Manual for the Laboratory-based Surveillance of Measles, Rubella, and Congenital Rubella Syndrome

Chapter 6. Detection of viral RNA by RT-PCR for the confirmation of measles and rubella infection

Overview

Conventional or real-time RT-PCR may be used to confirm the isolation of measles or rubella virus following inoculation and passaging of clinical specimens in cell culture, described in chapter 6. However, many of the laboratories in the GMRLN are equipped and trained to detect measles- and rubella-specific RNA directly from clinical specimens by real-time RT-PCR, which is much more sensitive than conventional RT-PCR. Protocols for performing real-time RT-PCR for the laboratory confirmation of measles or rubella infection have been developed and evaluated over many years and the assays continue to be improved.

When appropriate virologic specimens are available for testing, virus-specific RNA detection by RT-PCR for case confirmation is performed in many laboratories in combination with testing serologic or oral fluid specimens for virus-specific IgM. This approach has greatly improved the ability of the laboratory to provide timely confirmation of measles and rubella cases [1-4]. In addition, detection of viral RNA can provide a more reliable method for case confirmation than testing for IgM where disease prevalence is low, particularly in post-elimination settings [5].

Compared to conventional RT-PCR assays, real-time RT-PCR offers improved analytic sensitivity and the capacity for higher throughput without the need for post-amplification processing. However, the incorporation of adequate assay controls and the maintenance of physical separation of activities that reduce the risk of contamination are crucial. While this chapter provides guidance and recommendations for the diagnostic use of real-time RT-PCR, each laboratory in the GMRLN should work with their national surveillance programme and regional laboratory coordinator before incorporating RT-PCR as a tool for routine case confirmation.

Note: *The abbreviation, RT-PCR, may be used herein to refer to both conventional (endpoint) reverse transcription PCR and to real-time (kinetic) RT-PCR, when describing characteristics of RT-PCR in general. Because real-time RT-PCR can be used for quantification of RNA in clinical specimens, real-time RT-PCR is sometimes referred to as quantitative real-time RT-PCR and abbreviated as RT-qPCR. However, in the context of the diagnostic use of RNA detection from measles and rubella samples specifically employing the use of real-time RT-PCR protocols, the term real-time RT-PCR will be used.*

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6.1 Best practices for collection and processing clinical specimens and extraction of RNA

The timely and proper collection and processing of virologic specimens from suspected cases of measles and rubella is crucial for the genetic characterization of measles and rubella viruses, and increasingly, to aid in case confirmation. Because the integrity of the viral RNA can be compromised at any point during specimen collection, processing, and transport, the procedures and recommendations described in chapter 3 should be followed to minimize degradation of the samples.

Oral fluid (OF), throat swabs (TS), or nasopharyngeal (NP) aspirates or swabs are good sources of RNA for measles or rubella virus detection [3,4,6]. Urine samples are acceptable but may be more difficult to transport and may contain substances that are inhibitory to RT-PCR. When a suspected measles or rubella case has been detected in an elimination setting, particularly cases that have no known source or contact with other cases, the collection of more than one clinical specimen may improve the likelihood of successful RNA detection and identification of the genotype.

Serum specimens (including eluate from dried blood spots) have been utilized as a source of virus-specific RNA when routine virologic specimens could not be obtained. However, successful detection of viral RNA in serum specimens requires that the blood sample is collected very close to rash onset (<3 days). Even when collected early, the number of virus particles in serum is low, making RNA detection very challenging. A protocol utilizing nested amplification primers can increase the sensitivity, but its use is limited as a last option for determination of the genotype (not for case confirmation) from specimens that had tested positive by real-time RT-PCR. This approach should only be used by laboratories with comprehensive containment and control procedures in place to mitigate any inadvertent cross-contamination.

The most important factor for successful amplification and identification of measles or rubella RNA by any detection method is the timing of the sample collection. The specimens should be collected as soon as possible after rash onset as the sensitivity of detection decreases with time after rash onset. The recommended timing for collection of clinical specimens is provided in Chapter 3. The sensitivity of RNA detection methods from most types of specimens declines considerably after 5-7 days. As discussed in Chapter 3, different types of clinical specimens may be collected beyond 5 days post rash onset for detection of measles RNA if a delay is unavoidable. Successful amplification of measles virus-specific RNA by real-time RT-PCR from oral fluid specimens collected as late as 21 days after rash onset has been reported [2].

