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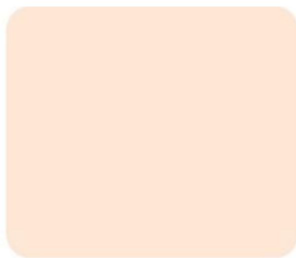
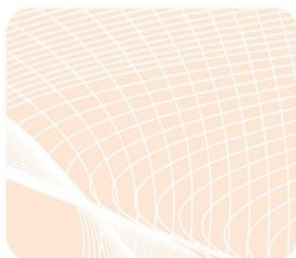
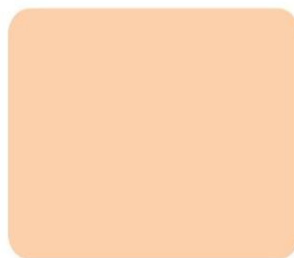
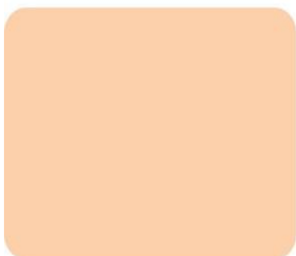
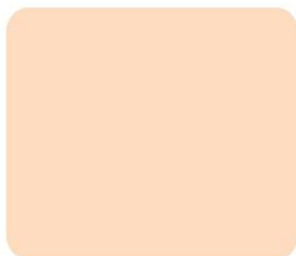
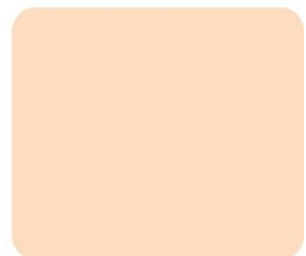
UK Standards for Microbiology Investigations

Good practice when performing molecular amplification assays





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Contents

Acknowledgments 2

Amendment table 4

1 General information 6

2 Scientific information 6

3 Scope of document 6

4 Introduction 6

5 General considerations 7

6 Specimen processing 8

7 Handling of master mixes 11

8 Selection of assay controls 11

9 Other considerations to avoid contamination 12

10 Quality assurance 13

11 Public health responsibilities of diagnostic laboratories 14

Algorithm: Diagram showing workflow in a PCR laboratory 15

References 16

Amendment table

Each UK SMI document has an individual record of amendments. The amendments are listed on this page. The amendment history is available from standards@ukhsa.gov.uk.

Any alterations to this document should be controlled in accordance with the local document control process.

Amendment number/date	11/20.03.25
Issue number discarded	5
Insert issue number	5.1
Section(s) involved	**Amendment**
Whole document.	**This is an administrative point change.** **The content of this UK SMI document has not changed.** **The last scientific and clinical review was conducted on 19/02/2018.** Hyperlinks throughout document updated to Royal College of Pathologists website. Public Health England replaced with UK Health Security Agency throughout the document, including the updated Royal Coat of Arms Partner organisation logos updated. Broken links to devolved administrations replaced. References to NICE accreditation removed. Scope and Purpose replaced with General and Scientific information to align with current UK SMI template.

Amendment number/date	10/19.02.18
Issue number discarded	4.4
Insert issue number	5
Anticipated next review date*	19.02.21
Section(s) involved	**Amendment**
Whole document.	Hyperlinks updated to gov.uk.

	Title of the document has been slightly amended. Reorganisation of some text. Professional body logos have been reviewed and updated. Flowchart in the Appendix updated.
References.	References updated.

∗Reviews can be extended up to five years subject to resources available.

1 General information

View general information related to UK SMIs.

2 Scientific information

View scientific information related to UK SMIs.

3 Scope of document

This UK SMI describes key elements of how to organise facilities for molecular amplification assays. This includes designated rooms, workflow plan, reagents, consumables and staff within a molecular diagnostic laboratory[1].

This UK SMI should be used in conjunction with other UK SMIs.

4 Introduction

The ability of polymerase chain reaction (PCR) to produce large numbers of copies of a target sequence from minute quantities - sometimes single copies of DNA has provided the exquisite sensitivity that makes PCR a powerful diagnostic tool. However, this ability also necessitates that extreme care needs to be taken to avoid the generation of false positive results.

