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ORIGINAL PAPER

Methods for Preparation of MS2 Phage-Like Particles and Their Utilization as Process Control Viruses in RT-PCR and qRT-PCR Detection of RNA Viruses From Food Matrices and Clinical Specimens

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Abstract RNA viruses are pathogenic agents of many serious infectious diseases affecting humans and animals. The detection of pathogenic RNA viruses is based on modern molecular methods, of which the most widely used methods are the reverse transcription polymerase chain reaction (RT-PCR) and the real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). All steps of RT-PCR and qRT-PCR should be strictly controlled to ensure the validity of obtained results. False-negative results may be caused not only by inhibition of RT or/and PCR steps but also by failure of the nucleic acid extraction step, particularly in the case of viral RNA extraction. The control of nucleic acid extraction generally involves the utilization of a non-pathogenic virus (process control virus) of similar structural properties to those of the target virus. Although in clinical samples the use of such process control virus is only recommended, in other kinds of settings such as food matrices its use is necessary. Currently, several different process control viruses are used for these purposes. Process control viruses can also be constructed artificially using technology for production of MS2 phage-like particles, which have many advantages in comparison with other used controls and are especially suited for controlling the detection and quantification of certain types of RNA viruses. The technology for production of MS2 phage-like particles is theoretically well established, uses the knowledge gained from the study of the familiar bacteriophage MS2 and utilizes many different approaches for the construction of the various process control viruses. Nevertheless, the practical use of MS2 phage-like particles in routine diagnostics is relatively uncommon. The current situation with regard to the use of MS2 phage-like particles as process control viruses in detection of RNA viruses and different methods of their construction, purification and use are summarized and discussed in this review.

Keywords MS2 phage-like particle · RNA virus · Process control virus · Detection · Quantification · Armored RNA

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Introduction

Reverse transcription polymerase chain reaction (RT-PCR) and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays are widely used methods for detection and quantification of RNA viruses. Detection methods based on RT-PCR were rapidly replaced by qRT-PCR methods which are nowadays considered as the gold standard in detection and quantification of RNA viruses (Vermehren et al. 2008). qRT-PCR is based on the method of PCR which was developed in the 1980s (Mullis et al. 1986; Saiki et al. 1985). Invention of real-time PCR (qPCR) in the early 1990s allowed monitoring of the course of the PCR reaction in real time, and added the dimension of nucleic acid quantification to microbial diagnostics (Higuchi et al. 1993; Higuchi et al. 1992). A logical evolution of PCR methods also witnessed the combination of the initial RT step with qPCR, which led to the establishment of qRT-PCR, a powerful assay for analysis of RNA molecules. In comparison with other methods

for the detection of RNA viruses, among which the cell culture methods are the most widespread, qRT-PCR has many advantages. Propagation of viruses using cell culture methods is a time-consuming process because each virus type and/or even strain has different characteristics. Furthermore, detection is very problematic in the case of those viruses which cannot be grown in conventional cell culture: e.g., human norovirus (NoV) or hepatitis E virus (HEV) (Barnaud et al. 2012; Rodriguez et al. 2009). On the other hand, qRT-PCR is capable of detecting as few as ten genome copies of viral nucleic acid in the sample (Puig et al. 2002). Although qRT-PCR is widely used nowadays, it is still not a perfect detection method. The main disadvantage lies in its inability to distinguish between infectious and non-infectious viral particles. It is also necessary to strictly control all steps of the qRT-PCR analysis of RNA viruses using a system of negative and positive controls, which would guarantee the validity of the results. The RT step is extremely unpredictable and needs to be monitored. The efficiency of the RT step is 20 % on average, and can vary within not only different qRT-PCR protocols, but also in the same qRT-PCR experiments (Curry et al. 2002). False-negative results may be not only caused by a wide range of different factors such as the presence of inhibiting substances in the sample, incorrect composition of the enzyme mixture, poor activity of the reverse transcriptase and DNA polymerase, and thermal cycler failure but also by the failure of the first step of viral RNA analysis—the extraction of nucleic acid.

The nucleic acid extraction step is a crucial point in each molecular biological analysis and therefore must be under strict control. Many reagents traditionally used for the isolation of nucleic acids such as chelating compounds, detergents, and guanidium hydrochloride can inhibit enzymatic reactions (Monteiro et al. 1997). Inhibitory substances such as complex polysaccharides, phenolic and organic compounds and metabolic products are also naturally present in the samples and can partially or completely inhibit RT and/or PCR (Abu Al-Soud et al. 2000; Das et al. 2009; Wilson 1997; Schrader et al. 2012). Also, working with low-quality nucleic acids strongly affects experimental results. Quantity and quality may vary due to the degradation of nucleic acids (especially RNA) during the isolation or storage processes. Therefore, every nucleic acid preparation should be assessed for quality and quantity (Fleige and Pfaffl 2006). In addition, the detection of RNA is a more sensitive and complicated process than the detection of DNA, especially because of the need of reverse transcribing the RNA and preventing it from contamination with ribonucleases. The stability of nucleic acids depends on their ability to resist the action of ubiquitous nucleases, DNases, and ribonucleases. While DNases can be easily heat-inactivated, ribonucleases can withstand high temperature, even under increased pressure and are stable in different conditions such as extremes of pH (Spackman et al. 1960; Zale and Klibanov 1986). For this reasons, it is inappropriate to use synthetic RNA constructs, RNA transcribed in vitro or reference RNA pools as controls for the nucleic acid extraction step. Also plasmid DNA, although more stable than RNA, is not representative of an authentic template in the RT procedure (Cartwright 1999). In the past, some authors have described methods for how to increase the stability of RNA in the presence of ribonucleases. It was shown that chemical modification such as phosphate modification (Black et al. 1973) and modification of ribose (Pieken et al. 1991) contribute to the stability of RNA molecules. Although these modifications can increase the resistance of RNA to ribonucleases, there is no practical example of the use of such modified RNA molecules as control material. Another potential method for ensuring RNA stability in the presence of ribonucleases is through utilization of RNA secondary structure (Chen et al. 1986). A control G+C rich rod-like RNA molecule with an extensive secondary structure, based on a modified hepatitis delta virus (HDV) genome was tested and shown to exhibit enhanced stability and resistance against ribonucleases (Dingle et al. 2004). However, a major disadvantage of this approach is that extensive secondary structure has a self-stabilizing effect and together with the high G+C content makes such a molecule a difficult template for RT and PCR. Therefore control of the nucleic acid extraction step generally involves the utilization of a non-pathogenic virus—process control virus—which protects the control RNA molecule inside its capsid and further mimics the target virus during the extraction step. Analyzed samples are spiked prior to processing with a defined amount of the process control virus and control RNA inside the capsid is extracted together with target RNA. This approach allows the control of the efficiency of RNA extraction and concentration steps, the removal of RT and PCR inhibitors and of the whole qRT-PCR assay. Currently, many different process control viruses are used in the detection of RNA viruses (see next chapters). MS2 phage-like particles represent one type of such process control viruses. Using the technology for the production of MS2 phage-like particles, it is possible to construct such a process control viruses. The technology for production of MS2 phage-like particles is theoretically well established, uses the knowledge gained from the study of the familiar bacteriophage MS2 and utilizes many different approaches for the construction of the various process control viruses. Nevertheless, the practical use of this technology in routine diagnostics is relatively uncommon.