Upon arrival at the laboratory, the contents of the package containing the clinical specimen for RT-PCR testing is inspected and the condition of the clinical specimen is recorded (see Chapter 11). If not accessioned and processed immediately, the specimens should be transferred to either a refrigerator (4°-8°C) or frozen at -70°C, according to the SOP for the type of specimen. However, repeated cycles of freezing and thawing should be avoided to preserve the integrity of the RNA.

The extraction of RNA from a clinical specimen (or cell culture lysate) must be performed carefully to maximize the yield of target RNA. The best approach for RNA extraction is to use one of the commercial RNA extraction kits (e.g. Qiagen) or commercially prepared reagents.

RNA extraction protocols for clinical specimens that are in use by GMRLN laboratories are available in Annex 6.1. When choosing an RNA extraction method, it is important to consider the source and type of specimen. For example, RNA extraction kits that are designed to extract RNA directly from clinical specimens may be unsuitable for extracting RNA from virus-infected cell culture lysates.

6.2 Considerations for the use of molecular diagnostic methods

The development and use of molecular testing specifically for case confirmation (virus-specific RNA detection) involves a wider range of RT-PCR methodologies than those used for genotyping. Different protocols produce amplicons of various lengths and may target different viral genes. Both conventional RT-PCR and real-time RT-PCR may be used to detect measles- or rubella- specific RNA in clinical specimens. Regardless of the assay selected, the specific target amplified by the primers (for either measles or rubella virus) must be demonstrated to be sensitive for detection of all genotypes.

Conventional RT-PCR is limited for diagnostic use due to its relative lack of sensitivity compared to real-time RT-PCR. In addition, the requirement for post-amplification analysis by agarose gel electrophoresis for conventional RT-PCR increases the turn-around time and is not amenable to high specimen throughput. While nested RT-PCR reactions usually offer a substantial increase in sensitivity over single round conventional RT-PCR, the greater potential for cross-contamination from positive specimens included in the test is a major concern. In general, nested PCR reactions are performed only in Global Specialized or Regional Reference Laboratories for molecular surveillance purposes to obtain a genotype from specimens that have a low copy number of viral RNA. Nested RT-PCR assays should not be used for case confirmation.

The reagents and equipment required for real-time RT-PCR are more expensive than the materials required for conventional RT-PCR. Real-time RT-PCR assays require a dye-labelled oligonucleotide probe. Synthesis of the probes is more complex than synthesis of non-labelled primers and it is critical to find a reliable source for probe synthesis. Because of the increased sensitivity of real-time RT-PCR assays compared to conventional assays, precautions to

minimize the possibility of contamination most be followed rigorously. This requires strict adherence to directional workflow and the use of appropriate controls.

Most real-time RT-PCR assays are designed as one-step assays which combine the reverse transcription and PCR amplification steps in a single reaction. This reduces the repeated pipetting steps that increase the likelihood of cross-contamination. An advantage to real-time RT-PCR is that the assays can be configured as multiplex assays for detection of multiple targets in the same reaction [7]. For example, multiplexed real-time RT-PCR assays have been developed to simultaneously detect measles- and rubella-specific RNA and to target reference (cellular) genes.

Whether utilizing conventional RT-PCR or real-time RT-PCR, it is important to use a validated assay and to include positive and negative controls in all RT-PCR assays. The negative controls, or no-template controls (NTCs) are essential to detect extraneous nucleic acid contamination. Laboratories should develop SOPs using validated RT-PCR protocols with established performance characteristics:

- Defined lower limit of detection: the analytic sensitivity has been measured using samples with a known RNA copy number or virus concentration (e.g., pfu/ml, copies/reaction)
- Diagnostic sensitivity: the demonstrated ability to detect measles or rubella RNA in clinical samples from infected individuals
- Specificity: the absence of a signal when tested against other pathogens causing febrile rash illness and other respiratory pathogens. Specificity can be evaluated *in silico* by submitting the primer sequences to a BLAST search on GenBank
- Repeatability and reproducibility: results show limited intraassay and interassay variation
- Reaction kits include positive control RNAs of known sequence, preferably with genetic markers that clearly identify control reactions
- Optimized and defined reaction conditions

- Demonstrated ability to detect all known circulating genotypes of measles and rubella
- Flexible platform/chemistry

The laboratory must ensure that validation and appropriate QC/QA plans are in place. The quality indicators to be monitored for each assay are indicated in the laboratory SOP.

