit any aerosol formation caused by frequent up-and-down pipetting.

The working area is to be equipped with a −20 °C freezer, a 4 °C refrigerator, a precision balance, a pH meter, a chipped ice maker, and stationery. The surfaces of the workbenches should be able to withstand decontamination procedures with such chemicals as sodium hypochlorite *[3]*. Ultraviolet irradiation of work surfaces is also effective. Given the critical importance of the distance and the energy of irradiation for decontamination, we use in our laboratories pull-down UV lights (254 nm wavelength) that can be adjusted to within ~60–90 cm (~2–3 ft.) above the workbenches after the work in the work area is finished. Also, because of the small size of a few hundred basepairs and the fact that dried aerosols are less susceptible than "wet" aerosols to UV damage, PCR fragments must be irradiated for extended periods—optimally, overnight *[4–6]*. Finally, a logbook (or a similarly suitable means of documentation) for recording use of the laboratory space and equipment has to be provided.

1.1.5. Consumables. Consumables include cleansing liquid and disposable tissues, disposable gloves, disposable caps (where used), autoclavable reagent vessels of various sizes, disposable weighing paper, reagents for nucleic acid preparation, and reagents for reactions (e.g., enzymes, buffers, dNTPs).

1.2. Sample/template preparation and cDNA synthesis

1.2.1. Operations. The sample preparation work area serves as specimen storage area. In addition, the extraction of nucleic acids (RNA, DNA), their storage, and their delivery into prepared reaction vessels (see above) are performed in this area. Single-strand cDNA synthesis for RNA analyses is also done in this work area.

1.2.2. Job description, flow of work. The reagents and consumables from this working area must not be taken into the PCR pipetting area, because they might be contaminated with unamplified nucleic acids. Also, leftover master mixes, enzymes, or reagents must not be transported back to the reagent storage and set-up area. Sample preparation must not be started after previous work in the PCR product analysis area (see below).

Good pipetting technique is instrumental for untroubled amplification procedures. Because contaminations from aerosol formation may occur, unnecessary moving around the laboratory should be avoided. Aerosol contaminations from the access area to the sample preparation area can be reduced by positive pressure conditions inside the laboratory. To avoid cross-contamination between samples, reaction vessels containing reaction mixes must be closed after addition of the test nucleic acids. For potentially infectious materials, established recommendations for handling and disinfection must be observed.

Used pipette tips must be disposed of exclusively in suitable decontamination containers, e.g., containing sodium hypochlorite solution. Laboratory bench tops must always be cleaned at the end of work, and any spillage of test material must be recorded.

Appropriate UV radiation of laboratory tables (254 nm wavelength, short distance to work bench surface) is suitable for decontamination. For safety reasons, we recommend a UV radiation source that can be switched on/off either from outside the room or by a timer. A pull-down UV tubular lamp mounted over the laboratory bench can be used to assure efficient irradiation of laboratory bench surfaces after work.

Use of suitable commercial systems based on liquid extraction/precipitation, adsorption of DNA to silica surfaces, or anion-exchange chromatography permits fast and trouble-free recovery of sufficient quantities of DNA or RNA from a variety of specimen types. Alternatively, the nucleic acids can be prepared according to accepted procedures with home-made reagents (e.g., *[7–9]*). The recovery of RNA is performed in a manner similar to DNA preparation. However, important differences are implied by the instability of the analyte and the omnipresence and stability of RNases. Moreover, several DNA

extraction methods and commercial preparation kits require a RNase digestion of the DNA sample. If these reagents are used in DNA extraction and both types of nucleic acids are handled in the same area, particular care must be taken to guarantee RNase-free conditions and solutions for RNA work by using dedicated consumables and pipettes.

For various reasons, it is practical to carry out a cDNA synthesis immediately after the RNA preparation. The cDNA synthesis should be carried out in the sample preparation area to help avoid contamination. Being more stable than RNA, the storage of a first-strand cDNA is less critical. Also, performance of the first-strand synthesis in the "downstream" working area for PCR assembly entails storage of the samples, because their transport back into the sample preparation area is, by definition, prohibited. To achieve the flexibility required for this RT reaction, one or more thermoblocks should be set up in the sample preparation area.

The optimal temperatures for cDNA synthesis depend on the enzyme chosen. One-step methods are preferable; i.e., use of heat-stable polymerases with RT activity under PCR buffer conditions (example 1, below) is safer than methods that follow cDNA synthesis by requiring opening of the reaction vessels for purposes of buffer adjustment (example 2, below) or polymerase addition (example 3, below). When using enzymes that possess both RT and DNA polymerase activity, one should assess the RNA dependence of the assay separately in the examination of intron-free genes as well as possible interference from processed pseudogenes. Examples of assay strategies currently in use are:

1) Addition of a polymerase with RT activity (e.g., Retrotherm from Epicentre Technology) → cDNA synthesis → no opening of vessels → PCR

2) Addition of a polymerase with RT activity (e.g., rTth from Perkin-Elmer) → cDNA synthesis → opening of vessels → adjustment of buffer to PCR conditions (here, chelate buffer) → PCR

3) Addition of RT (e.g., AMV-RT from Pharmacia) → cDNA synthesis → opening of vessels → addition of polymerase and PCR components → PCR

The cDNA copies of the test material are kept in the sample preparation area. PCR amplification from specimens is not allowed in this area.

1.2.3. Clothing. Marked laboratory clothing as described above; frequent change of gloves.

1.2.4. Equipment. Workbench with hood and UV radiator (see *Sections 1.1.4* and *3.2.2*) and positive-displacement pipettors or regular pipettors in conjunction with aerosol-proof disposable pipette tips. The pipettors should be autoclavable. Dispensers, freezer (−20 °C and −80 °C), refrigerator (4 °C). Additional equipment includes a vortex-type mixer and a waterbath or heating block. A logbook (or similarly suited means of documentation) must be available.

For RNA work, two additional pieces of equipment are recommended:

1) A cooling microcentrifuge (e.g., from Eppendorf) or centrifugation in a cold cabinet is preferred for RNA work for the following reasons: First, RNA preparations are best done on ice because of the low stability of the analyte and, depending on the specimen material, because the precipitation of RNAs is a usual step in preparation protocols *[8]*. Second, when stored for extended periods of time, RNA is usually kept as an ethanol precipitate, also for reasons of stability, and requires centrifugation before use in RT reactions. Centrifugation in the cold minimizes the risk of degradation while handling RNA.

2) Depending on the method used and the specimen investigated, shearing of the high-molecular-mass DNA, commonly done by passing the samples through a syringe needle *[8]*, may be necessary to reduce the viscosity of the material. However, this bears a substantial risk of contaminating the work area with unamplified nucleic acids, is hazardous with respect to handling, and is also impractical for routine purposes. Instead, high-molecular-mass DNA may be degraded by using a suitable ultrasonic water bath, obviating the need to open the sample tubes.

1.2.5. Consumables. Cleansing liquids and disposable tissues, aerosol-tight pipette tips, autoclavable reagent vessels, gloves, disposable caps, reagents for preparation of samples (prepared in reagent set-up area).