False positive results can occur from sample-to-sample contamination and from the carry-over of amplicons from a previous amplification of the same target. Another significant risk is through cross-contamination of different reactions prepared at the same time and contamination of master stocks (for example oligonucleotide stocks) by DNA templates which is a major risk to be considered[2]. In addition to cloned DNA and virus cell cultures, microorganisms within the environment are a significant source of contamination. This can be limited by meticulous attention to good laboratory procedures[3].

Thought should be given to facility design and operation within clinical laboratories in which polymerase chain reactions are performed. Laboratories should be designed to prevent exposure of pre-PCR reagents and materials to PCR products/contaminants. Consideration needs to be given to other activities that could cause contamination such as shared specimens between laboratories as well as handling of “cultured” samples in molecular laboratory workspace. Cultured isolates present a contamination risk that could compromise primary molecular diagnostic assays from primary samples. This document describes procedures that will help to minimise the carry-over of amplified nucleic acid.

Whilst the guidance concerns the majority of PCR applications, they are most relevant where ‘in-house’ assays are in use and applicable to many modifications of the basic PCR protocol for example nested PCR, although specific details are not provided. The guidance may be less relevant when using commercial kits or performing other amplification procedures. However, the greatest threat of contamination lies in

laboratories that practise techniques that involve manipulation of amplified product or cloned DNA such as plasmids containing DNA target regions. Laboratories exclusively performing real-time PCR (qPCR) and discarding all amplified product without opening the tubes or sealed plates containing product are less liable to contamination. Further reassurance can be provided in many commercial or in-house systems by the enzymatic anti-contamination features described in section 5. Even in laboratories that avoid manipulation of PCR product, the good practice described in this document should be standard practice, especially in the clean room. Similar guidelines are available from other organisations[4].

Next-Generation Sequencing (NGS) technology involve one or more PCR amplification steps, so the practices described also apply to NGS[5]. Additional contamination risks are present in NGS, particularly surrounding the use of adaptor/index molecules throughout an NGS workflow. This potential contamination can be due to a failure to remove free adapters or primers from prepared libraries which may result in index hopping (also known as index switching) and misassignment
of libraries from the expected index to a different index in the pool, leading to misalignment and inaccurate sequencing results. However, to reduce the effects of index hopping, it is essential that users adhere to sequencing system manufacturers' instructions on best practices, as well as the library preparation workflow[6].

5 General considerations

5.1 Organisation of work[7]

For reverse transcription (RT)-PCRs, specific local precautions are necessary to prevent contamination of equipment, consumables and reagents with RNases, as these will lead to false negative results.

A common cause of contamination is poor technique by staff. Staff must be trained and signed as competent on local policies before performing these procedures[8]. All new members of staff, visitors and students must be trained in the appropriate use of the PCR facilities. It is recommended that a formal induction process must be established for these laboratory workers, regardless of prior experience.

Good housekeeping policy must be practised at all times. Tubes or reagents should not be kept any longer than necessary. All reagents, reaction tubes etc. should be clearly labelled. Records of batch numbers of all reagent batches used in individual assays should be kept.

Entry into pre-amplification rooms, in particular those used to prepare/aliquot PCR stock reagents (Pre-PCR room), after working in rooms where products, cloned materials and virus cultures are handled should be avoided. If working with these materials is unavoidable, judicious use of clean laboratory coats, gloves and hand washing is necessary. Gloves should be changed frequently for example in between the processing of individual patient samples or when moving from one room to another such as from extraction room to the Pre-PCR room[7].

It is important that the area where specimens are received into the testing facility remains PCR 'clean', with no cloned or PCR-amplified material being handled. If such material is received by the testing laboratory, a separate, dedicated area for processing should be available, with its own equipment, lab coats, etc[7].

Ensure that all equipment, including documentation, pens and lab coats are dedicated for use only in that particular laboratory for example a designated laboratory coat for each of the PCR rooms/section. Any documentation that have been in contaminated areas should not be taken into clean PCR areas[7].

Laboratory equipment such as pipettes should be clearly identified to show the section that they belong to (for example may be colour coded) and never taken from one area to another to avoid cross-contamination and confusion among staff. In cases in which the equipment is potentially contaminated, thorough cleansing or even replacing the equipment may be necessary.