6.3 Measles RNA detection by RT-PCR

The conventional RT-PCR for measles that amplifies a fragment to generate the N-450 sequencing window is used by some laboratories for case confirmation. Several sensitive conventional RT-PCR assays that target the N or H genes for either RNA detection and/or genotyping have been described with lower limits of detection in the range of 1,000-10,000 copies of measles viral RNA per reaction [8]. Real-time RT-PCR assays that target regions of the measles N or H genes can typically detect as few as 10-100 copies of viral RNA per reaction and can provide quantitative results [9,7,10].

Intercountry laboratory workshops sponsored by WHO to support molecular detection and genotyping for measles and rubella may offer different protocols for conventional or real-time RT-PCR. As with EIAs for antibody detection, various validated in-house and commercial kits may demonstrate acceptable performance, but all RT-PCR protocols must meet validation criteria. Regional workshops may utilize different kits, but all workshops emphasize the technical aspects of RT-PCR as well as quality assurance and quality control components. Some examples of the protocols that are widely used are described below.

Many of the intercountry laboratory workshops have provided training for the real-time RT-PCR method utilized at the CDC (Atlanta) that amplifies a 75-nucleotide region of the measles N gene [9]. Kits containing primers, probes and positive control RNA are provided by CDC to GMRLN laboratories. The kits produced at the CDC include two positive controls consisting of synthetic measles RNA of known copy number. The acceptable range of Ct values for both controls are provided in the kit instructions. The primers and probe for detection of the reference gene RNase

P are also included. The protocol for the CDC measles real-time RT-PCR is available in pdf format (Annex 6.2).

6.4 Vaccine-specific RT-PCR for identification of measles vaccine strains

A vaccine-related rash may occur among approximately 5% of recipients of measles-containing vaccine, typically 7-14 days after vaccination. A wild type measles infection and a rash due to vaccination cannot be distinguished by measles-specific IgM testing. In outbreak settings, enhanced surveillance for rash illness may detect suspected cases among potentially exposed individuals who were vaccinated as a control measure. The rapid confirmation of a vaccine-related rash is important to avoid unnecessary public health responses. A vaccine-specific, real-time RT-PCR has been developed to provide a rapid method to identify the vaccine genotype (genotype A) from RNA extracted from clinical specimens collected from suspected cases of measles with a recent history of vaccination prior to rash onset [19] (Annex 6.3).

The real-time RT-PCR that selectively amplifies genotype A, measles vaccine strains (MeVA) includes a probe with four 'locked' nucleotides that improve the specificity of the probe. The target for the MeVA assay is the measles N gene, nucleotides 478- 548. Validation studies of the assay have demonstrated that this target region provides an assay that is highly specific for genotype A. The lower limit of detection is about 10X higher than the CDC measles real-time RT-PCR assay. Compared to standard genotyping to determine the genotype and thus discriminate vaccine from wild-type infection, the clinical sensitivity of the MeVA assay is approximately 97%.

Note: When the investigation of a suspected measles case indicates a high probability that the individual may have a rash due to a recent vaccination, the MeVA assay (if available) is performed in parallel with the measles real-time RT-PCR.

6.5 Rubella RNA detection by RT-PCR

Conventional RT-PCR methods are performed to amplify the 739-nt region of the E1 gene for assignment of rubella genotypes. Many variations of RT-PCR have been used to detect rubella

RNA in clinical specimens for case confirmation. Currently, real-time RT-PCR is the most commonly used confirmatory method for rubella in the GMRLN laboratories. Several laboratories in GMRLN have developed real-time RT-PCR assays that target different regions and amplify amplicons of various lengths of the rubella genome [1,11-14]. A different system, the RT-loop-mediated isothermal amplification (RT-LAMP) has been described for rubella detection [15].