1.3. PCR REACTION MIX ASSEMBLY

1.3.1. Operations. Dispensing of sample material (from sample preparation area, see *Section 1.2*) and master mix solutions (from reagent storage and set-up area, see *Section 1.1*) into the reaction mixtures as well as the amplification reactions is performed exclusively in this working area. In nested PCR assays, it is usually necessary to open the vessels after the first round of PCR. Consequently, nested PCR possesses a substantially higher contamination risk and therefore demands particularly strict attention. The setting aside and installation of this working area should be obligatory for all nested PCR applications.

1.3.2. Job description, flow of work. Amplification of DNA fragments or of first-strand cDNA fragments is to be done exclusively in this working area. There should be no access from this section to any "upstream" working areas. Working under conditions of reduced atmospheric pressure is advisable to impede any leaking of aerosol contaminants from this area.

To avoid contaminations by aerosol formation, work should be set up beforehand, and any moving around the laboratory should be kept to a minimum. Pipetting should be performed in a hood. The opening of predispensed reaction mixes must be performed with great care, partic-

ularly between nested PCR steps. A good procedure is to briefly centrifuge all liquids in the reaction tubes before opening. Very small centrifuges (e.g., Picofuge™ from Stratagene) are ideally suited for this purpose; they take up very little bench space, are easily operated with one hand, and fit into most work cabinets. Moisture barriers, such as paraffin wax or light mineral oil, provide additional protection against contamination. However, one must be aware that mineral oil itself can be the cause of persistent contaminations. Used pipettes must be disposed of, preferably in containers filled with decontamination liquids (see *Section 3.2.2*). We also recommend analyzing samples in duplicates or amplifying two different DNA sequences.

Commercially available wax pellets can be used for hot-start assays. Alternatively, a chemically denatured (and, in this state, inactive) DNA polymerase (TaqGOLD™; Perkin-Elmer) has recently been introduced. Upon prolonged exposure to high temperatures near 94 °C, the enzyme will reactivate, thereby enabling an elegant hot start of PCR. For nested PCR, increased attention must be given to possible splashes when opening vessels. A brief centrifugation before opening reaction vessels is especially important. For pipetting purposes, reaction vessels should be opened under a safety hood.

Laboratory bench surfaces must always be cleaned and decontaminated after finishing work and at the end of the workday. Again, any spilling of solution is to be recorded.

1.3.3. Clothing. Marked laboratory clothing as described above, and wearing of gloves and caps. In this area, gloves should be changed frequently. Clothes must be left behind when leaving the room.

1.3.4. Equipment. Workbench with hood and UV radiator (see *Sections 1.1.4* and *3.2.2*), autoclavable pipettors, thermocycler, waterbath, stationery, electric micropipettor, and one or two Picofuges (see above).

1.3.5. Consumables. Cleansing liquids and disposable tissues, aerosol-tight pipette tips, autoclavable reagent vessels, gloves, caps, and reagents for assay preparation (produced in pre-PCR area).

1.4. PCR product analysis

1.4.1. Operations. Operations depend on the sensitivity (detection limit) of the method for detection of PCR fragments.

1.4.2. Job description, flow of work. For detection of amplified PCR fragments, a variety of methods are available, including those listed here. The method to use depends on the analytical problem to be solved.

- Agarose gel electrophoresis
- Polyacrylamide gel electrophoresis
  - Nondenaturing
  - Denaturing gradient
- Hybridization method (radioactive, nonradioactive)
  - Solid phase
    - Dot–blot
    - Southern transfer
  - In solution
    - ELISA
- Sequencing methods (radioactive, nonradioactive)
- Other methods

The analysis of amplification products inevitably leads to a contamination of this area by PCR fragments. In contrast to the other PCR working areas, the following safety aspects have to be considered to protect the laboratory personnel in this area:

1) Common use is made of mutagenic and toxic substances, e.g., ethidium bromide, acrylamide, formaldehyde, or radioisotopes (the pertinent radiation protection recommendations are applicable, including wearing of personal dosimeters, where necessary).

2) When handling fragments previously amplified from genes with an oncogenic potential, appropriate protective measures should be considered. Laboratory staff should be informed accordingly.

1.4.3. Clothing. Normal laboratory and protective clothing.

1.4.4. Equipment. Depends on method used.

1.4.5. Consumables. Depend on method used.

2. Preanalytical Aspects

The techniques used in the amplification of genetic information require special emphasis and a clear definition of the preanalytical steps, especially when these techniques are applied to diagnosis. On the one hand, nucleic acids (especially RNA) can easily be destroyed through ubiquitous nucleases; on the other, the extremely high sensitivity of PCR necessitates the adoption of special precautions to protect the sample against contamination by unrelated nucleic acids (e.g., from laboratory personnel).

The quality of the test results is influenced to a high degree by factors that affect sampling and dispatch of samples. Aspects of sampling, sample storage before dispatch, and dispatch itself must be seen as important elements of the preanalytical stage. Minor mistakes in connection with preanalytical steps that are not standardized may have severe consequences for the test result. In the individual case it will be difficult, if not impossible, for the laboratory to reconstruct any such mistakes made by the sender.

In the following sections, sample preparation is differentiated from the preanalytical step and is defined as part of the analytical stage, because the choice of method depends on the intended application, and the sensitivity of the assay will depend on the preparation procedure.

2.1. SPECIMENS

In principle, PCR analysis can be applied to a wide variety of materials, including whole blood or bone marrow containing an anticoagulant such as EDTA or citrate, serum or plasma, dried blood (filter-paper cards), buffy coat, sputum, mouthwash, bronchial lavage, cerebrospinal fluid, urine, stool, biopsy material, cell cultures, fixed tissue, embedded tissue, tissue sections, and so forth. Because heparin inhibits PCR reactions, specific precautions have to be considered when the use of heparinized material is intended (see *Section 2.2*). Depending on the test material, pretreatment of the sample may be necessary before stabilization, e.g., liquefaction of sputum with hyaluronidase.

2.2. SAMPLING

Sampling is best done in closed, disposable sampling systems, as is customary with other clinical test material. New disposable plasticware can be considered nuclease-free and used without further pretreatment. Where non-closed sampling systems are used, e.g., for urine, secretions, stool, or bone marrow, or when particulate specimens like hair are obtained, special attention must be given to protecting the sample against contamination with (e.g.) hair, epidermal scale, or sputum from the sampler. At the very least, disposable gloves must be worn.

With respect to reusable general glassware, the following should be considered: Glassware should be autoclaved or even better, heat-sterilized, because (*a*) sterile glass equipment is not necessarily free of contaminating DNA, and (*b*) glassware is often a source of highly resistant RNA-degrading enzymes, a major source of which is the hands of the investigator. RNases can be permanently inactivated through high temperatures, e.g., by baking at 250 °C for 4 h or more *[9]*.

In blood and bone marrow specimens, clotting must be inhibited. EDTA and citrate are commonly used and are the preferred inhibitors. The citrate-containing specimen collection systems (also used for routine blood coagulation tests) will dilute the specimen by 10%. In contrast, heparin (routinely 14.3 IU/mL of whole blood) reportedly inhibits amplification in concentrations as low as 0.05 IU per reaction volume *[10]*. For heparinized specimens the following must be taken into consideration:

1) For simple PCR tests not requiring high sensitivity, dilution of the prepared nucleic acids is ordinarily sufficient to overcome the inhibition. If heparinized material has to be used and a more sensitive DNA PCR is required, nucleated cells should be isolated first and washed repeatedly in physiological buffers before further processing.

