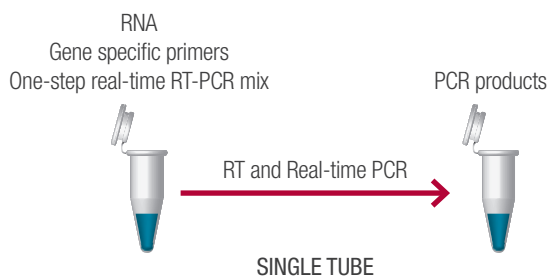


One-step real-time RT-PCR versus two-step real-time RT-PCR

Real-Time PCR has become an increasingly popular technique for analysis of gene expression. There are two primary methods of real-time PCR that can be performed. The first involves including the reverse transcriptase step in the same tube as the PCR reaction (one-step). The second method involves creating cDNA first by means of a separate reverse transcription reaction and then adding the cDNA to the PCR reaction (two-step). There are advantages and disadvantages to both systems that you should consider before choosing the best one for your application, these include the ease of use and cost of reaction to the resulting yield and sequence representation. One-step reactions are certainly easier to set up with less overall hands-on time, but do not provide the flexibility and control that is possible with two-step reactions.

ONE-STEP REAL-TIME RT-PCR - ideal for high throughput screening



Advantages

- Accurate representation of target copy number
- Simple and rapid
- Fewer pipetting steps (reducing possible errors and contamination)
- Best option for high-throughput screening
- Best method when only a few assays are run repeatedly
- Multiplex PCR of gene of interest and control can be done in single well, from same RNA sample

Gene-specific primers are used for generating the cDNA and for subsequent amplification in one tube reducing experimental variation since both enzymatic reactions take place under the same conditions, making one-step real-time RT-PCR highly reproducible. There are several other advantages of one-step reactions, these include limited sample handling and reduced bench time, which helps to decrease chances for pipetting errors and cross contamination between RT and real-time PCR steps. This method is quick to set up and makes processing multiple RNA samples easy (especially when using liquid

handling robotics), when you are amplifying only a few genes of interest. It is therefore ideal for high throughput screening laboratories where only a few assays are run repeatedly, using well-established reaction conditions, with the added advantage that multiplex PCR of the gene of interest and control genes can be done in single well, from same RNA sample.

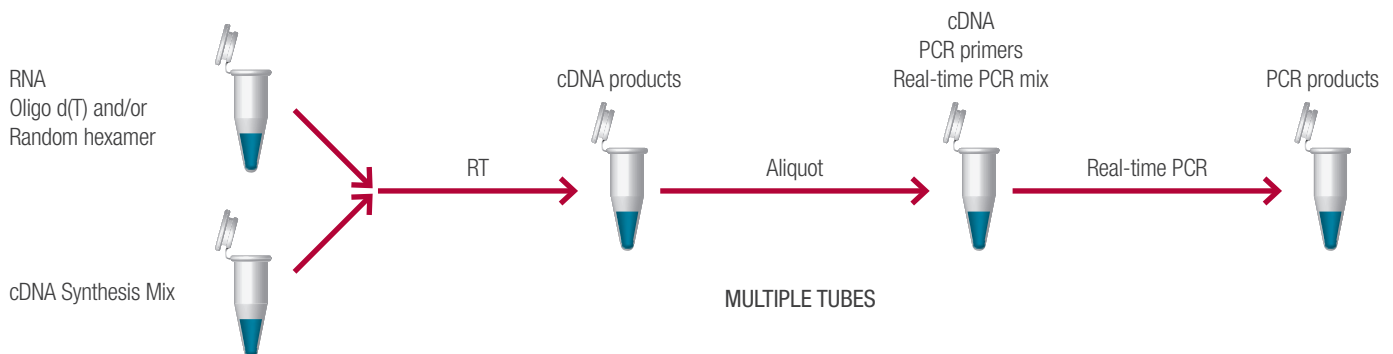
Considerations

- Usually less sensitive as it is impossible to optimize the two reactions separately
- Difficult to troubleshoot RT step
- No stock of cDNA

With one-step real-time RT-PCR, the quality of the RNA used in the reaction is very important, as all of the cDNA is used for the subsequent PCR step, also the reaction conditions needed to support both the RT and PCR may not be optimal for either reaction, affect efficiency and yield. Because of this, one-step reactions may require substantially more RNA in your initial samples if you are performing multiple amplifications and variation between these different RT reactions can complicate assay interpretation significantly. One-step real-time RT-PCR is therefore generally less sensitive than two-step RT-PCR.

One-step real-time RT-PCR also requires careful evaluation to prevent primer dimer formation because the primers will be present during the lower temperature conditions of the RT reaction as well as the PCR cycling.

TWO-STEP REAL-TIME RT-PCR - flexible



Advantages

- Two buffers optimized for independent RT and real-time PCR
- Highly sensitive
- Potentially more efficient because random primers and oligo d(T) can be used
- Possibility to stock cDNA to quantify several targets
- Recommended when the reaction is performed with a limiting amount of starting material.

When performing a reverse transcription reaction for a two-step assay, it is possible to use different primer strategies. Random hexamer primers, which bind anywhere on the RNA allow full coverage of total RNA (including ribosomal, bacterial and viral RNA), oligo d(T)_n (or anchored oligo d(T)_n), which binds to the poly-A tail of the mRNA which is located at the extremity of the transcript, leading to full transcripts, a mixture of random hexamer and oligo d(T)_n (such as in the SensiFAST™ cDNA Synthesis Kit) that reduces the risk of bias in cDNA synthesis and specific primers, which bind to the transcript of interest.

The ability to optimize both cDNA and real-time PCR reactions separately and the higher cDNA yields, two-step reactions can be more sensitive than one-step reactions. The ability to separately optimize these reactions is also beneficial when performing real-time PCR on challenging sequences.

Because the process of reverse transcription is notorious for its highly variable reaction efficiency, using dilutions from the same cDNA template ensures that reactions from subsequent assays have the same amount of template as those assayed earlier. The two-step method is therefore particularly useful when the goal is to detect multiple messages from a single sample or perform multiple PCR amplifications from a single sample.

Considerations

- Time consuming
- More pipetting steps (increases possible error and contamination)
- Requires more optimization

With two-step real-time PCR, the use of several tubes means that it is more time consuming and less adaptable to liquid handling robotics and so more difficult to adopt for high throughput screening assays. The use of several tubes and pipetting steps also exposes the reaction to a greater risk of DNA contamination.

IN SUMMARY

Using gene specific primers, one-step real-time RT-PCR such as the SensiFAST™ One-Step kits offer a quick and simple method to detect mRNA and so are useful when analyzing a few genes over a large number of samples as less pipetting and sample manipulation reduces variation and potential contamination. However reaction conditions needed to support both the RT and PCR may not be optimal for either reaction and it is not possible to archive the cDNA produced during the reverse transcription reaction.

Two-step real-time RT-PCR such as the SensiFAST™ kits in contrast, offers a truly accurate determination of mRNA and is useful when analyzing a large number of transcripts over a few samples. SensiFAST™ kits have flexibility in the priming strategy, allowing for oligo-dT, random primers or gene specific primers and are generally more sensitive than one-step as the RT and PCR occur separately and can be optimized individually. Also, the cDNA produced is more stable than the initial RNA sample and can be more easily archived for future use.