The original protocol for rubella real-time RT-PCR, included in training workshops provided by CDC (Atlanta), amplified a 185-nt region that lies within the 739-nt region of the E1 gene [11,16]. A modification of the protocol has been provided to improve the sensitivity of the assay for the rubella viruses in genotype 2B. The modification consists of the addition of second reverse primer, based on the consensus sequence of 151 genotype 2B rubella viruses. The additional reverse primer was described in a study of rubella viruses in Uganda in 2014 [17]. The kits produced at the CDC that amplify the 185-nucleotide region within the E1 gene include two synthetic positive controls, a high copy control and a low copy control. Primers and probe for the reference gene, RNase P, are also included. The protocol for the conventional RT-PCR for rubella detection using the 3-primer system is provided in Annex 6.4.

While the CDC rubella real-time RT-PCR assay described above may still be in use, an alternative target has been identified for a real-time assay that further improves detection of 2B genotype viruses. This assay can detect as few as three to seven copies of rubella-specific RNA of both clade 1 and clade 2 viruses. The assay targets the 5’ terminus of the rubella genome and amplifies a 154- nucleotide region of the p150 gene. This assay was used in an investigation of a rubella outbreak in Romania [18]. The protocol for the CDC rubella real-time RT-PCR is available in pdf format (Annex 6.5). Additional assays that have been described include a one-step TaqMan assay that targets a highly conserved region in the other rubella non-structural gene (p90 gene) [14].

6.6 Quality assurance and quality control of RT-PCR

RT-PCR is a sensitive method and should be performed following strict quality control and quality assurance procedures. A description of the quality assurance programme administered

through WHO for molecular external quality assurance (mEQA) is provided in Chapter 12. All the laboratories that use RT-PCR as a method for case confirmation and/or report genotypes to MeaNS and RubeNS participate in the annual mEQA programme. In addition, the WHO accreditation process for the GMRLN includes an assessment of the facilities for RT-PCR and documentation of QA/QC processes for laboratories using molecular techniques.

To avoid cross-contamination of samples tested by RT-PCR, unidirectional workflow proceeding from the area for RNA extraction/reagent preparation (pre-amplification area) to the PCR amplification room (post-amplification area) must be implemented and maintained. It is important that appropriate facilities and dedicated rooms and equipment for RT-PCR pre-amplification and post-amplification steps are provided to reduce the possibility of cross-contamination with amplification products. There should be designated areas for RNA extraction, preparation of the pre-mix, addition of test RNA, and addition of control RNAs. The initial steps of RNA extraction must be performed in a class II BSC because clinical samples are potentially infectious until the RNA lysis buffer is added. Subsequent steps in RNA extraction and RT-PCR can be performed in a BSC or a dedicated PCR cabinet.

Each work area should have dedicated laboratory coats and equipment and the equipment should not be moved from one work area to another. All personnel must be familiar with the protocols and instruments used. Equipment should be properly maintained, with documentation that verifies that routine preventive maintenance and calibration are regularly performed. Following these guidelines will help minimize the chance of false-positive results. Important practices that should be included in RT-PCR SOPs are listed below.

- Maintain separate areas, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips, gowns and gloves) for each of the following activities:
    - RNA extraction in a class II BSC
    - Preparation of the RT-PCR pre-mix
    - RNA template addition
    - Addition of positive controls
    - RT-PCR amplification
    - Manipulation of the products of amplification

- Wear clean, previously unworn, disposable gowns and new, powder-free gloves during pre-mix setup and handling of extracted nucleic acids; gloves should be changed frequently
- Store primer/probes and enzyme master mix at appropriate temperatures (see package inserts).
- Do not use reagents beyond their expiration dates
- Keep reagent tubes and reactions capped as much as possible
- Clean work surfaces and equipment (pipettors, tube racks, etc.) using a commercial product that destroys DNA and RNA or a freshly prepared chlorine bleach solution (10%)
- Use aerosol barrier (filter) pipette tips only