2) Where highly sensitive RT-PCR methods are required, additional measures are necessary to overcome heparin inhibition. Methods shown to fail at this are boiling, Sephadex chromatography, pH shifts with subsequent gel filtration, repeated ethanol precipitations, and treatment with protamine sulfate. Although treatment with heparinase restores the amplification *[11, 12]*, this enzymatic purification step is costly. In addition, RNA may be degraded during enzyme incubation by traces of RNase still present in the sample or by heparinase preparations contaminated with RNase.

3) As demonstrated recently, lithium chloride can separate heparin from RNA, thus reversing the inhibition. This method, which reliably restores amplification from heparinized blood samples, is easily incorporated into a routine RNA preparation procedure without additional effort *[13].*

2.3. SAMPLE FRACTIONATION

When necessary, target cells can be enriched before sample stabilization and dispatch. Enrichment is useful when specimens contain low numbers of cells (e.g., urine, ascites, secretions, excretions). The sample should be centrifuged at low speed before stabilization.

In specimens containing high numbers of nonnucleated cells, e.g., blood or red bone marrow, stabilization may be preceded by a selective lysis of erythrocytes and followed by recovery of the nucleated cells through centrifugation at low speed. Another common method of sample fractionating is Ficoll density-gradient centrifugation. When performed at the site of specimen sampling, problems may arise from poor standardization, variable recovery of target cells, and danger of specimen contamination. Problems with standardization of Ficoll gradient enrichment of nucleated cell populations may be circumvented in the future by using combined sampling/fractionating systems, e.g., the Vacutainer Tube CPT tubes™ (Becton Dickinson).

2.4. SAMPLE STABILIZATION

Stabilization of test material is essential because nucleic acids degrade rapidly and is especially important when RNA has to be analyzed. Instant inactivation of DNases and RNases is reliably achieved by chaotropic substances (especially guanidinium isothiocyanate, GITC). GITC has been increasingly used in concentrations of 4 mol/L as originally described *[8]*. Organic solvents, e.g., phenol, may be added in parallel. Extraction systems based on these additives are now commercially available, e.g., RNAzol, Trizol. However, the limited stability of reducing agents (β-mercaptoethanol or dithiothreitol) and their requirement for sample stability need to be considered. Therefore, the user must be aware that batches of ready-to-use extraction solutions have a limited shelf life because of instability of some of their individual components, e.g., β-mercaptoethanol. Moreover, the handling of organic solvents is hazardous to human health and due care must be taken during handling.

At the site of specimen collection, original or enriched material is lysed by addition to reagent tubes containing GITC. The appropriate concentration leading to an irreversible denaturation of RNases has recently been determined to be 5 mol/L. Use of GITC concentrations $<$4 mol/L leads to very rapid RNA degradation *[14]*.

After proper stabilization, the material usually need not be cooled before mailing for analysis. At temperatures cooler than room temperature, GITC will crystallize, thereafter requiring complete thawing before addition of the specimen. Depending on the abundance of the RNA target to be amplified, chelating agents, e.g., citrate (combined with low temperatures), may be appropriate to inhibit RNases, given that the enzyme activity of the RNases depends on free divalent cations *[9]*. In our experience, GITC is preferable where maximum sensitivity is required and where delays in transport to the laboratory cannot be excluded. In any case, the suitability of a stabilization system should be documented with respect to the sensitivity of the subsequent RT/amplification reactions.

For extraction of DNA from leukocytes, blood containing EDTA as anticoagulant requires no special stabilization; nonetheless, samples should be dispatched without delay to the laboratory.

2.5. SAMPLE DISPATCH

Samples stabilized appropriately may be dispatched by regular mail at ambient temperatures. This applies to EDTA-containing whole blood for DNA preparation and GITC-stabilized specimens for RNA recovery. Cooling is not necessary but depending on the application, prolonged storage at room temperature will result in a critical loss in sensitivity *[14]*. In general, samples should be dispatched in breakproof containers. RNA targets to be investigated in nonstabilized samples must be shockfrozen and then dispatched in solid CO_2. Samples that reach the laboratory in the state of thawing should be invariably rejected.

2.6. SAMPLE STORAGE

Specimens for DNA analysis should be stored in buffers of 10 mmol/L Tris, 1 mmol/L EDTA (pH 7.5–8.0), at 4 °C. Specimens for RNA analysis should be kept in buffered solution preferably at −80 °C or in liquid nitrogen. Equally suitable is storage as an ethanol precipitate at −20 °C. GITC-stabilized RNA samples may be stored for ~7 days at room temperature. In cases of longer storage, less-sensitive limits of RNA detection have been observed. Such findings must be taken into account when only a few viruses or cells are to be detected.

3. Factors Interfering with Analytical Procedures

3.1. PREPARATION OF SPECIMENS

RNA or DNA (e.g., from human cells or viruses) may be isolated from a multitude of different specimens (see *Section 2.1*). For DNA analysis, no special measures are usually necessary if sample transport to the laboratory and DNA preparation are performed without delay. When RNA has to be analyzed, degradation of the analyte may be prevented through stabilization as described above.

3.1.1. Interferences related to DNA preparation. DNA preparations of inferior quality are often characterized by incomplete removal of inhibitors, either from the sample itself (e.g., heme, its precursors, or degradation products *[7]*) or introduced during inadequate sampling (e.g., heparin *[10]*). When phenol is not completely removed, the subsequent enzymatic steps (e.g., PCR, restriction digest) may be inhibited *[15]*. In this case, the DNA should be taken up in a larger volume of buffer before phenol extraction, chloroform extraction, and repeated precipitation. Although usually not required, traces of chloroform may be removed by ether extraction *[9]*. When starting with very small amounts of sample material (e.g., Guthrie filter cards, tissue sections), losses in nucleic acid may occur during preparation. Such losses can be minimized by adding carrier tRNA (Gibco BRL) or glycogen (Boehringer Mannheim) during the precipitation steps *[9]*. Other reagents of suitable quality (molecular biology-grade) can be purchased from several commercial sources. Where used according to manufacturer's instructions, such reagents do not interfere with the subsequent enzymatic reactions.

Long-term storage of DNA should be done exclusively in buffered solutions. Tris–EDTA buffer (10 and 1 mmol/L, respectively, pH 8.0) is well suited. In water, autocatalytic processes through depurination can result in a completely degraded DNA after only a few weeks. DNA appropriately prepared may be stored in buffered solutions at 4 °C for years without any large losses in quality or quantity.

3.1.2. Interferences related to RNA preparation. The sensitive detection of intact RNA is frequently compromised by inhibitors that have not been eliminated completely, e.g., heme and heparin, as described above. RNA degradation presents a major problem. Common causes for a failure of RT-PCR assays are insufficient sample stabilization before sample dispatch and RNase contamination of reagents for preparation. For the former, the logistics of the preanalytical phase need to be checked; if there is evidence for RNA degradation, the specimen should be rejected by the laboratory, which should order a new specimen and give detailed instructions to the sender regarding the proper procedure. For the latter, use of commercial preparation kits, quality-checked by manufacturers, is recommended for routine tests.