The aim of the present review is to provide insight into the currently used process control viruses in RT-PCR and qRT-PCR detection of RNA viruses focusing on the possibility of using MS2 phage-like particles. Current knowledge regarding the possibilities of MS2 phage-like particle preparation and utilization will be discussed.

The Use of Different Control Process Viruses in Detection of RNA Viruses from Food Matrices and from Clinical Specimens

The Use of Different Control Process Viruses in Detection of RNA Viruses from Food Matrices

Using process control virus in detection of viruses from food matrices is necessary (Fig. 1). The European Committee for Standardization (CEN) released the ISO technical specifications (ISO/TS) ISO/TS 15216-1 and ISO/TS 15216-2 (The methods for determination of HAV and NoV in food using qRT-PCR), which require the use of process control virus in qRT-PCR detection of these viruses. According to these technical specifications, cultivable non-enveloped positive-sense single-stranded RNA (+ssRNA) virus shall be used as such a control (ISO/TS 1526-1 2013; ISO/TS 1526-2 2013). Furthermore, the process control virus should be of a similar size to the target virus to provide a good morphological and physicochemical model, should be genetically distinct form the target virus to avoid cross-reactivity and should not be naturally present in the analyzed sample. The above-mentioned ISO/TS standards recommend the use of genetically modified mengovirus as a process control virus in detection and quantification of HAV and NoV (Costafreda et al. 2006; Le Guyader et al. 2009; Butot et al. 2014). Mengovirus is a murine virus of the *Picornaviridae* family. This virus has structural and physicochemical properties close to those of HAV and NoV. It is a non-enveloped virus with single-stranded RNA. It has resistance properties in the environment close of those of the targeted viruses and can be used with all types of matrices. The genetically modified Mengo virus strain MC_0 is a recombinant virus which lacks the poly(C) tract and thus has an avirulent phenotype (Martin et al. 1996). The growth properties of this strain are identical to those of the wild-type virus. The MC_0 strain is a genetically modified organism (GMO); thus, it is

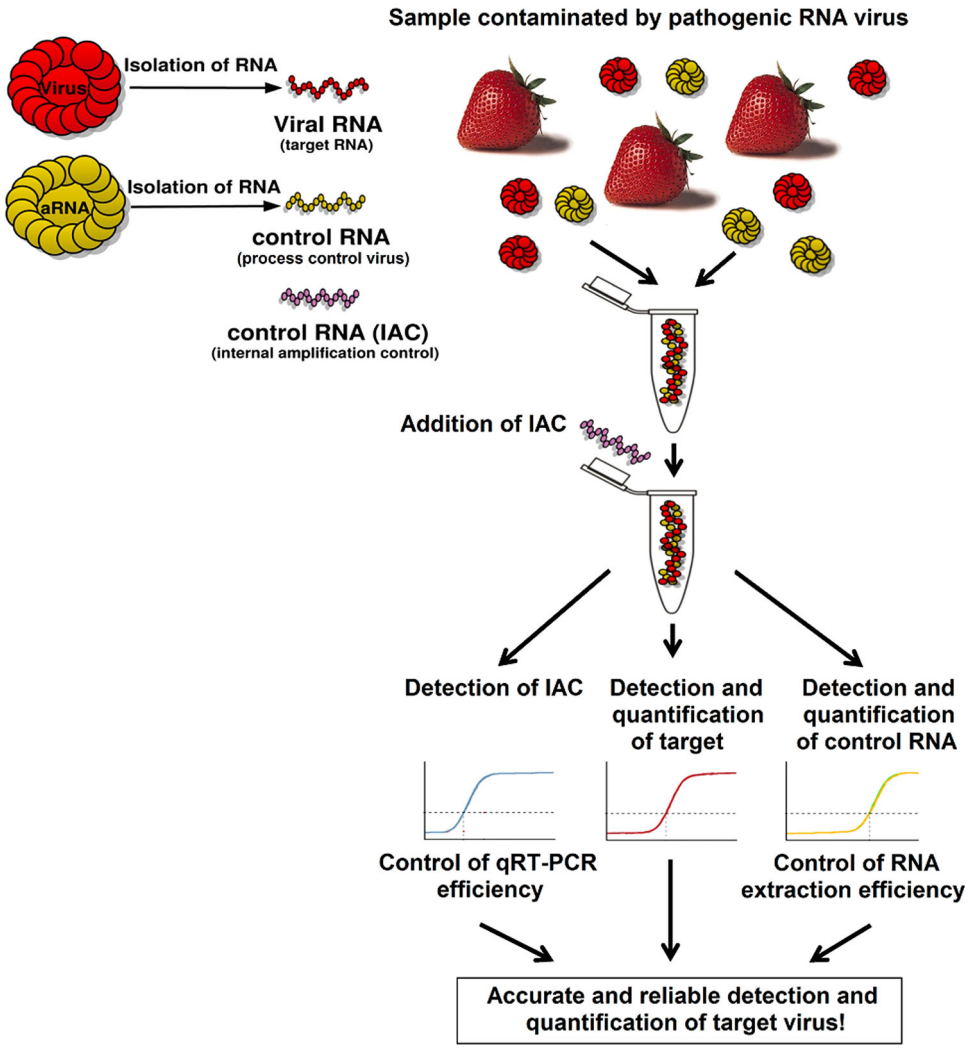

Fig. 1 Schematic representation of the analysis of viral RNA utilizing both an internal amplification control and process control virus to ensure the validity of obtained results. Adapted from Costafreda et al. (2006), edited

recommended that in laboratories where the use of a GMO is prohibited or problematic a different process control virus should be used in qRT-PCR assays (ISO/TS 1526-1, 2013). In the literature, there are many examples of the use of other process control viruses. Nishida et al. used echo type 9 virus as the process control virus in qRT-PCR detection of NoV from oysters (Nishida et al. 2007). Murine norovirus 1 (MNV-1) was used as a process control virus in qRT-PCR detection of HAV from lettuce (Coudray et al. 2013), semi-dried tomatoes (Martin-Latil et al. 2012), and NoV from bottled and tap water (Hennechart-Collette et al. 2014). Feline calicivirus (FCV) was used as a process control virus in qRT-PCR detection of HAV and calicivirus from bottled natural mineral water (Di Pasquale et al. 2010). Mattison et al. even suggested that the FCV become a standard process control virus for methods aimed at the extraction and detection of RNA viruses from food and water matrices (Mattison et al. 2009). However, comparison of whole genome sequences of different strains of FCV showed high genetic diversity and relatively few target sites to which qRT-PCR primers and probes can be designed (Coyne et al. 2012; Radford et al. 2007). In the case that primers and probes for the detection of target viruses interact with those for detection of FCV, FCV cannot be used as a process control virus. Also another non-human calicivirus—San Miguel sea lion virus serogroup 17 (SMSV-17)—was used as a process control virus in qRT-PCR detection of HAV and NoV from oysters (DePaola et al. 2010). These natural RNA viruses are resistant to ribonucleases and allow control of decapsulation during RNA extraction. The disadvantages of such viruses are that they are pathogenic, and not all of these viruses (MNV-1) are easily accessible to private companies. Moreover, most routine laboratories are not equipped for the cultivation of such viruses and therefore are not able to maintain a steady supply of process control virus.