Single-use PCR reagents should be aliquoted to avoid excessive freeze-thawing and to protect stock reagents from contamination as soon as they are received into the laboratory. Where contamination is detected, all diagnostic work should be suspended or relocated until the source of contamination has been eliminated. In many situations, discarding all suspected reagents may be necessary to address the immediate source of potential contamination but a full investigation should be considered to determine why the contamination occurred. Pulse centrifuge tubes briefly before opening the reagents. Uncap and close tubes carefully after use to prevent aerosols.

Bench work areas in PCR laboratories should be wiped daily with disinfectant solution (for example hypochlorite) before and after use[9]. Validated chemical or a suitable alternative may be preferred. Containment areas can additionally be decontaminated using ultra-violet radiation if fitted. It is of vital importance that weekly monitoring of UV bulb strength is performed to ensure sufficient decontamination effect[1,8].

6 Specimen processing

All accreditation schemes stipulate some requirements for the storage and retention of specimens and records[10].

Avoid molecular contamination problems of PCR through good housekeeping practice and following the unidirectional workflow[11].

6.1 The unidirectional workflow[12,13]

According to WHO Good Laboratory Practice principles, it emphasises that facilities and equipment must be sufficient, adequate and spacious enough to avoid problems such as overcrowding, cross contamination or confusion between projects[14,15].

A laboratory workflow plan consists of the sequential processes: pre-examination, examination and post-examination and their respective sequential sub-processes. Laboratories follow these processes to deliver the laboratory’s services. Where possible PCR facilities should be organised into four discrete areas/rooms as described below. Requirements may vary with the assay format and platform. For example, for real-time PCRs only 3 areas may be required as post-PCR analysis is not required. However, for nested PCR assays, the additional steps require that four rooms/areas are available.

Workflow between these rooms/areas must be unidirectional, that is, from clean areas to contaminated areas, but not from contaminated areas to clean labs. See appendix. Dedicated laboratory coats should be supplied for each area and gloves should be

changed between areas. Staff will have to leave product analysis areas and go back to the earlier rooms eventually. It is here that rigid adherence to good practice is most essential. Laboratory coats, gloves and any other personal protective equipment should be changed, and hands washed. No working materials can be brought back to earlier stages, not even notebooks or pens, ideally all qPCR machines will be remotely connected to the laboratory reporting systems. If this is not possible it may be necessary to take memory sticks and worksheets back to areas for result reporting. However, this should not be into clean areas.

6.2 Separation of pre-PCR and post-PCR assay stages

To prevent carry-over of amplified DNA sequences, PCR reactions should be set up in a separate room or containment area ('PCR workstation' cabinet) from that used for post-PCR manipulations[16]. However, it should be noted that there are newer molecular working set ups where total enclosed automated platform systems are utilised in a unidirectional way from specimen processing, extraction to PCR set up as they are becoming commonplace in molecular UK laboratories[17].

A complete separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for the specific use of pre - or post-PCR manipulations according to the area designation. Care must be taken to ensure that amplified DNA, virus cultures or DNA clones other than low copy number control material do not enter the 'Pre-PCR area'.

Reagents and supplies should be taken directly from (clean) storage into the pre-PCR area and should never be taken or shared with areas in which post-PCR analyses are being performed. Similarly, equipment such as pipettes should never be taken into the containment area after use with amplified material.

6.3 Reagent preparation and PCR setup clean room

This room is also known as the "Pre-PCR room". In this room/area, PCR reagents are stored. It is important to keep this room/area free of any biological material (this includes DNA/RNA extracts, samples, cloned materials and PCR products).

Procedures carried out in this area include preparation and aliquoting of reagent stocks and preparation of reaction mixes prior to the addition of the clinical nucleic acid (which takes place in the nucleic acid extraction room or, in the second round of a nested PCR, in the PCR amplification room). Aliquoting of primers and other reagents is recommended to minimise any consequence of contamination and reduce assay downtime. It may be useful to clean the workspace/surface when changing between different primers and other reagents.

It should be noted that materials from other rooms (including amplified, template, and target nucleic acid or positive controls, reagents, supplies, or equipment) should not be brought into the reagent preparation room.

There should be retrievable documentation records for the preparation of PCR reagents stock. The records should include the following:

- manufacturer's name and date of despatch
- number of containers, type, and amount of reagent contents received

- batch number(s)
- number of aliquots prepared and date prepared
- reagent expiry date

The prepared aliquots should be clearly labelled with the reagent name with concentration if appropriate and date prepared[18]. The other consumables used in the preparation of the reagents such as sterile water bottles should also be labelled with the date opened.