The use of appropriate controls is essential for both conventional RT-PCR and real-time RT-PCR assays for detection of measles or rubella. Synthetic RNA controls for conventional RT-PCR are available for GMRLN laboratories (contact the GLC and CDC for instructions). The synthetic positive control RNAs for conventional RT-PCR are designed with insertions or deletions in the target regions. The PCR product from the control reaction will migrate with a slightly different molecular weight in agarose gel electrophoresis. Use of the synthetic positive controls enables the laboratory to visually detect cross-over of the positive control RNA into the test samples or negative control samples when running the amplification products on the agarose gel. Because of this ability to discriminate the synthetic controls from wild or vaccine viruses, the synthetic positive controls should be used exclusively when available.

Real-time RT-PCR assays are usually designed to detect cellular RNA from a reference gene, or "housekeeping gene". The most frequently targeted reference genes, human ribonuclease P (RNase P) and glyceraldehyde 3-phosphate dehydrogenase (GADPH), are present in all human cells and are used to evaluate the adequacy of the clinical sample and the quality of the RNA extraction. Detection of a reference gene is performed either in a multiplex format or as a separate reaction. Failure to detect the reference gene indicates that insufficient cellular material was collected, inhibitory substances are present in the sample, or the sample was transported or

processed improperly. WHO strongly recommends the use of reference genes for real-time RT-PCR assays that are used for case confirmation.

The incorporation of adequate in-house RNA positive controls provides evidence that the sensitivity of the assay was within accepted parameters. Documentation of the use of appropriate controls is necessary to ensure accuracy. Synthetic viral RNA or viral RNA from infected culture are commonly used as positive controls. Negative controls should include sterile, nuclease-free water for no-template controls (NTC) and mock-extracted RNA samples (extraction control). For real-time RT-PCR, multiple virus-specific and single cellular gene-specific reactions are often included in the test protocol. The SOP should identify quality indicators that will be monitored for each assay to help identify trends that may affect assay performance. For example, many laboratories plot the Ct value of one of the standard positive control reactions.

6.7 Test validity, data interpretation and assay limitations

A valid assay requires that all positive and negative controls yield the expected results. The assay is not valid if any positive control is negative or any of the negative controls are positive. If any negative control has a positive result, the following steps should be taken:

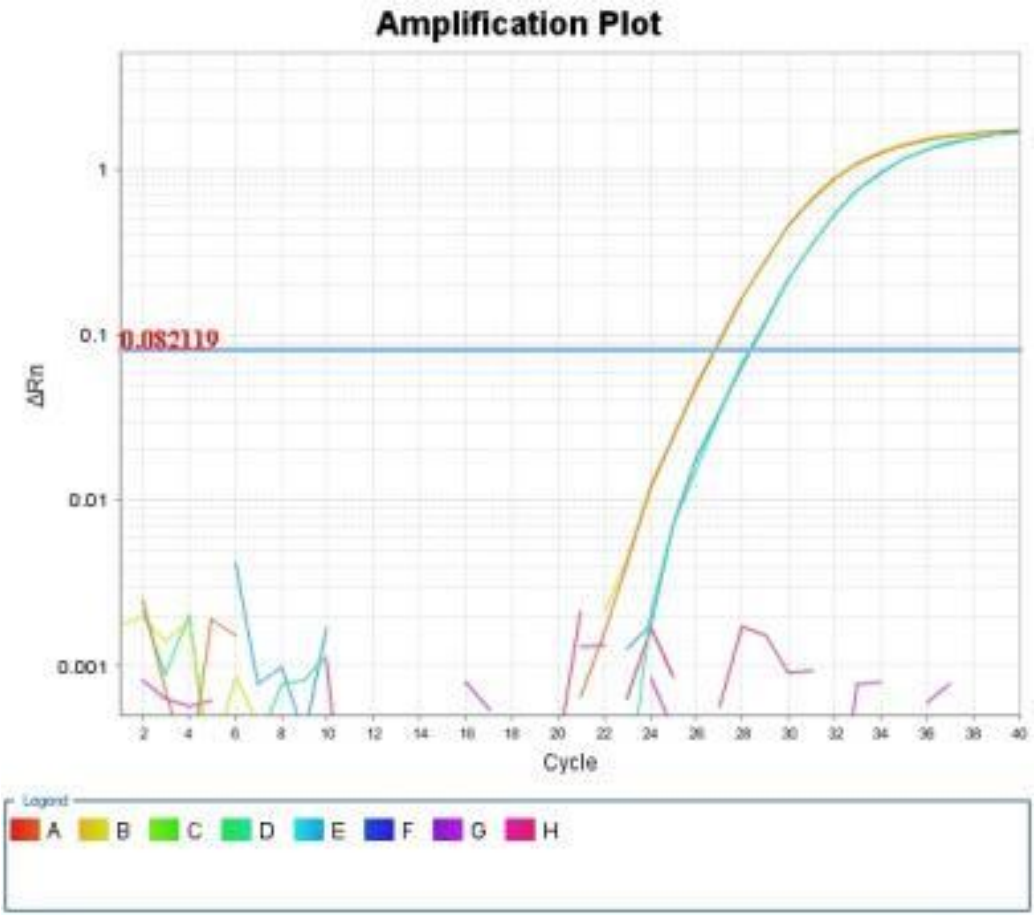
- Immediately discard all working stocks of reagent dilutions and remake from fresh stock
- Bench surfaces, pipettes, and other equipment should be cleaned with bleach or a commercial product to destroy nucleic acids in the reagent setup and template addition work areas