Another interesting example from the literature relating to process control viruses describes the use of bacteriophage MS2 for these purposes. Wild-type MS2 bacteriophage was used as the process control virus in qRT-PCR detection of HAV from food and water matrices (Blaise-Boisseau et al. 2010) and was successfully tested in qRT-PCR assays for the detection and quantification of other viral pathogens, e.g., NoV GI and GII (Rolfe et al. 2007). The data showed that the MS2 bacteriophage offered a very reliable and simple way to monitor the nucleic acid extraction step, making it a valuable tool in the routine diagnostics laboratory. Moreover, wild-type MS2 bacteriophage has also been used as the process control virus for the evaluation of different systems for isolation of viral RNA and for estimation of the amount of co-purified inhibitors of qRT-PCR (Shulman et al. 2012). Cultivation of a bacteriophage such as MS2 is much easier than that of animal RNA viruses and requires no additional special laboratory equipment. In comparison with pathogenic animal RNA viruses, wild-type bacteriophage MS2 does not constitute a safety problem for laboratory personnel, but its use still has two major disadvantages. First, wild-type MS2 bacteriophage has the ability to proliferate. Theoretically, in specific samples such as those with fecal contamination that naturally contain *Escherichia coli*, MS2 bacteriophage can proliferate and exceed the number of detected pathogenic RNA viruses present in the sample. Second, the wild-type MS2 bacteriophage cannot be used as a competitive process control virus because its genome does not contain the specific target sequences. One possible solution to this problem would be the production of recombinant MS2 bacteriophages which carry in their genomes sequences that serve as targets for established detection methods. However, the production of viable, recombinant, and infectious MS2 bacteriophage that would serve as competitive process control virus was rejected for several reasons (Pasloske et al. 1998). The main obstacle to this is that recombinant bacteriophage is not genetically stable and heterologous sequences are quickly deleted. Finally, MS2 RNA replicase fidelity is very poor and thus a high number of point mutations and deletions could be introduced into the target sequence. Therefore, recombinant MS2 bacteriophage is not suitable as a competitive process control virus. However, using the technology for the production of MS2 phage-like particles, it is possible to create such a genetically stable and homogenous process control virus carrying the specific control RNA sequence inside its capsid.

The Use of Different Control Process Viruses in Detection of RNA Viruses from Clinical Specimens

The use of process control virus in RT-PCR and qRT-PCR detection of RNA viruses from clinical specimens is necessary, but so far there is no ISO/TS, which would describe the use of specific process control viruses. As in the case of food matrices, different types of process control viruses have been used in clinical specimens. Cleland et al. used bovine viral diarrhea virus (BVD) as the process control virus in RT-PCR detection of HCV from blood (Cleland et al. 1999). FCV, which is used as a process control virus in food and water matrices, was also used in qRT-PCR detection of HEV form swine fecal and blood samples (Ward et al. 2009). Wild-type MS2 bacteriophage was successfully used as a process control virus in qRT-PCR detection of HCV in blood donor screening (Dreier et al. 2005). Also Chidlow et al. used MS2 bacteriophage as the control process virus to monitor the efficiency of sample extraction, the removal of RT and PCR inhibitors and of cDNA production in qRT-PCR detection of pandemic (H1N1) and seasonal influenza A/H1, A/H3, and B viruses from deep nasal and throat swabs (Chidlow et al. 2010).

Also MS2 phage-like particles have been used as the process control virus for the detection of pathogenic RNA viruses in clinical samples. For example, Pasloske et al. prepared MS2 phage-like particles, also called armored RNA (aRNA), that carried the consensus RNA sequence from human immunodeficiency virus type 1 (HIV-1) packaged in the capsid which can serve as quantitative standard in detection of HIV-1 (Pasloske et al. 1998). Beld et al. used MS2 phage-like particles carrying the 5′ non-coding region of enterovirus (EV) as the process virus control in detection of human enteroviruses (Beld et al. 2004). The MS2 phage-like particles contained the same primer binding sites as detected enteroviruses but had a different probe region. MS2 phage-like particles carrying the plant-specific ribulose-1,5-bisphosphate carboxyl small subunit (*rbcs*) gene fragment were used as the process control virus in qRT-PCR detection of severe acute respiratory syndrome coronavirus (SARS-CoV) (Cheng et al. 2006). MS2 phage-like particles were also used as the process control virus in qRT-PCR screening of HCV in blood donors (Meng and Li 2010). These studies confirm that MS2 phage-like particles are suitable process control viruses for the detection of RNA viruses in clinical specimens.

Bacteriophage MS2 and the Technology for Production of MS2 Phage-Like Particles

The technology for production of MS2 phage-like particles is based on knowledge gained from the study of the familiar bacteriophage MS2. RNA bacteriophages have long been used as model systems for studying RNA transcription and translation. They are simple to grow in vitro and genomic RNA can be easily extracted. Bacteriophage MS2 was first isolated and described in 1961 (Davis et al. 1961). According to virus classification, bacteriophage MS2 belongs to the family *Leviviridae* and genus *Levivirus*. It is a +ssRNA virus. The MS2 genome is one of the smallest known, consisting of only 3569 nucleotides (nt) which encode only four genes: the maturase protein (A-protein), coat protein, lysis protein, and replicase protein (Fig. 2). MS2 bacteriophage only infects male *E. coli* strains, which possess the F plasmid and create the F pilus for conjugation. Each MS2 phage particle has one copy of A-protein and uses this to connect to the bacterial pilus (Wong and Paranchych 1976). Inside the bacteria viral RNA operates as messenger RNA and can be immediately translated into bacteriophage proteins which form new phage capsids. The MS2 bacteriophage particle has an icosahedral structure and lacks a tail or any other obvious surface structure (Stockley et al. 1994). The MS2 phage particle consists of 90 copies of coat protein dimers, one copy of the maturase protein (A-protein) and one molecule of viral +ssRNA. The coat protein possesses all the information needed for assembly into a viral capsid. Packaging of the viral RNA into the capsid starts with binding of a coat protein dimer to a specific stem-loop structure, also called the operator or "*pac*" site. The stem-loop structure is located 5′ to the phage replicase gene. The stem-loop structure consists of only 19 nucleotides and the residues A-4, U-5 and A-7 constitute key recognition sites in the loop important for packaging (Fig. 3) (Parrott et al. 2000; Grahn et al. 2001). The maturase protein (A-protein) is not required for packaging of viral RNA, but the presence of A-protein in the capsid structure is important for the resistance of viral RNA to ribonuclease digestion (Argetsinger and Gussin 1966; Heisenberg 1966). Detailed research into bacteriophage MS2 not only elucidated basic processes, such as RNA transcription, translation, and sequencing, but has also enabled the application of MS2 in RNA detection. This application lies in the utilization of the MS2 bacteriophage packaging system for production of ribonuclease-resistant process controls which can be used in RT-PCR and qRT-PCR detection methods.