6.4 The nucleic acid extraction room

Ideally, laboratories should have separate rooms for DNA extraction of the clinical specimens and addition of DNA extracts to molecular mixes, however due to lack of laboratory space, most laboratories will perform both stages in the same room but at separate areas of the room.

Extraction of nucleic acid from clinical samples must be performed in areas where PCR products and stocks of cloned materials have not been handled. A second clean area is therefore required for this purpose. The second area is where the samples are processed, the reverse transcriptase step of RT-PCRs is performed and where the extracted DNA or cloned DNA and positive control is added to the PCR reaction mixes (previously prepared in the reagent preparation room).

Specimens for PCR should come directly from the specimen reception into the extraction laboratory; the samples should never enter rooms where PCR products and cloned DNA are present.

6.5 The amplification room

The amplification room is the area in which the PCR machines are housed. It may also contain a containment area in which, for nested PCRs, the second round reaction mixes are inoculated with the primary reaction product. Cloned DNAs should not be brought into this area.

Ideally, where PCR machines are shared, a clear booking system is recommended to provide a cohesive system for the assays as there could be variations in workload at times. Individual users' PCR programs in the thermocyclers should not be edited by other users (even temporarily) without notification to the program owner.

6.6 The product analysis rooms

This is known as the post-PCR room. This is the room in which post-PCR manipulations are performed (for example agarose gel electrophoresis of products, PCR-ELISA detection systems, sequencing, nested PCR testing). This is a contaminated area and therefore no reagents, equipment, laboratory coats etc. from this room should be used in any of the other PCR areas.

7 Handling of master mixes

Master mixes should be aliquoted in appropriate volumes for the usage requirement of an assay and to minimise the number of freeze-thaw cycles.

Master mixes should be subjected to minimal thawing and ideally should be thermostable (handled as per manufacturer instructions). If this is not the case, then all master mix reagents should be handled using a cold block rather than using ice, as that is a potential source of contamination.

Master mixes containing either fluorophores, as in probe-based assays, or DNA-binding dyes, such as SYBR Green, should not be exposed to excessive light in order to prevent degradation by photo-bleaching[19].

Different batches of master mixes should never be mixed and used together for setting up molecular assays. This is so as to allow traceability of any problems that may arise with any molecular assay at any time.

Note: Laboratories looking to use in-house molecular assays should ensure that during validation of these assays, stability studies associated with the aliquoting of master mixes must be included.

8 Selection of assay controls

Assay controls are included according to the individual assay protocol that will be specific to the pathogen targeted. However, as general guidelines, the following are suggested.

A positive amplification control: this should normally be an extract that amplifies weakly but consistently within an acceptable defined range. A decline in assay performance may not be detected when using a high copy-number positive as this may still give a signal. Use of a strong positive is an unnecessary risk as it can be a possible reservoir of contamination. Positive control should be a whole process control.

A positive amplification control derived from a plasmid should always be diluted to give a detection endpoint expected from a weak positive.

A negative or “no template” amplification control, for example nuclease free water, should always be included to control the reagent master mix during the whole process.

Extraction run controls: known positive and negative specimens for an assay may be extracted and tested to act, respectively, as a process control for successful nucleic acid extraction and a check on contamination during extraction respectively. Positive controls can be contrived specimens containing clinical relevant amounts of target (quantified organism or nucleic acid) specific to the disease and specimen tested as well as commutable.

Internal amplification controls: these should be used to control for inhibitors of the PCR process and for failures of extraction or the PCR process including potential technical errors, for example failure to add the extracted sample to the PCR reaction. Internal amplification controls should ideally be added to the primary extraction reagent at the same time as the test sample, so that they are used to control the whole process of sample extraction and PCR amplification. In this scenario, the internal amplification

control could be used in place of a positive control provided the concentration of internal amplification control is of similar concentration to the positive control. Demonstration of the internal amplification control sequence by PCR in a multiplexed reaction with the target sequence can therefore confirm potential to amplify the target sequence and validate a negative result. They should ideally represent the target organism as closely as possible, providing a quantification cycle (Cq) value that would be typically encountered in a positive clinical sample. For RNA targets detected through reverse-transcriptase PCR, an RNA control should be used to control for this step. A range of approaches have been used including addition of bacteriophages such as MS2 or lambda phage or addition of DNA or RNA transcripts. Where human DNA or RNA is co-purified with the target organism, detection of human gene targets especially 'house-keeping genes' such as β-globin have been used as internal amplification controls. This has an added advantage of controlling for the adequacy of the sample, although assessments of partial inhibition are more difficult where expected levels of human DNA are not known and Cq values will be inconsistent and no reference Cq value will be available for comparison. Samples containing potentially large amounts of human material and subsequent DNA (for example, bronchoalveolar lavage) can be inhibitory to PCR amplification of the target sequence.