In addition, RNA may be lost during precipitation or long-term storage of prepared RNA. A loss, especially of polyA-mRNA, may be prevented through coprecipitation with glycogen (10 g/L in diethylpyrocarbonate-treated water), which gives a clearly recognizable visible precipitate. Because RNA is unstable in water, long-term storage as an ethanol precipitate at −20 °C or −80 °C is recommended.

3.2. REVERSE TRANSCRIPTION AND AMPLIFICATION OF TARGET SEQUENCES

3.2.1. Interferences with cDNA synthesis. cDNA synthesis is the first enzymatic step in RT-PCR. The cDNA generated

as the reverse complement from the target mRNA then serves as the template for the subsequent amplifications.

cDNA synthesis is convenient when the amplification of single exons from DNA is not feasible, when no information is available regarding genomic sequences or gene organization, or when disease-associated or disease-specific transcripts are to be detected. In addition, analysis of a few cDNA fragments instead of many genomic fragments may be advantageous. For prospective studies, it may be safer to translate mRNA into cDNA to achieve better conditions for long-term storage.

The following factors commonly affect the efficiency of cDNA synthesis:

1) Reduction or complete absence of RT activity. Inadequate cDNA synthesis caused by low enzyme quality, decomposed reagents, or pipetting errors must be excluded.

2) Inhibitors of the RT or of the heat-stable polymerase (e.g., phenol, heme). These are to be suspected when no amplification can be achieved even though the RNA appears to be intact. Amplification of a sufficiently expressed housekeeping gene (e.g., β_2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase) may serve as control if the sample, and thus the inhibitor, is appropriately prediluted.

3) Degradation of the RNA (which can be checked through appropriate controls during or after preparation). A degraded mRNA may be assumed when the ribosomal RNA populations 28S, 18S, and 5S are no longer clearly defined, or are possibly completely absent. A quick check with electrophoresis in a nondenaturing, ethidium bromide-prestained agarose gel is usually sufficient. This simple test requires ~1 μg of total RNA for reasons of detectability; thus it is not suited for all methods. A more thorough assessment of whether the RNA species is intact requires agarose gel electrophoresis under denaturing conditions.

4) Contamination of RNA with genomic DNA. Primers for the amplification of cDNA fragments usually can be positioned to hybridize in different exons, which will warrant RNA-dependent amplification. Where this is the case, PCR products from contaminating non-mRNA nucleic acids, should they occur, are larger than those obtained from cDNA. Where this design is not possible, e.g., with intron-free genes or intron-free pseudogenes, the RNA preparation is treated with RNase-free DNase. For such targets, an amplification without a preceding RT step must be performed to control sufficiency of this step. This control is not recommended when one is using enzymes that possess both RT and DNA polymerase activity (e.g., rTth).

5) Residual DNase activity. If RNA samples are treated with RNase-free DNase before first-strand cDNA synthesis, care must be taken to remove the DNase activity before the reverse transcription step. Usually, 10 min at 65 °C is sufficient and will also aid in breaking up secondary structures in the RNA template that can interfere with efficient reverse transcription *[9]*. The RNA sample can be extracted with organic solvents/precipitation, but this may result in loss of material or inhibition of the subsequent enzyme step because of trace amounts of phenol in the reverse transcription reaction.

6) Insufficient priming of mRNA in the first-strand cDNA synthesis attributable to, e.g., the type of priming. Three different methods for the initiation of first-strand cDNA synthesis exist: oligo(dT) priming, random priming, and transcript-specific priming. *Oligo(dT) priming* starts the reverse complementary first-strand cDNA synthesis at the 3′-end of the mRNA. An unfavorable secondary structure of the mRNA or a long mRNA sequence can lead to the first-strand cDNA being not fully reverse-transcribed towards the 5′ end of the mRNA. Consequently, amplification is not successful in the subsequent PCR amplification, even if the mRNA has been successfully prepared from the specimen. *Random priming* starts cDNA synthesis from short primers with random sequences (hexamers or octamers). The cDNA synthesis initiates at random sites and, theoretically, will thus cover all RNA species present in the sample. The average length of the cDNA depends on the molar ratio of random primers and mRNA, with a high excess of random primers favoring short DNA fragments. If this is the case, subsequent PCR amplification may be compromised. For *target-specific mRNA priming*, the suitability of the respective 3′ primer for cDNA synthesis has to be established.

3.2.2. Factors affecting PCR. Various factors can lead to false-negative or false-positive results in PCR, e.g., inhibitors or the absence of enzyme activity (see above), inappropriate annealing temperature, suboptimal magnesium concentration, or contamination of patients' samples or reagents, each of which will be discussed separately.

Of particular importance is the selection of the proper primer pairs. Usually, commercially available primers are high in quality, especially when further purified by the vendor, e.g., by HPLC chromatography. Today, numerous 5′-end modifications can be ordered, allowing labeling of the oligonucleotides with fluorochromes, biotin, amino linkers, and others. If one wants to design a test "from scratch," however, much thought should be given to the initial primer design. Several computer programs—either part of a regular DNA analysis software package or a stand-alone program that can be ordered—can help avoid unfavorable secondary structures, predict the occurrence of primer dimer formation, allow approximations of optimal annealing conditions, and so forth. (Evaluation of the algorithms underlying primer calculation or program features is beyond the scope of this document.) Once a primer is identified, the investigator should "run" it through the genetic database to check for cross-hybridization. This can be done conveniently through the Internet by using the BLAST programs displayed in the National Center for Biotechnology Information homepage (http://www.NCBI.NIH.NLM.gov). Certainly, however,

the performance of a chosen primer pair identified through a software still requires careful evaluation with respect to specificity and amplification efficiency during the phase of test establishment.

Inhibitors of PCR are to be avoided in the preanalytical phase and have to be eliminated during sample processing within the laboratory.

The annealing temperature is dependent on the sequence, i.e., length and base composition, of the amplification primers. In assessing the optimal temperature, one may apply the Wallace rule as a rule of thumb; i.e., the melting temperature of an A–T bond is in the range of 2 °C and that of a G–C bond is in the range of 4 °C. As with the use of computer programs (see above), the optimal primer performance always has to be investigated systematically by suitable pilot experiments.

The optimal magnesium concentration has to be established by appropriate titration experiments. The optimal concentration window may be very narrow. As a rule of thumb, one should initially start by using the standard concentration recommended by the manufacturer of the enzyme. Because the free magnesium ion concentration determines the efficacy of the enzymatic nucleic acid polymerization, the nucleic acid content or the individual concentrations of other magnesium-binding polyanions in the preparation will influence the amplification result. An optimal concentration of magnesium should result in a maximum yield of PCR, and no unspecific bands should be detectable by agarose gel electrophoresis.

Uniform temperature transition is an important aspect for a successful amplification. For example, the wall thickness, the material making up the reaction vessels, and a good fit of the reaction vessels into the thermoblock well are essential.

The homogeneity of heat conduction in the reaction block is of crucial importance. The heat performance of the cycler and the uniformity of heat conduction in the heating block must be controlled regularly to avoid false-negative results.

During test evaluation or in case of uncertain interpretation, the identity of the PCR product must be certified by proper means, i.e., by restriction fragment polymorphism, hybridization, or, ideally, by DNA sequencing of the purified PCR fragment.

3.3. CONTAMINATIONS

For practical reasons, the following types of contamination are distinguished here: contamination with PCR fragments (product contamination); contamination with native, genomic DNA; reagent contamination (stock solutions or working solutions); and cross-contamination (e.g., spreading of aerosols from a positive sample into an originally negative sample).