The first study in which non-bacteriophage RNA was encapsidated by MS2 coat protein was carried out at the beginning of the 1990s (Pickett and Peabody 1993). The main aim of this study was to determine whether the stem-loop nucleotide sequence would confer MS2-specific packageability on heterologous RNA in vivo using the advantage of a two-plasmid expression system. *E. coli* was co-transformed with two plasmids: the first encoded MS2 coat protein and the second encoded the β-galactosidase (*lacZ*) gene. The *lacZ* gene was modified so that the MS2 stem-loop sequence was cloned upstream of it. The MS2

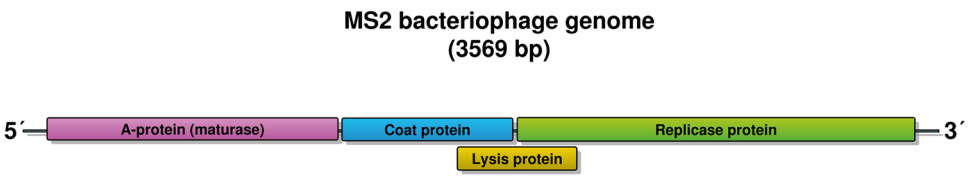

Fig. 2 The MS2 bacteriophage genome consists of 3569 nucleotides and encodes only four genes. The lysis gene overlaps the coat and replicase genes and is translated in the +1 reading frame

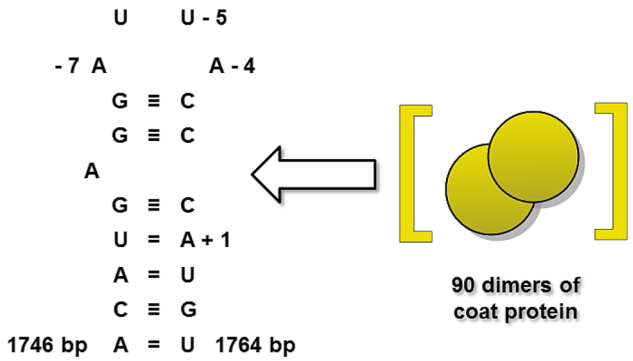

Fig. 3 Schematic representation of the interaction of the 19 nucleotide stem-loop structure (*pac* site) with the coat protein dimer. Numbers of bases are relative to the start of the MS2 replicase initiation codon AUG where A is +1. Adapted from Wei et al. 2008a, edited

coat protein and the stem-loop-*lac*Z hybrid RNA were co-expressed in *E. coli* and the encapsidation of the stem-loop-*lac*Z RNA into phage-like particles was investigated. The phage-like particles were purified and their different distribution in the gradient suggested substantial heterogeneity of RNA content. The stem-loop-*lac*Z RNA purified from phage-like particles was degraded to a major species of ~500 nt in contrast to the expected full length of around 3000 nt. The authors of the above study suggested that phage-like particles could be subjected to ribonuclease digestion because such a ribonuclease sensitivity was observed earlier with maturase-defective mutants of MS2 bacteriophage (Argetsinger and Gussin 1966) and their plasmid-produced particles also lacked maturase. It was also found that most of the packaged RNA molecules were 200 nt and 1800 nt in length and were easily detectable on agarose electrophoretic gels. The 500 nt stem-loop-*lac*Z RNA fragment was only detectable by Northern blotting using a specific *lac*Z probe. The authors hypothesized that 200 nt and 1800 nt packaged RNA fragments were derived from the pre-16S rRNA of *E. coli*. The packaging specificity of this two-plasmid expression system was very poor, because it was not possible to reach and maintain the appropriate molar ratio of coat protein to stem-loop-*lac*Z RNA fragments (Pasloske et al. 1998; DuBois et al. 1997). The packaging system of non-bacteriophage RNA based on a mechanism *in trans*, where the coat protein is continually translated from a different RNA than that of the packaging is not sufficiently effective and specific. Since there is no stem-loop sequence on the coat protein RNA, the coat protein is continually translated and similarly there is no control of the transcription of the stem-loop containing RNA. Therefore, the transcription of both RNAs as well as translation of the coat protein is constitutive. Because of this, production of the coat protein is not regulated at the level of translation and the level of coat protein becomes so high that RNA is packaged nonspecifically (DuBois et al. 1997).

Plasmid-Driven Packaging Systems for Production of MS2 Phage-Like Particles

Currently, the production of MS2 phage-like particles is based on various plasmid packaging systems (Table 1). The historical developments leading to the design of these systems, as well as their advantages, disadvantages and concrete examples of their use are summarized in the following sections.

One-Plasmid Packaging System and the Length of Packaged RNA

The packaging system of non-bacteriophage RNA based on an *cis* mechanism, where the sequences of the coat protein, maturase, and the stem-loop containing control sequence are localized on the same RNA molecule [recombinant RNA (reRNA)], eliminate the problem of overproduction of coat protein and thus the problem of nonspecifically packaged RNA molecules (DuBois et al. 1997). The so-called plasmid-driven packaging system is based on a vector, which contains maturase and coat protein bacteriophage MS2 sequences together with target RNA sequence containing a stem-loop sequence cloned downstream of an inducible *lac* promoter (Fig. 4). The presence of the maturase gene in the vector is not mandatory; however, its presence in the capsid stabilizes the whole structure and contributes to ribonuclease resistance. This strategy uses the high-fidelity *E. coli* RNA polymerase to transcribe the cloned sequence into reRNA using isopropyl-β-D-thiogalactopyranoside (IPTG) induction. Once the coat protein is translated it binds to the stem-loop sequence and initiates the packaging of the reRNA-containing control sequence into MS2 phage-like particles. Because the expression vector does not contain lysis and replicase genes of bacteriophage MS2 that are not essential for assembling the bacteriophage capsid, the MS2 phage-like particles are localized in the cytoplasm of *E. coli*.

The length of reRNA, which can be wrapped into MS2 phage-like particles, represents one of the most important parameters in the one-plasmid packaging system. However, in the first study to use this system only the de novo-constructed 172 nt RNA fragment was packaged into MS2 phage-like particles (Pasloske et al. 1998). Other authors packaged a 412 nt RNA fragment using the one-plasmid packaging system (WalkerPeach et al. 1999). It was suggested that because of the icosahedral structure of the MS2 bacteriophage capsid, the maximal size of reRNA, that can be packaged is theoretically around 4 kb in total (DuBois et al. 1997). From these 4, 1.7 kb are reserved for

Table 1 Plasmid packaging systems for production of MS2 phage-like particles

Packaging system	Maximum length of packaged RNA	Localization of phage and control sequences	Sequences packed in phage-like particles	Advantages	Disadvantages
One-plasmid system	Up to 2 000 nt	*cis*	Coat protein, maturase and control sequence	Simple construction	Limited capacity for packaging of control sequence
Two-plasmid system	Up to 3600 nt	*trans*	Only control sequence	Specificity	Lower efficiency of packaging
One-plasmid double-expression system	Up to 3600 nt	*cis*	Only control sequence	High efficiency of packaging, specificity	Complicated construction