In real-time RT-PCR assays, a clinical sample that is positive by both virus- and cellular- gene specific RT-PCR (or RT-qPCR) is considered positive. In multiplex assays, if the virus-specific RT-PCR is positive, it is acceptable for the cellular gene to have a negative result- this does not invalidate the positive virus-specific result when expected results are obtained for the positive and negative controls. Specimens that are negative by the virus-specific RT-PCR but positive by the cellular-gene specific RT-PCR are considered negative. If both the cellular-gene specific RT-

PCR and the virus-specific RT-PCR have negative results, the quality of the RNA in the sample has may have been compromised and the result is reported as undetermined (inconclusive).

It is critical to carefully evaluate the data for each sample. The amplification curves must cross the set threshold for the virus-specific RT-PCR to be considered positive and visual inspection of the amplification curves is suggested to verify positive results. (See Figure 6.1).

Figure 6.1 Evaluation of true positive in real-time RT-PCR scan

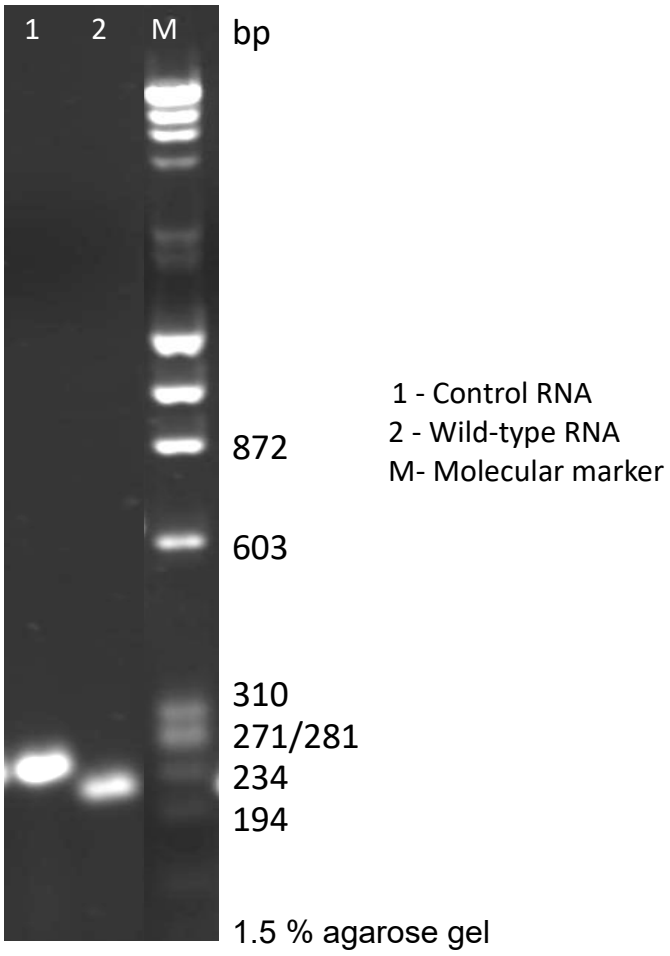


Graph shows signal strength over the course of 40 cycles, each colored line represents one sample. The cycle in which the signal of a sample crosses the threshold and becomes positive is the Ct value. The lower the Ct value, the more target RNA was present in the sample. If the graph of a sample does not cross the threshold within 40 cycles, the result for Ct value is termed either 'undetermined' (Applied Biosystems) or 'undetected'. Non-template controls have water instead of RNA template and should produce negative results.

Extremely weak positives (high Ct values close to the cut-off Ct) should be interpreted with caution. Ideally, the test samples should be tested in triplicate. If there are inconsistent results (positive/negative, positive/undetermined), the samples should be retested.

For conventional RT-PCR, the PCR products are usually visualized by agarose gel electrophoresis on a 1.0-2.0% agarose gel with an appropriate molecular weight marker. Figure 6.2, Rubella diagnostic RT-PCR control and wild-type products, shows the difference in migration of the amplicons from the rubella synthetic positive control and that from a wild-type rubella virus sample.

Figure 6.2 Rubella diagnostic RT-PCR control and wild-type products



The instructions for agarose gel preparation and clean-up of PCR products are provided in Annex 6.6. Gels are stained by ethidium bromide or GelRed™ and bands are visualized on a uv transilluminator. Positivity is determined by the presence of a fluorescent band on the gel of the expected size. The image of the gel should be recorded with the results. If any of the controls fail to produce the expected result, the assay is invalid and must be repeated.