For all amplification techniques, greatest attention is directed towards the prevention of contamination, because locating its source around the laboratory is time-consuming and tedious. Once a contamination has occurred, testing has to cease, until the source is identified. Without exception, test results must be rejected, even if only one of the accompanying contamination controls reveals contaminating PCR fragments.

3.3.1. Contamination sources in the preanalytical phase. Nucleic acids not originating from the patient may contaminate the test material during the preanalytical phase.

3.3.2. Contamination sources in the analytical phase. As a general rule, contamination of the sample may occur anywhere during the different steps of the analytical phase. Accordingly, any component of the reaction mixture and any piece of laboratory equipment coming into contact with the reaction during nucleic acid preparation and reaction set-up is suspicious as a source. Examples include: contaminated reagents (e.g., bovine serum albumin, gelatin, or mineral oil); commercially available enzyme preparations *[16]*; consumables (e.g., reaction vessels, pipet tips); and laboratory equipment (e.g., pipettors, centrifuges).

One origin of contamination is cross-contamination with unamplified DNA during the simultaneous preparation of many specimens. Most contaminations, however, will consist of the specific PCR fragments generated during previous amplifications.

A contamination of reagents, consumables, or laboratory equipment used in the first three work areas (see *Section 1*) may indicate improper laboratory procedures. In contrast, contamination of the product analysis area is inevitable when PCR products are pipetted. Great care must be taken to identify this sort of contamination. Specifically, one main source is the microdrops that cross-contaminate samples, e.g., when these are loaded onto agarose gels or onto dot–blot equipment. Whether or not such contaminations are detected and thus influence the test result depends on the detection limit of the method used to detect the PCR fragments. For example, a contamination not detected in ethidium bromide-stained agarose gels may very well become apparent if the method is changed to a sensitive hybridization protocol. Multiple analyses of the same sample, run at different positions on a gel or in different runs, will help to exclude such cross-contaminations as the cause.

3.3.3. Avoidance of contamination. As a general rule, prevention rather than removal must be given highest priority. Individual aspects have already been dealt with earlier in the context of dividing the working areas and the logistical separation of assay steps.

Several methods exist to destroy amplification products generated in previous assay runs before they can evolve into a detectable contamination. As a general safeguard, for example, amplification reactions can be performed with reaction mixes, in which dTTP is partially replaced by dUTP, thereby generating uracil-containing specific amplification fragments. A preamplification di-

gestion of reaction mixes with uracil-*N*-glycosylase will therefore destroy contaminations carried over from previous assays *[17]*. An alternative "post-PCR sterilization" is photochemical generation of DNA adducts by isopsoralen compounds in the presence of longwave UV light *[18, 19]*. These also prevent contamination, because the DNA adducts are refractory to amplification but do not interfere with post-PCR hybridization procedures.

However, the aforementioned methods are not generally recommended and should be viewed with caution, because they can generate a false feeling of safety. In particular, these measures will not prevent contaminations from foreign, nonamplified native DNA.

3.3.4. Decontamination measures. Efficient decontamination at regular intervals after termination of work is mandatory. A combination of various methods promises the best results. Decontamination measures include, but are not limited to: chemical cleaning of surfaces with 100 mL/L sodium hypochlorite *[3]*; permanent UV radiation (254 nm) of laboratory benches and other surfaces after use *[4]*; autoclaving of laboratory equipment, e.g., pipettors; and flaming of laboratory equipment, where possible.

3.4. analysis of amplification products

Various techniques can be used to evaluate amplification products, including electrophoresis, restriction digestion, blotting, hybridization, sequencing, and mass spectrometry, each of which may be subject to specific disturbances. Assessing the specificity of a PCR only on the basis of the product's fragment length determined by simple agarose gel electrophoresis is to be reserved for well-established assays.

3.4.1. Interferences in electrophoresis. In general, two different types of electrophoretic separation techniques are used in molecular biological diagnosis: submarine agarose gel electrophoresis and polyacrylamide gel electrophoresis. Both techniques can be applied under either denaturing or nondenaturing conditions. As with the electrophoretic characterization of proteins, the following factors affect the electrophoretic characterization of PCR fragments:

1) False gel concentrations, ionic strength, or pH. A careful control of reagents or the use of commercially available ready-to-use reagents checked for their quality by the manufacturer is suggested.

2) Imperfect sample preparation. High salt content of the sample (e.g.) can affect the electrophoretic mobility and the band pattern of the DNA fragments and may lead to incorrect estimation of the fragment size.

3) Overstaining DNA products or using insufficiently sensitive stain. The intercalating fluorescent stain ethidium bromide can differ in sensitivity between manufacturers or lot-to-lot. The right stain concentration is easily established through use of amplification products as controls. (*Note:* Intercalating DNA stains are strong carcinogens; therefore, contaminated buffers must be properly disposed of.)

3.4.2. Interferences related to restriction digestion. Restriction digestion may precede a PCR reaction so as to increase the specificity of the reaction (e.g., cutting a processed pseudogene in a RT-PCR) or to detect the methylation of target sequences (e.g., X-inactivation). The main application of restriction digestion, however, is either to verify the specificity of a PCR product or to detect gene mutations via defined restriction fragment lengths (restriction fragment length polymorphism, RFLP). In comparison with restriction of unamplified DNA, the following must be considered when amplification products are to be digested:

Usually, the unit definition, as given by the manufacturer, is the basis for assessing the enzyme concentration required for the restriction digestion. One unit is defined as the quantity of enzyme that completely cuts 1 μg of DNA at a defined temperature within 1 h. The abundance of a restriction site in the test DNA—assuming a uniform base distribution—statistically depends on the length of the recognition sequence of the enzyme: e.g., for a recognition sequence of 4 bases, 1 site/4^4 = 256 bases; for a recognition sequence of 6 bases, 1 site/4^6 = 4096 bases. Obviously, this does not apply to a PCR product, because the "cutting site density" in the digestion reaction is very high compared with the naturally occurring sequences, thereby rendering the mass-related enzyme concentration a limiting factor. Accordingly, the unit definition is not valid for amplification products, and the amount of enzyme necessary for digestion must be determined empirically. The manufacturers provide information on the characteristics of restriction enzymes, e.g., inhibition by glycerol or star activity, and the related quantity per volume to be used.

Further major complications are insufficient or absent activity of the restriction endonuclease, possibly caused by the presence of certain compounds in the amplification reaction mix. For example, the salt conditions required for cutting may be incompatible with the salt conditions used for amplification. If in doubt, use of organic solvent extraction and subsequent precipitation most often will solve a cutting problem.

Another cause for insufficient or absent digestion is decreased activity of these temperature-sensitive enzymes.

The assessment of a quantitative restriction digestion must be assured through appropriate internal and external controls, because a partial digestion can entail misinterpretations of the banding pattern.

3.4.3. Interferences related to nucleic acid transfer (blotting). There are essentially two reasons for the transfer and immobilization of DNA from a gel to a solid matrix (e.g., nylon membrane, nitrocellulose), namely, to increase the specificity of detection of a PCR product and to decrease

the detection limit relative to simple staining with fluorescent dyes.