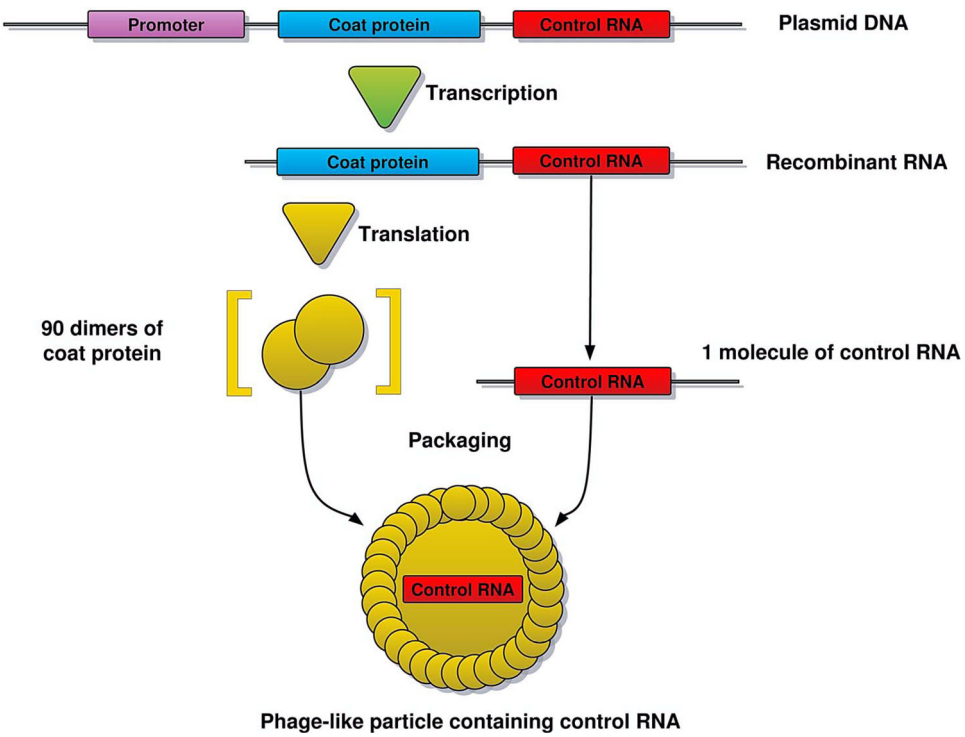

Fig. 4 Schematic representation of the one-plasmid packaging system

bacteriophage sequences encoding the maturase, the coat protein, and the stem-loop structure. Therefore, only about 2 kb are dedicated for control reRNA sequences.

To define the maximum size of the reRNA that can be packaged constructs designed to package bacteriophage λ RNA sequences of different size were created (Pasloske et al. 1998). It was found that only the construct encoding the 0.5 kb bacteriophage λ RNA contained a reRNA of the expected size. It was postulated that the efficiency of packaging decreased quickly as the size of the reRNA increased beyond 500 nt. Later, Huang et al. tried to directly package a 1200 nt control reRNA sequence (Huang et al. 2006). The MS2 phage-like particles containing the entire 1200 nt control sequence were successfully assembled. They found that by deleting some of the disposable sequences between the multiple cloning site and the transcription terminator they were able to increase the packaging capacity of the original vector without affecting packaging efficiency. So far, the largest target RNA that could be packaged was 1200 nt, using one stem-loop wild-type sequence in reRNA.

Not only the presence of a stem-loop sequence in the reRNA but also its composition is crucial for the formation of MS2 phage-like particles. It was shown that substitution of U-5 with C in the stem-loop structure increases the affinity between the coat protein and reRNA to six-fold or even as high as 50-fold that of the wild-type stem-loop (Lago et al. 1998; Stockley et al. 1995; Lecuyer et al. 1995; Romaniuk and Uhlenbeck 1985; Horn et al. 2004; Talbot et al. 1990; Lowary and Uhlenbeck 1987; Stockley et al. 1994). It was

also confirmed that the presence of a second stem-loop structure enables the cooperative binding of the coat protein dimer to the reRNA and results in a higher affinity compared with a single stem-loop structure (Pickett and Peabody 1993; Witherell et al. 1990). Wei et al. constructed a 1891 nt chimeric control reRNA sequence containing two C-5 variant stem-loop structures in order to explore whether reRNA longer than 1200 nt could be packaged using a one-plasmid packaging system (Wei et al. 2008a). They demonstrated that a reRNA with a length of 1891 nt can be packaged into MS2 coat protein utilizing a one-plasmid packaging system with two C-5 variant stem-loop structures and also showed that vectors with two C-5 variant stem-loop structures exhibited the highest expression efficiency. Therefore, the maximal length of reRNA that can be packaged using the one-plasmid packaging system is around 2000 nt.

Two-Plasmid Packaging System and the Length of Packaged RNA

The two-plasmid packaging system was developed in order to package sequences longer than 2000 nt, which cannot be packaged using the one-plasmid packaging system. In the two-plasmid packaging system the maturase and coat protein are expressed from one plasmid vector and the target control RNA sequence with modified C-5 stem-loop structure is transcribed from another plasmid vector (Fig. 5a) (Wei et al. 2008b). In the first study, where non-bacteriophage RNA was encapsidated Pickett and Peabody used the two-plasmid packaging system (Pickett and Peabody 1993). However, the system lacked specificity due to the inability of determining an appropriate ratio of coat protein to stem-loop-*lac*Z RNA. The system designed by Wei et al. differs from the Pickett and Peabody system in several ways (Wei et al. 2008b). The MS2 bacteriophage sequences located on the first plasmid in the Pickett and Peabody system consisted only of the coat protein sequence (Pickett and Peabody 1993). This is in contrast to the system of Wei et al., which has in addition the maturase sequence on the first plasmid (Wei et al. 2008b). The maturase protein is a very important component of MS2 phage-like particles because its presence is required to protect the integrity of RNA against the effect of ribonucleases (Argetsinger and Gussin 1966; Heisenberg 1966).