A positive result indicates that measles or rubella RNA was present in the clinical sample. However, if the suspected case had been vaccinated within 7-14 days prior to rash onset, the positive result may be due to a vaccine-associated rash. A set of criteria is available for identifying vaccine-associated rash cases (chapter 4, text box section 4.3), when there was no potential exposure to wild-type measles or rubella virus.

In those instances where the patient received an MR/MMR vaccination following potential exposure to a confirmed case or was vaccinated as part an outbreak response, it is necessary to identify a vaccine strain (genotype A) by sequence analysis or by use of a validated vaccine-specific RT-PCR assay to discard the case. For more information on distinguishing vaccine strains from wild-type infection by sequence analysis, refer to Chapter 7.

The interpretation of real-time RT-PCR test results must consider the possibility that the test result may not reflect the true case status of the suspected case. Not only can false positive (from a non-case) results arise due to cross contamination, a negative result may be obtained from a true case of measles or rubella. The abundance of virus-specific RNA is generally much lower in infected cells compared to the cellular RNA of the reference gene. Therefore, a positive signal from the reference gene may be obtained from specimens collected from true cases while the virus-specific real-time RT-PCR result is negative. A ‘false negative’ result may be obtained due to:

- Poor sample collection, transportation or processing
- Partial degradation of RNA from excessive freeze-thaw cycles, reducing viral RNA below detection levels
- Timing of specimen collection
- Failure to follow the assay protocol or equipment failure

- Failure to use specified extraction kit and platform

Therefore, a negative RT-PCR result should be interpreted carefully. A negative RT-PCR result can be helpful to corroborate other negative laboratory results but should never be the sole basis for ruling out a suspected case. In addition, a negative RT-PCR result obtained from a suspected case with a doubtful (positive) IgM result does not provide support for concluding that a positive result for IgM was due to a nonspecific reaction. The final classification of suspected cases that are negative by RT-PCR with inconclusive IgM results (i.e. negative IgM from serum collected ≤3 days after rash for measles or ≤5 days after rash for rubella) must be based on clinical assessment or discarded based on laboratory confirmation of other rash-causing illness. In elimination settings, additional testing may be necessary or additional sample collection may be indicated. The recommendations for testing in elimination settings are provided in Chapter 8.

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