Even in cases of a professional performance of blotting, the presence of membrane regions in which the binding of nucleic acids is reduced or even absent may compromise results. Avoidance of membrane artifacts is a demand addressed to manufacturers.

3.4.4. Interferences related to hybridization. Inadequate hybridization results can be caused by inappropriate gene probes, inappropriate labeling methods, inadequate labeling of probes, or inappropriate hybridization or washing methods.

The gene probes most commonly used are: DNA fragments, synthetic oligonucleotides, and in vitro transcripts (antisense RNA probes). Probe production or labeling is mostly done with commercially available enzymes that are suitable for the respective labeling strategy. The enzymes most frequently used are: Klenow polymerase; T7 DNA polymerase; thermostable enzymes, e.g., Taq polymerase; T_4 polynucleotide kinase; terminal deoxy nucleotidyltransferase (tdT); T7 RNA polymerase; and SP6 RNA polymerase.

The efficiency of the labeling of a gene probe must be determined after each labeling reaction, because the sensitivity of the hybridization and thus the possibility of false-negative findings depends, among other factors, on the specific activity of the hybridization solution.

With radioactive labeling, the activity of a fraction of the labeled preparation is measured in a β-scintillation counter. The specific activity of the gene probe (counts/minute per microgram of probe) is then adjusted according to the total incorporated counts and the amount of probe used for labeling. For example, radioactively labeling a synthetic oligonucleotide by using T_4 polynucleotide kinase with [γ^{32}P]ATP typically generates a specific activity of 1×10^9 to 5×10^9 counts/min per microgram. With nonradioactive labeling, the specific activity can be determined via a dilution series with subsequent detection in dot–blot. With both labeling methods, one must make sure that none of the measured signal is from nonincorporated label, e.g., radionuclide, fluorescence label, or a biotin-derivatized base.

To ensure reproducibility, the specific activity at time of labeling, the age of the probe at time of use, and the quantity of probe used in the hybridization preparation all must be recorded.

Hybridization conditions must always be ascertained empirically in the laboratory when a particular probe is first introduced into the test program. Accepted conditions as well as melting temperatures computed by oligonucleotide software programs can usually be used for a first approximation during the evaluation process. We recommend strict adherence to a fixed hybridization program once it has been established.

Both a temperature too low or an ionic strength too high will reduce the stringency of hybridization and may negatively affect the specificity of the detected signal. In contrast, raising the temperature, decreasing the ionic strength, or both, will increase stringency. Thus, a tight control of temperature and of reagents is the prerequisite to avoiding false-positive as well as false-negative results. Note that temperature and ionic strength should never be changed at the same time.

3.4.5. Interferences related to sequencing. DNA sequencing, the most accurate method of determining the primary base composition of a DNA fragment, is imperative when controlling the authenticity of PCR products and has the highest analytical specificity for detection of point mutations in PCR products. Sequencing should be performed directly from the amplification reaction after PCR.

Because of absent 3′ exon nuclease activity, Taq polymerase and some other heat-stable polymerases cannot correct primary incorporation errors (absent proof-reading activity). According the amplification conditions used, misincorporations of nucleotides into the growing DNA chain (Taq polymerase errors) will occur with various frequencies. A considerable fraction of the DNA molecules generated will differ from the original sequence as a result of these misincorporations. For Taq polymerase, the magnitude of these substitutions ranges from ~2 to 4 nucleotides per 10 000 synthesized bases. Other enzymes will have different error rates.

A misincorporation randomly introduces a mutation into the sequence that will subsequently be amplified during the remaining PCR cycles. Still, at the end of the amplification run, errors will be underrepresented in comparison with the authentic sequence at any position in the fragment, unless a very low target number was amplified in the first place and the misincorporation occurred within the first cycles. Because the risk for the latter will increase with the length of the DNA segment amplified, the amplification of shorter fragments should be favored. During direct sequencing of the fragments in the PCR reaction, the authentic signal will be the highest at any given position in the amplified fragment.

In contrast, when the amplification products are cloned into plasmids and the recombinant clones (harboring individual PCR amplicons) are subsequently sequenced, incorrect base sequences will result as a consequence of the misincorporations "singled out" by the cloning process. The importance of this problem is aggravated for assays that are designed for the amplification of a very low number of target nucleic acid molecules, the use of a high number of cycles, or both.

Enzymes with low error rates, so-called high-fidelity (or proofreading) polymerases, are commercially available. They should be used for amplification where the cloning of the PCR product is desirable and the probability of isolating the authentic gene segment has to be guaranteed. However, these enzymes are generally more expensive and do not possess the same processivity as the nonproofreading enzymes.

In summary, only the sequence analysis determined directly from the uncloned PCR products (direct sequencing) can determine the correct DNA sequence and is the method of choice for detecting mutations in a heterozygous state.

Poor template quality, defective sequencing reagents, or poor primer specificities are easily detectable by the ambiguous or absent sequence ladder. Where nonradioactive sequencing methods and software-supported base-calling is used, the software design or settings should be appropriate to allow the identification of samples with heterozygous genotypes, i.e., allelic polymorphisms or point mutations. Towards this end, sequencing reactions must be optimized for low background signals. Results that are not unequivocally clear must not be used for interpretation; instead, the procedure needs to be repeated. While it is a good idea for increased confidence to routinely sequence both strands of the amplified fragment, sequencing of the opposite strand is mandatory when problems with the base-calling persist.

4. Quality Controls

As mentioned above, a variety of controls should be implemented to assess the quality of single steps during analysis, e.g., for RNA integrity, its suitability for amplification, sensitivity. With increasing automation and standardization, the number of controls to be performed has to be reassessed. As in conventional clinical chemistry, internal and external quality control can be distinguished.

4.1. INTERNAL QUALITY CONTROL

A considerable number of controls must be carried out in the laboratory to monitor the complex steps during DNA or RNA analysis. Such controls are mandatory for arriving at a conclusive interpretation of findings and serve to avoid false-positive and false-negative results. The production of reagents, the preparation of test material, PCR preparation itself, and each of these steps in its own right require thorough control because of the high analytical sensitivity of the PCR; the same applies, with appropriate alteration of details, to other enzymatic steps that may precede or follow amplification reactions.

4.1.1. Controls related to preparation of the test material. For control of DNA preparation, agarose gel electrophoresis is most commonly used. The average length of the DNA is ~100 kb in routine manual preparation methods. In DNA preparation kits suitable for PCR, the average range is between 30 and 40 kb. However, clearly degraded DNA also generates strong fluorescent signals after electrophoretic separation and staining with ethidium bromide in a lower-molecular-mass range between 1 and 10 kb. Through digestion of the DNA with a methylation-insensitive restriction endonuclease, e.g., *Eco*RI, followed by electrophoretic separation, one can control for inhibitors of enzymatic activities (in the presence of inhibitors, high-molecular-mass fragments remain uncleaved). The presence of potential inhibitors is usually assessed by using photometry at 260 and 280 nm. In a good DNA preparation, the A_{260}/A_{280} ratio should be in the range of 1.75–2.0; otherwise, the contamination (e.g., with residual protein or phenol) may be too high. Photometric measurement alone does not allow conclusions to be made regarding the integrity of the DNA.