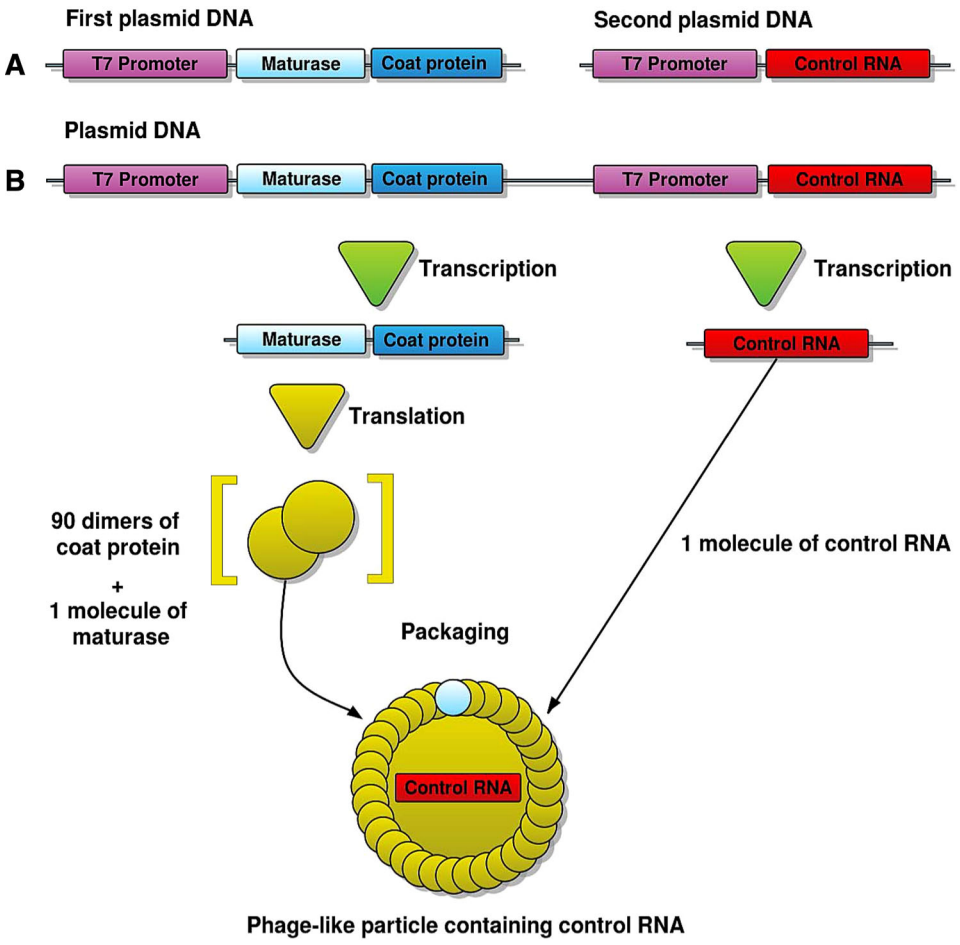

Fig. 5 Schematic representation of the two-plasmid packaging system (**a**) and one-plasmid double-expression packaging system (**b**)

In addition, the maturase protein interacts specifically with phage RNA at two sites (Shiba and Suzuki 1981) and may therefore play a substantial role in packaging.

Another difference is that Wei et al. chose two low-copy plasmids with almost equivalent copy numbers and containing the same T7 bacteriophage promoter (Wei et al. 2008b). Given this equivalence, the ratio of coat protein and the target RNA with the stem-loop sequence should be appropriate. Moreover, the target RNA sequence contains a C-5 variant stem-loop structure, which further increases the affinity between the coat protein dimer and target RNA. The biggest difference between the two-plasmid packaging system and the one-plasmid packaging system is that MS2 phage-like particles produced by the two-plasmid packaging system contain only control RNA sequences without any other bacteriophage MS2 sequences. The advantage of using control RNA of several kb in length is that such a long sequence can encompass multiple target control sequences for a variety of detected viruses. Accordingly, it is not necessary to construct different MS2 phage-like particles containing controls for each diagnostic assay. With these multiple controls, different research groups and clinical laboratories could directly compare their quantitative data.

Using the two-plasmid packaging system, Wei et al. were able to package a sequence of 2248 nt in length (Wei et al. 2008b). Theoretically, the length of packaged RNA using the two-plasmid packaging system could reach approximately 3.6 kb since the MS2 bacteriophage genome is 3569 nt in length. The same authors also tried to package a 2700 nt long sequence and were successful. In conclusion, the two-plasmid packaging system can be used to effectively package sequences longer than 2000 nt.

One-Plasmid Double-Expression Packaging System and the Length of Packaged RNA

The one-plasmid double-expression packaging system represents the latest and most advanced system for the packaging of different RNA sequences into MS2 phage-like particles (Zhan et al. 2009). In comparison with the two-plasmid packaging system, the one-plasmid double-expression system does not have the disadvantage of lower expression efficiency because all the necessary sequences for packaging and control sequences are located on one plasmid vector. The one-plasmid double-expression system consists of one plasmid vector with two cloning sites under the control of two T7 bacteriophage promoters (Fig. 5b).

Like in the case of the two-plasmid packaging system, MS2 phage-like particles produced using the one-plasmid double-expression packaging system contain only defined control RNA sequences without any other MS2 bacteriophage sequences. Wrapped control RNA contains more C-5 variant stem-loop structures, which ensures its recognition by coat protein dimers during encapsidation. This arrangement ensures maintenance of the optimal ratio of maturase and coat protein to the stem-loop containing RNA control sequence.

Zhan et al. used the one-plasmid double-expression packaging system to package a control RNA sequence 3034 nt in length containing three C-5 stem-loop structures (Zhan et al. 2009). Zhan et al. also discussed the enhanced packaging efficiency they observed, which was induced by increasing the number of stem-loop structures (Zhan et al. 2009). They assumed that it may be due to one or more of the following mechanisms. First, the initiation complex is able to form more quickly and is more stable with an increasing number of stem-loop structures (Johansson et al. 1998; Valegard et al. 1997), thus triggering packaging more efficiently. Second, the initiation complex promotes the continuation of the packaging with higher efficiency and at a higher rate (Valegard et al. 1997; Toropova et al. 2008). Third, the presence of a second stem-loop structure presumably makes the two coat protein dimers bind to the RNA in a cooperative manner, which results in higher affinity and lower sensitivities to pH, ionic strength and temperature than RNA with only a single stem-loop structure (Pickett and Peabody 1993; Witherell et al. 1990).

In Vitro Systems for the Production of MS2 Phage-Like Particles

Plasmid-driven packaging systems are based on the production of MS2 phage-like particles in vivo. In these protocols, a suitable *E. coli* strain (e.g., *E. coli* BL21 DE3 strain) is transformed by a recombinant vector bearing all the necessary sequences for the production of MS2 phage-like particles. Production is induced by the addition of IPTG into the *E. coli* culture. Subsequently, MS2 phage-like particles are assembled spontaneously in the cytoplasm of bacteria. Understanding the natural assembly process is useful for the production of MS2 phage-like particles in vitro.

In vitro synthesis of MS2 phage-like particles containing the sequence of choice is possible because the wild-type or MS2 phage-like capsid can be disassembled by treatment with acetic acid and then reassembled in the presence of a variety of stem-loop structure-containing RNA molecules simply by raising the pH toward neutrality (Sugiyama and Nakada 1970; Stockley et al. 2007; Sugiyama and Nakada 1967). In comparison with in vivo production of MS2 phage-like particles, the efficiency and fidelity of these in vitro reactions is lower and optimization of these reactions is difficult. Thus, the MS2 phage-like particles used as process control viruses in RT-PCR and qRT-PCR are synthesized mostly in vivo. In vitro systems for the

production of MS2 phage-like particles currently find application in methods for targeted drug delivery (Wu et al. 1995; Mastico et al. 1993; Wu et al. 2005; Brown et al. 2002; Galaway and Stockley 2013; Li et al. 2014).

Stability of MS2 Phage-Like Particles

Process control viruses should be very stable in long-term storage and their stability depends especially on the ability to resist the action of the ubiquitous nucleases. Pasloske et al. demonstrated that reRNA packaged within MS2 phage-like particles was completely resistant to DNase and ribonuclease treatment under conditions in which naked DNA and RNA were both degraded rapidly (Pasloske et al. 1998). These stability findings of MS2 phage-like particles are consistent with those of other authors (Song et al. 2011; Beld et al. 2004; Hietala and Crossley 2006; Wei et al. 2008b; WalkerPeach et al. 1999; Yu et al. 2008; Zhan et al. 2009). All these results demonstrate the high stability and durability of MS2 phage-like particles under different storage conditions.