Internal amplification controls added at the PCR stage will control for inhibitors in the PCR but will not control for sample extraction.

9 Other considerations to avoid contamination

A number of additional measures and procedures can be included in an assay protocol to minimise further the likelihood of contamination, the most common of which is the use of Uracil-DNA Glycosylase (UDG)[12]. While use of such measures is recommended (as per assay protocol), it is to be noted that these are used in addition to the good practices outlined above, not as an alternative as these have their own limitations. Inclusion of UDG may reduce amplification efficiency and thereby delay or prevent detection, when only one or a few target DNA molecules are present. Heat-labile forms of the enzyme are available to minimise residual UDG activity after PCR.

Decontamination is performed using UV-irradiation, 10% sodium hypochlorite or 1M HCl[7,9,20].

Regular environmental monitoring of work areas for example swabbing may be considered.

The use of aerosol-barrier pipette tips (preferably DNase- and RNase-free) during preparation of molecular assays will help prevent reintroduction of aerosols of a sample into other samples being processed at the same time[1].

Equipment used should be periodically inspected, cleaned, maintained, and calibrated, as appropriate. Any equipment that is out of use for any reason should be clearly identified as such[8,15].

Laboratory coats should be cleaned regularly to reduce the possibility of contamination of the designated workspace and the PCR reaction[4,8].

Staff awareness of these issues and how they play an important role in the prevention of contamination is essential.

Ensuring that standard operating procedures (SOPs) used by staff in the laboratory are up to date and are the most current. It should be noted that these SOPs should have been approved and authorised by management prior to being used in the laboratory for work.

Reviewing staff competency through internal audits or participation in external quality assessment schemes also ensure that staff are following the local SOPs that are in place hence avoiding any contamination issues.

10 Quality assurance

Contamination is a potential threat when using sensitive nucleic acid amplification techniques and regular environmental monitoring serves as a useful indicator of potential problems. Many commercial systems now recommend environmental monitoring as part of the housekeeping and maintenance procedures but this should also be carried out for in-house assays. In the event of major laboratory disruption, for example during emergency evacuations, servicing of equipment by external contractors or the entrance of building contractors into the PCR suite, 'deep clean' decontamination procedures should be put in place and employed.

It is important to demonstrate that assays are performing consistently and that results reported are reliable and accurate. Methodology will be dependent on platform or assays used. Where available it is advisable to run external controls for commercial and in-house based assays[7,21]. For many viral targets, quantitated controls can be obtained from commercial and other sources (for example National Institute for Biological Standards and Control (NIBSC)). Keeping a regular record of these results will help to identify problems at an early stage.

Assays should be appropriately validated before introduction into routine use (see UK SMI UK SMI Q 1 - Evaluations, validations and verifications of diagnostic tests). Note that the validation of an in house assay (as for a CE marked assay) is a validation of the total process. Any change in that process, be it in extraction procedure, reagents, cycling parameters, introduction of internal controls for inhibition, will necessitate a documented revalidation of the whole process.

New batches of reagents for example primers, probes PCR mix etc need to be assessed for performance against such well characterised control material and recorded as an auditable record (acceptance testing).

Participation in quality assurance programmes is essential if a scheme exists for example United Kingdom National External Quality Assessment Scheme (UK NEQAS) and Quality Control for Molecular Diagnostics (QCMD). However, where no scheme exists, section 5.6.3.2 of ISO 15189:2012 recommends interlaboratory collaboration for the exchange of samples to ensure that the test is performing correctly[22]. For PCR methods specifically targeting fungal disease, the Fungal PCR initiative (formerly the European *Aspergillus* PCR initiative) has been formed to standardise methods for fungal PCR through quality control evaluations (https://fpcri.eu/).