The fastest method for controlling the quality of the total RNA preparation is agarose gel electrophoresis under nondenaturing conditions, as was applied in the separation of DNA. In case of doubt, however, the RNA should be run on an agarose gel under denaturing conditions to check its integrity. Ideally, the three major ribosomal RNA species (28S, 18S, and 5S) will be detectable as relatively sharp bands. Bands smeared to lower molecular masses or absence of the bands strongly indicates decay of RNA. A densitometric measurement of ribosomal RNA bands with indexing may become an in-laboratory standard for assessing the quality of RNA preparations; assessment of peak asymmetry with peaks trailing to lower molecular masses also is a suitable indicator of RNA integrity. In addition, agarose gel electrophoresis will indicate the degree of DNA contamination in the RNA preparation. For these reasons, the photometric measurement alone does not allow one to draw conclusions as to the integrity of the RNA.

4.1.2. Controls for cDNA synthesis and amplification. Preparations for PCR amplification must be controlled carefully with reaction-internal as well as reaction-external positive control specimens to check for enzymatic activity and to exclude the presence of putative inhibitors. Negative controls are essential for monitoring contaminations. In general, the following control reactions should be distinguished: The positive-control reaction supplies information about enzymatic activity and inhibitors in the sample; the negative-control reaction detects contamination with human DNA (e.g., from the investigator) or with PCR product; and the reagents-control reaction indicates whether any of the reagents are contaminated.

4.1.2.1. cDNA synthesis. The crucial control for monitoring the performance of cDNA synthesis is the use of an internal reaction control. cDNA synthesis can proceed either from mRNA transcripts, which are present in each RNA preparation (i.e., mRNA of ubiquitously expressed, so-called housekeeping genes such as transcripts for ribosomal proteins), or from RNA, which is added to the sample as an internal standard at the time of preparation. Internal controls for synthesis are positive controls that lead to a defined product; if the amplification is not successful, the controls indicate a degradation of the RNA, faulty priming during the cDNA synthesis, or absence of enzyme activity.

The addition of a defined number of molecules of an amplification control permits determination of the lesser sensitivity of the RT-PCR. This is particularly important in PCR applications of high sensitivity. A mandatory con-

tamination control for RT-PCR assays consists of a reaction with the RT omitted. In summary, equivalent amplification characteristics of control and template must be assured, and the control must be clearly distinguishable from the template.

4.1.2.2. PCR reaction. *Internal reaction controls* are positive controls and are particularly important in cases where the presence or absence of an amplification product is diagnostically relevant, i.e., with gene deletions or Y-chromosomal sequences. The negative result must be clearly distinguishable from a technical failure of the assay. Thus, to guarantee that amplification is successful under a given set of reagents and DNAs, one can use a control target gene essential for the organism to survive. Such genes are present in at least the hemizygous state, because their absence from the genome is lethal (i.e., so-called lethality factor). A good example is the gene for the vitamin D-binding plasma protein *[20]*; its polymorphisms have been extensively examined in many ethnic groups worldwide, and no homozygous loss of gene activity has ever been identified. Therefore, a coamplification of a segment of this gene by the PCR will be in all cases successful and will demonstrate the successful amplification reaction.

External positive controls of appropriate DNA and appropriate dilutions thereof allow the quality of the reaction solution to be checked, and information regarding the detection limit and specificity of the PCR are obtained. The same master mix solutions used for the diagnostic test (i.e., patient's material) must be used in the external positive controls. Amplification controls are to be performed with each reaction if the detection limit of the procedure is suspected to be inadequate to detect the product. Controls of this type increase the contamination risk in the test series, if the nucleic acid used corresponds to a positive control sample. Vector-cloned target sequences or a genomic DNA of known copy number is suitable.

An *external negative control (contamination control)* must be performed in each PCR test; indeed, several controls should be contained in one run, one for each primer pair applied. In general, these controls are to furnish information about the point in time at which a contamination occurs in the course of a PCR-based test. A blank reaction vessel taken through the entire course of the sample preparation comes into contact with all solutions used in the preparation, but contains no amplifiable material (so-called mock preparation). If necessary, different mock preparations can be integrated at various preparation stages in the course of the nucleic acid preparation, if necessary. In this manner the individual steps at which contamination can occur can be identified. Mock controls allow assessment of the overall quality of the PCR test.

In addition, during the pipetting process, when bringing the samples into contact with the enzymatic amplification solution, water samples have to be added: These contain all reaction components, but water is used instead of the sample. Water samples must be present at least at the start and at the end of a pipetting series in which sample material is processed. The widely practiced method of detecting the absence of contamination solely by means of the water sample is not admissible, because this does not detect contamination sources in the course of sample handling; water can serve as a control only of the amplification reagents.

If the same amplification assay is to be repeated—as will be the case in most diagnostic tests—all contamination controls must be checked regularly by means of specific hybridization with use of Southern transfer or dot–blot. In PCR tests that amplify RNA targets, a contamination control should be performed with the RT step omitted. In this way contamination caused by DNA fragments from previous amplifications can be detected.

4.1.3. Controls for the evaluation of test results.

4.1.3.1. Control of restriction digestion. Digestion of genomic DNA can be reduced by inhibitors of the activity of enzymes present in the preparation, by inappropriate reaction conditions, or by low enzymatic activity. The digestibility of genomic DNA can be assessed through agarose gel electrophoresis. The following criteria serve for an assessment of a successful restriction:

1) After restriction digestion with most enzymes, a continuous, mostly smeared band is observed from the high- to low-molecular-mass range. This reflects the size heterogeneity of the restricted genomic fragments. Where digestion is not successful, a pronounced high-molecular-mass fraction will persist at the molecular range corresponding to the uncut DNA. With some enzymes, a high-molecular-mass fraction may be observed even after extended digestion incubation times. Thus, although disappearance of the high-molecular-mass fraction indicates full enzymatic activity, persistence of this fraction does not necessarily indicate insufficient digestion.

2) An elegant method to assess inhibitors in the sample is to branch off an aliquot of the digestion mix, and then add to this a known amount of high-molecular-mass molecular marker (see *Section 4.1.3.2*). For example, together with the genomic nucleic acids, the 48-kb large λ-bacteriophage (e.g., 1 μg) is digested into its predicted fragments. One can monitor the suitability of the sample DNA on the basis of the digestibility of the indicator DNA in the aliquot by agarose electrophoresis after different time intervals and extend the digestion times of the genomic DNA if necessary *[9, 21]*.

3) The human genome contains repetitive sequences, e.g., mitochondrial DNA, which may appear as distinct sharp bands (satellite bands) within the background smear of heterogeneous fragments. Satellites, therefore, do indicate successful restriction; however, not all enzymes will generate a satellite band pattern.

Restriction digestion of PCR products is often applied to identify mutations on the basis of various restriction fragment lengths. Compromised enzymatic activity in

these cases leads to false interpretation of results. Complete digestion of PCR products can be controlled only by a second invariant restriction site of the same enzyme in the PCR product to be tested. This must be strictly observed in the construction of the diagnostic fragment, to assure a correct interpretation of results.

4.1.3.2. Control of electrophoresis. Quality assessment of electrophoretic methods involves, in the first place, calibrators for length and suitable control fragments at defined concentrations; controls must be subject to the same sample preparation procedures as the diagnostic specimens. Length calibrators permit determination of the size of PCR products, and defined concentrations help control the detection limit of the visualization process used. Many manufacturers offer good calibrators for length in different molecular mass ranges, so-called basepair ladders; these often permit an exact size determination of the electrophoretically separated fragments.