Isolation and Purification of MS2 Phage-Like Particles

Expression vectors for the production of MS2 phage-like particles do not contain the lysis gene, which is not essential for assembling the bacteriophage capsid; therefore, the MS2 phage-like particles are localized in the cytoplasm of *E. coli* and must be released enzymatically by lysozyme (DuBois et al. 1997), or mechanically by ultrasonic disruption of the bacterial cells (Cheng et al. 2006; Wei et al. 2008a, b; Zhan et al. 2009; Yu et al. 2008). However, the major problem of current MS2 phage-like preparations is the purification procedure.

Like the MS2 bacteriophage, MS2 phage-like particles can be purified using traditional CsCl gradient ultracentrifugation. They band tightly at a concentration of 1.45 g/cm^3 (Pickett and Peabody 1993). However, the procedure is very laborious and expensive. Ultracentrifugation in a sucrose gradient can also be used to isolate MS2 phage-like particles; the particles localize at about the 35 % sucrose density layer (Cheng et al. 2006). Yu et al. used electroelution of MS2 phage-like particles from an agarose gel with a GeBAflex tube (Yu et al. 2008). Compared to purification of MS2 phage-like particles using traditional CsCl fractionation, the method of electroelution was efficient and easy to perform. In order to solve the problem of purification procedure a recombinant plasmid vector for the expression of MS2 phage-like particles harboring an affinity tag (polyhistidine-tag) at the surface of the capsid was constructed (Cheng et al. 2006). This allowed the isolation of extremely pure MS2 phage-like particles by affinity chromatography. This enhanced purification procedure is based on the finding that foreign peptides may be presented on the surface of the MS2 bacteriophage capsid (Mastico et al. 1993). The coat protein subunit does not have the conserved anti-parallel β-barrel topology seen in every other spherical RNA virus coat protein structure (Rossmann and Johnson 1989). Rather, coat proteins in the bacteriophage capsid are packaged in the form of non-covalent dimers, which are secured by interdigitation of C-terminal α-helices from each monomer. Underneath the helices lies an extensive β-sheet comprising five β-strands from each subunit, whereas at the N-terminus of the protein the polypeptide is folded into a β-hairpin structure, which protrudes from the surface of the bacteriophage capsid (Mastico et al. 1993). Thus, it was found that the insertion of foreign peptide sequences at the top of the β-hairpin structure does not lead to aberrant folding of bacteriophage capsids. However, the foreign peptide sequences must be inserted into the specific position of the coat protein sequence to avoid detrimental effects on phage capsid assembly. This position is located between residues 15 and 16 of the coat protein amino acid sequence. Foreign peptide sequences are inserted into this position using the method of site-directed mutagenesis (Sayers et al. 1988; Heal et al. 1999). Adenine at position 1380 in the sequence of the coat protein is replaced by thymine and thymine at position 1383 is replaced by cytosine. These changes lead to the creation of a *Kpn*I restriction enzyme cleavage site at position 1378 in the sequence of the coat protein without changes in the amino acid sequence. The foreign protein sequences can then be cloned into this *Kpn*I restriction enzyme cleavage site. This method of foreign peptide sequence insertion causes duplication of the codons for glycine and threonine at positions 14 and 15 in the coat protein thus leading to the inserts being flanked by the sequence Gly-Thr (Mastico et al. 1993). Cheng et al. used this system to produce His-tagged phage-like particles which can be easily purified using affinity chromatography on Co^{2+} (Fig. 6) (Cheng et al. 2006). They tried but failed to insert a StrepTag or Tat into the same position—no intact MS2 phage-like particles were obtained. In comparison with MS2 phage-like particles purified using gradient ultracentrifugation affinity-purified MS2 phage-like particles are extremely pure. Using the method of affinity chromatography greatly simplifies and reduces the cost of purifying MS2 phage-like particles.

Quantification of MS2 Phage-Like Particles

Transmission electron microscopy (TEM) is the most direct method for counting viral particles and can be

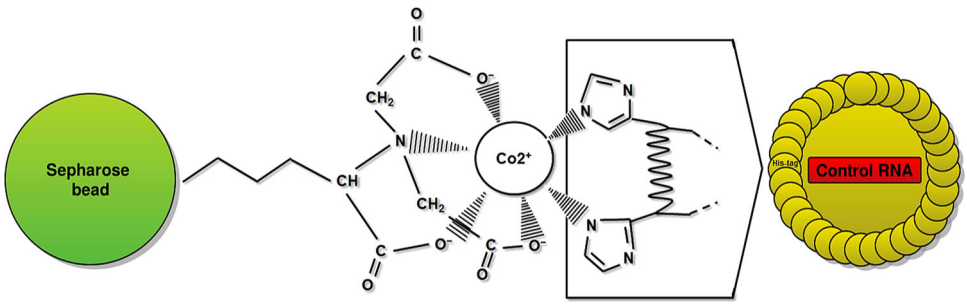

Fig. 6 Schematic illustration of the purification of His-tagged MS2 phage-like particles with a Co^{2+} affinity resin. Each MS2 phage-like assembly has 180 units of coat protein and each coat protein has a His_6 tag exposed outward from the phage-like assembly, allowing access of the chelated Co^{2+} on the Sepharose beads to the His-tag. Adapted from Cheng et al. 2006, edited

successfully used for determining the concentration of MS2 phage-like particles (Borsheim et al. 1990). However, the long analysis time required, as well as the high cost and difficulty of this method has led to the development of alternative methods.

One of the major advantages of MS2 phage-like particles is that they are not able to replicate. On the other hand, this major advantage is also a disadvantage with regard to their enumeration because MS2 phage-like particles do not form plaques. Therefore, their number cannot be easily determined using a conventional plaque assay. For this reason, some authors have used indirect methods of determining the number of MS2 phage-like particles. The number of MS2 phage-like particles may be determined using the Avogadro constant, extinction coefficient of 1 $OD_{260} = 0.125$ mg/ml of MS2 bacteriophage and the molecular weight of 3×10^6 (Cheng et al. 2006; Wei et al. 2008a; DuBois et al. 1997). Based on this procedure, approximately 1×10^{15} phage-like particles can be purified and counted from 1 L of *E. coli* production culture (DuBois et al. 1997; Yu et al. 2008). In some cases where the MS2 phage-like particles contain the sequence detected in the commercially available kits used for accurate quantification of viral load in the samples, these kits also can be used for quantification of MS2 phage-like particles. Pasloske et al. used the Amplicor® HIV-1 Monitor® kit for quantification of MS2 phage-like particles containing the 142 nt HIV *gag* sequence. This sequence serves as the target for primers used in this kit (Pasloske et al. 1998). Other examples of quantification of MS2 phage-like particles using commercial kits are quantification of MS2 phage-like particles containing nearly the full length HIV *pol* gene in the packaged sequence using the Versant HIV-1 RNA 3.0 assay (Zhan et al. 2009) and quantification of MS2 phage-like particles containing the 244 nt target consensus sequence using Amplicor® HCV Monitor® kit primers (WalkerPeach et al. 1999).