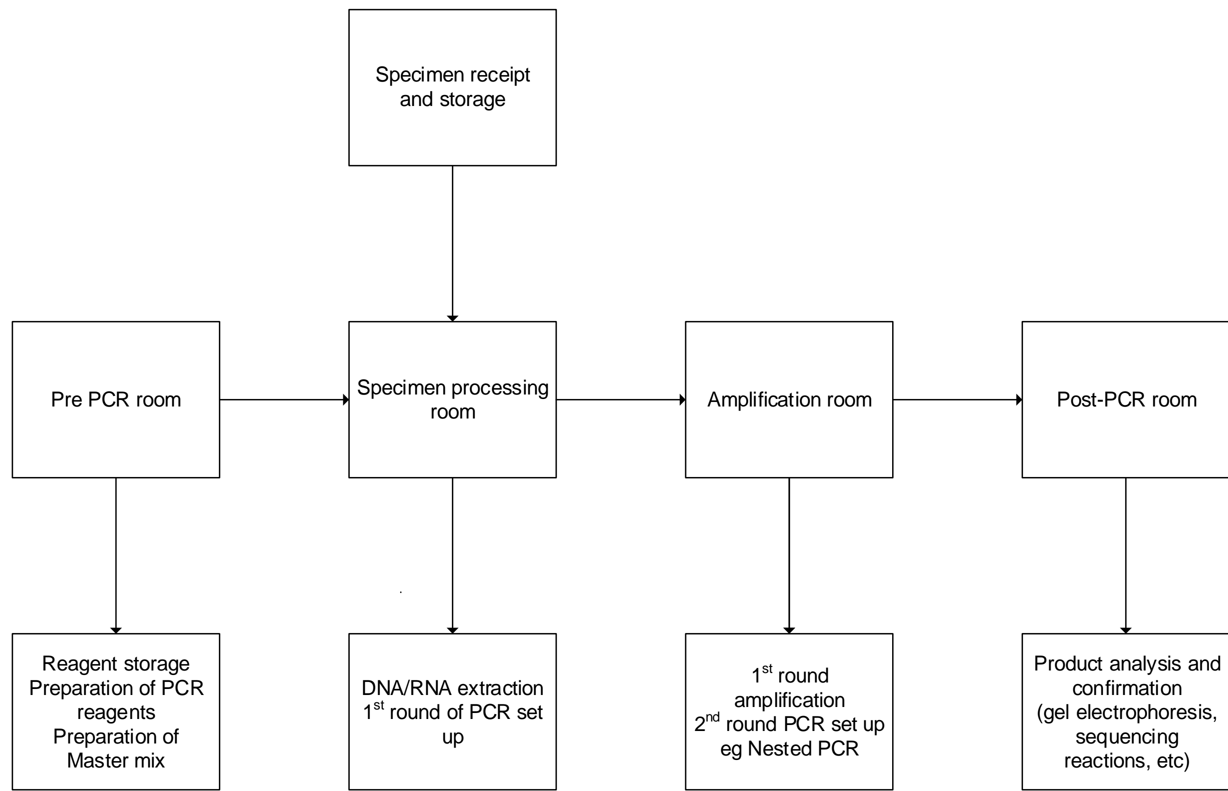
11 Public health responsibilities of diagnostic laboratories

Diagnostic laboratories have public health responsibility as part of their duties. Amongst these are additional local testing, or referral to further characterise the organism as required, primarily for public health purposes e.g. routine cryptosporidium detection; serotyping or microbial subtyping; and a duty to refer appropriate specimens and isolates of public health importance to a reference laboratory.

Diagnostic laboratory outputs inform public health intervention, and surveillance data is required to develop policy and guidance forming an essential component of healthcare. It is recognised that additional testing and referral of samples may entail some costs that has to be borne by the laboratory but in certain jurisdictions these costs are covered centrally.

Diagnostic laboratories should be mindful of the impact of laboratory investigations on public health and consider requests from the reference laboratories for specimen referral or enhanced information.

Algorithm: Diagram showing workflow in a PCR laboratory



Note: Although four rooms are ideal, many laboratories only have two rooms available.

Pre-PCR and extraction are therefore carried out within defined areas of a larger laboratory while amplification and product analysis are in a second laboratory.

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An explanation of the reference assessment used is available in the scientific information section on the UK SMI website.

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