Previously restricted vector DNAs also can be used as molecular mass markers, given that the digestion of vector genomes (plasmids or phages) results in defined band patterns. Because the DNA fragments are generated from the same molecule, all the fragments exist in equimolar concentrations. From the known DNA quantity added into the digestion, one can calculate the DNA quantity for each fragment as a fraction of the uncut DNA amount. Because the ethidium bromide fluorescence is proportional to the DNA quantity, and because the relative quantity of the individual bands of the calibrator is known, the concentration of PCR fragments can be judged in comparison with the fluorescence intensity of the bands of the calibrators. Many manufacturers or distributors of restriction enzymes list the precise restriction fragment lengths of vector genomes in the annexes to their manuals. Finally, basepair ladders and restricted vector calibrators can be mixed to increase the resolution of the calibration curve.

4.1.3.3. Control of blotting and of hybridization. In blot hybridizations, positive and negative controls should always be analyzed on the same membrane alongside the patient's sample reactions. This excludes misinterpretation from the use of different hybridization conditions in different reaction vessels.

4.1.3.4. Control of sequencing. Manufacturers usually supply suitable DNA amplification templates, and matching primers, as controls for their sequencing reagents. Moreover, this sequencing reaction can help control the quality of the sequencing gel electrophoresis. For analysis of mutations, the wild-type allele and samples from family members should be sequenced in parallel.

4.2. EXTERNAL QUALITY CONTROL (PROFICIENCY TESTING)

Considering the multitude of methodological variants and diagnostic approaches, it does not appear feasible to set up external quality-assessment trials for every diagnostic problem, especially if the diseases considered are rare. This problem is particularly pertinent in smaller countries. For this reason, the Central Reference Institution of the German Society of Clinical Chemistry has started to perform its first external quality-assessment trials with two major objectives: methodological proficiency testing and application-based proficiency testing.

Methodological proficiency testing is intended to control the quality of the elementary analytical steps in molecular genetic diagnosis: the DNA/RNA preparation, the performance of the PCR method with supplied "standard primers," and the agarose gel electrophoresis. Application-based proficiency testing is at this time suitable for relatively frequent diagnostic questions, e.g., the factor V Leiden mutation in thrombophilia, caused by the resistance of clotting factor V to activated protein C; hereditary hemochromatosis; α_1-antitrypsin deficiency; mutations in apolipoproteins B100, E2, E3, and E4; and mutations in LDL receptor, β-myosin heavy chain, and others. These will increasingly be included in nationwide quality-assessment trials in Germany. Obviously, the above-mentioned favorably complement the conventional routine panel of corresponding analytes/markers commonly determined in clinical chemistry laboratories. Continuous cell lines obtained from diseased individuals or stably transfected with genes coding for the respective gene products could be used as standardized template sources for proficiency testing in molecular diagnostics laboratories. For rarer genetic diseases, international trials make more effective use of resources and expertise.

In conclusion, during recent years laboratory science has faced an increasing interest in molecular diagnostics and a corresponding demand for routine genetic testing. Expectations are high for two reasons: First, much is expected from molecular testing—expectations nurtured by scientific progress in, e.g., the Human Genome Project. Second, physicians have become used to a high quality of test results from the routine clinical laboratory through their day-to-day use of more-conventional laboratory markers. Although molecular tests to support clinical diagnostics will arise and prove useful over time, the issues important for the laboratories are already defined. A rapidly increasing number of laboratories are now establishing molecular technologies to use for clinical diagnosis. This results in an obvious need for standardization of both the test systems and the laboratory procedures, and efforts should be made towards this end. Particularly for the amplification-based techniques, internal laboratory procedures have to be carefully controlled. Because both the technology and its applications are in constant change and development, we emphasize that this publication is intended to recommend, not define, good laboratory practice and internal quality control at this time and to guide troubleshooting, primarily in diagnostic amplification techniques. Communication of operating procedures and scientific discussion of them is important for such guidelines to evolve. Only then will

these procedures become general tools suitable for maintaining and strengthening confidence in molecular clinical testing.

References

1. Yap EP, McGee JO. Short PCR product yields improved by lower denaturation temperatures. Nucleic Acids Res 1991;19:1713.
2. Mazars GR, Moyret C, Jeanteur P, Theillet CG. Direct sequencing by thermal asymmetric PCR. Nucleic Acids Res 1991:19:4783.
3. Prince AM, Andrus L. PCR. How to kill unwanted DNA. BioTechniques 1992;12:358–60.
4. Fairfax MR, Metcalf MA, Cone RW. Slow inactivation of dry PCR templates by UV light. PCR Methods Appl 1991;1:142–3.
5. Sarkar G, Sommer SS. Parameters affecting susceptibility of PCR contamination to UV inactivation. BioTechniques 1990;9:590–4.
6. Ou CY, Moore JL, Schochetman G. Use of UV irradiation to reduce false positivity in polymerase chain reaction. BioTechniques 1991;10:442–6.
7. Higuchi R. Rapid and efficient extraction for PCR from cells or blood. Amplifications 1989;2:1–3.
8. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
9. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
10. Beutler E, Gelbart T, Kuhl W. Interference of heparin with the polymerase chain reaction. BioTechniques 1990;9:166.
11. Imai H, Yamada O, Morita S, Suehiro S, Kurimura T. Detection of HIV-1 RNA in heparinized plasma of HIV-1 seropositive individuals. J Virol Methods 1992;36:181–4.
12. Tsai M, Miyamoto M, Tam SY, Wang ZS, Galli SJ. Detection of mouse mast cell-associated protease mRNA: heparinase treatment greatly improves RT-PCR of tissues containing mast cell heparin. Am J Pathol 1995;146:335–43.
13. Jung R, Lübcke C, Wagener C, Neumaier M. Reversal of RT-PCR inhibition observed in heparinized clinical specimens. BioTechniques 1997;23:24–8.
14. Gillespie HH. Dissolve and capture: a strategy for analysing mRNA in blood. Nature 1994;367:390–1.
15. Greer CE, Peterson SL, Kiviat NB, Manos MM. PCR amplification from paraffin-embedded tissues: effects of fixative and fixation time. Am J Clin Pathol 1990;95:117–24.
16. Schmidt TM, Pace B, Pace NR. Detection of DNA contamination in Taq polymerase. BioTechniques 1991;11:176–7.
17. Longo MC, Berninger MS, Hartley JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. Gene 1990;93:125–8.
18. Cimino GD, Metchette KC, Tessman JW, Hearst JE, Isaacs ST. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Res 1991; 19:99–107.
19. Isaacs ST, Tessman JW, Metchette KC, Hearst JE, Cimino GD. Post-PCR sterilization: development and application to an HIV-1 diagnostic assay. Nucleic Acids Res 1991;19:109–16.
20. Braun A, Kofler A, Morawietz S, Cleve H. Sequence and organization of the human vitamin D-binding protein gene. Biochim Biophys Acta 1993;1216:385–94.
21. Keith DH, Singer-Sam J, Riggs AD. Active X chromosome DNA is unmethylated at eight CCGG sites clustered in a guanine-plus-cytosine-rich island at the 5′ end of the gene for phosphoglycerate kinase. Mol Cell Biol 1986;6:4122–5.