Determining the number of isolated MS2 phage-like particles is important for setting up the qRT-PCR reaction and for the entire nucleic acid extraction procedure. An excessive number of process control viruses can cause inhibition; conversely, too low an amount may cause problems with their detection limit. The exact number of added process control viruses must be evaluated individually for each detection and quantitative method. However, it was found that a number of MS2 phage-like process control viruses of approximately 10^4 copies/ml has a negligible influence on target amplification (Cheng et al. 2006). Therefore, commercially available MS2 phage-like particles are also offered in numbers of approximately 10^5 copies/ml.

Commercially Available MS2 Phage-Like Particles and Others Process Control Viruses

As discussed in previous sections, the production, isolation, purification, and quantification of MS2 phage-like particles requires knowledge of different laboratory methods. The requirements for laboratory expertise and sophisticated equipment may discourage many laboratories from producing their own MS2 phage-like particles for routine use as a process control virus in diagnostic methods based on RT-PCR and qRT-PCR. For these laboratories, there is the possibility to purchase these MS2 phage-like particles from commercial companies. Soon after the publication of the first article describing the technology for the production of MS2 phage-like particles, the wish was expressed that these process control viruses be commercially available as soon as possible (Cartwright 1999). The Asuragen company currently offers MS2 phage-like particles for diagnostic use under the trade mark of ArmoredRNA® and ArmoredRNA QUANT®.

ArmoredRNA® controls can be used for RNA extraction, amplification, detection, and as calibrating controls when new assays are being developed. These types of controls are available for use in RT-PCR detection systems for HIV (subtype B) (Mulder et al. 1994), HCV genotypes

1a, 1b, 2a/c, 2b, 3a (Young et al. 1993), Hepatitis G virus (HGV) (Schlueter et al. 1996), NoV GI and GII (Ando et al. 1995), West Nile virus (Briese et al. 1999; Briese et al. 2000; Lanciotti et al. 2000), Dengue virus (type 1) (Sudiro et al. 1997), Enterovirus (Schwab et al. 1995; Rotbart 1990), SARS BNI-1 (Drosten et al. 2003) and SARS CoV-NC (Emery et al. 2004).

ArmoredRNA QUANT® controls can be purchased in specified numbers of MS2 phage-like particles per volume units (5×10^5 copies/ml) and therefore can be utilized as process control viruses and for the establishment of standard curves in qRT-PCR. The number of particles is lot-to-lot consistent and these types of controls can be used in qRT-PCR detection and quantification systems for HIV (subtype B) (Mulder et al. 1994), HCV (genotype 2b) (Young et al. 1993), Enterovirus (Schwab et al. 1995; Rotbart 1990), and for the detection of the molecular marker of chronic myeloid leukemia BCR/ABL b3/a2, b2/a2, and e1/a2 (Burmeister et al. 2000). These commercially available controls are designed mostly like competitive controls compatible with commercial kits like the ArmoredRNA® Quant Human Immunodeficiency Virus (subtype B) with Amplicor® HIV-1 Monitor® kit or ArmoredRNA® Quant Hepatitis C Virus (genotype 2b) with Amplicor® HCV Monitor® kit.

The range of commercially available MS2 phage-like particles on offer is not wide. They are all produced using the one-plasmid packaging system, which means that the maximum length of packaged control reRNA does not exceed 500 nt (Pasloske et al. 1998; WalkerPeach et al. 1999). Thus, it is clear that these commercially available controls cannot be utilized in RT-PCR and qRT-PCR-based detection assays with specific requirements for control sequences. Moreover, the cost of these commercially produced MS2 phage-like particles is relatively high, especially in the case of ArmoredRNA® Quant products. However, anyone skilled in this field can relatively cheaply produce a huge amount of MS2 phage-like particles with strictly defined control sequences designed according to his/her needs.

ISO/TS 15216-1 and ISO/TS 15216-2 recommend the use of genetically modified mengovirus as the process control virus in detection of HAV and NoV from food matrices (Costafreda et al. 2006; Le Guyader et al. 2009; Butot et al. 2014). Genetically modified mengovirus can be bought from Ceeram Tools corp. which has an exclusive license for qRT-PCR mengovirus detection system. The Mengo Extraction Control® kit contains genetically modified mengovirus isolated from cell culture and the number of copies of mengovirus depends on batch number so the lot-to-lot number is not as consistent as in the case of ArmoredRNA QUANT® products. The Mengo Extraction Control® kit is designed for a rapid estimation of RNA extraction efficiency.

Conclusion

The appropriate use of process control viruses in the RT-PCR and qRT-PCR detection and quantification of RNA viruses from different matrices is a critical point of sample analysis. The use of process control viruses enables monitoring of the whole process of sample analysis including the nucleic acid extraction step. MS2 bacteriophage and MS2 phage-like particles are widely used to control the whole process of sample preparation, including RNA isolation. In particular, MS2 phage-like particles represent suitable process control viruses for the detection of RNA viruses and moreover, process control viruses based on MS2 phage-like particles are used in routine clinical diagnostics for a vast number of viruses. The key features of these particles are their ability to protect the control RNA contained in them from degradation by ubiquitous ribonucleases, non-infectivity, long-term storage stability under different conditions, their inability to replicate and the possibility of packing specific control sequences. The relative simplicity of MS2 phage-like particle preparation, the possibility of plasmid-driven packaging systems, the ability to pack ssRNA of variable lengths, cheapness, fastness, and the capability of producing huge quantities are also significant advantages of MS2 phage-like particles.

The situation regarding the utilization of MS2 phage-like particles as process control viruses in the detection of RNA viruses from food matrices differs from the scenario in clinical diagnostics. In the literature, there are no reports in which MS2 phage-like particles were used as process control viruses for the detection of RNA viruses in food matrices. Only wild-type bacteriophage MS2 was used for this purpose (Blaise-Boisseau et al. 2010; Rolfe et al. 2007; Shulman et al. 2012) and the results showed that the MS2 bacteriophage represents a very reliable process control virus. At the same time, MS2 phage-like particles have similar, if not the same physicochemical properties as the wild-type bacteriophage MS2. Therefore, nothing prevents the use of MS2 phage-like particles as process control viruses in the detection of RNA viruses from food matrices, similarly as in the case of clinical specimens. Currently, the only commercially available system for the process control in food matrices is based on the use of genetically modified mengovirus as the process control virus in detection of HAV and NoV from food matrices (ISO/TS 15216-1, 2013).

It should be stressed that MS2 phage-like particles cannot be used universally as process control viruses. MS2 phage-like particles are good process control viruses only in the detection of certain types of RNA viruses. In general, MS2 phage-like particles should be used as process control viruses in the detection of structurally similar RNA

viruses—non-enveloped, small ssRNA viruses with icosahedral structure—so as to closely mimic their physicochemical properties during the RNA extraction step. Therefore, MS2 phage-like particles can be good process control viruses in the detection of HAV, HEV, or NoV from food matrices. However, in the literature, we can also find examples of utilization of MS2 phage-like particles as the process control viruses in detection of coronaviruses, which are enveloped and in comparison with MS2 phage-like particles relatively larger ssRNA viruses (Cheng et al. 2006; Yu et al. 2008). Therefore, researchers should always consider the suitability of MS2 phage-like particles as process control viruses with regard to the target virus to be detected.

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Conflict of interest The authors declare that they have no conflict of interest.

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