**Real-time PCR: Principle, Procedure, Advantages, Limitations and Applications**

A technique used to quantify the nucleic acid (DNA/RNA) present in a sample, during the PCR reaction is called real-time PCR or quantitative (q)PCR”

or

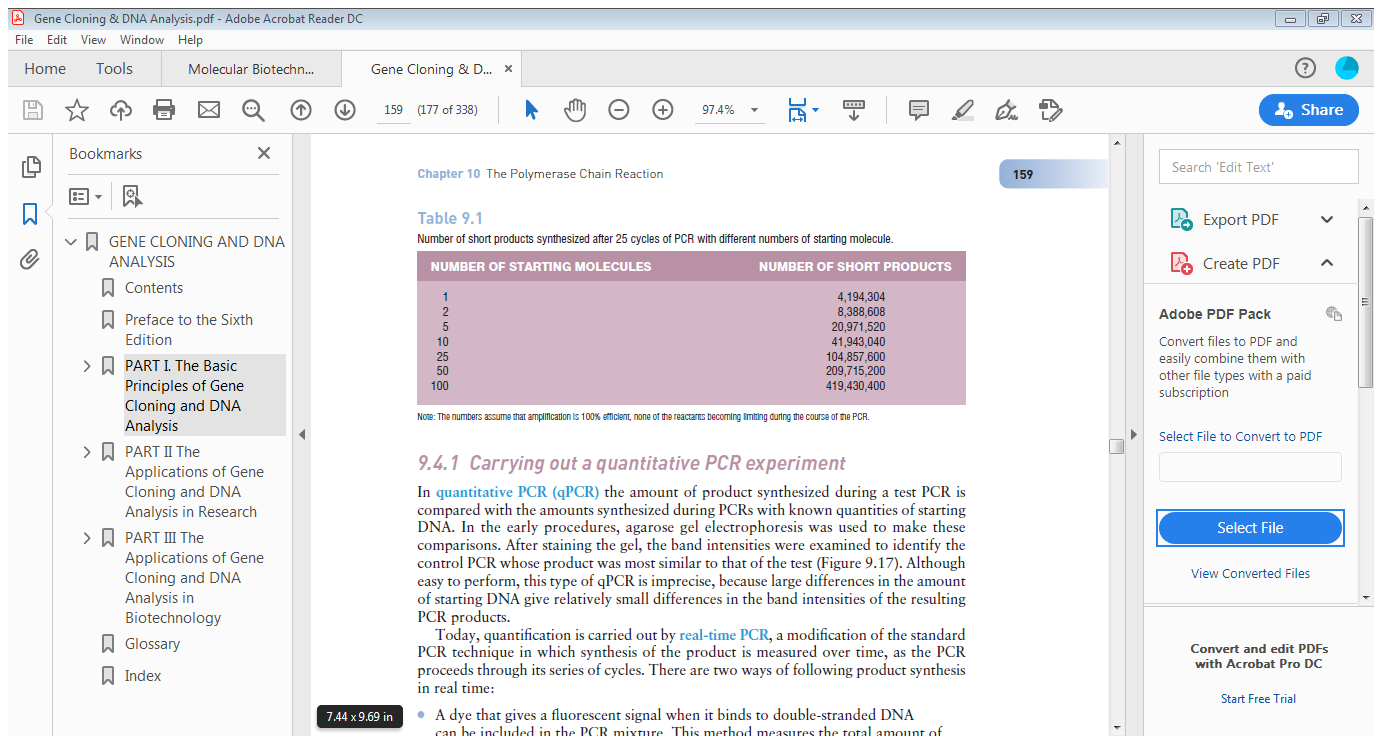
A molecular biology technique used to monitor the amplification of the target DNA/RNA sequence is referred to as real-time PCR or quantitative PCR.” This approach allows one to quantify the amount of a specific DNA fragment in the starting material. Labeling the DNA is achieved using any one of a variety of protocols.

Depending on the number of starting DNA molecule in PCR, number of amplified product varies

In the early procedures, to quantify the amplified products, agarose gel electrophoresis was used. After staining the gel, the band intensities were examined to identify the control PCR whose product was most similar to that of the test.

This technique is also called real time PCR, a modification of the standard PCR technique in which synthesis of the product is measured over time, as the PCR proceeds through its series of cycles.

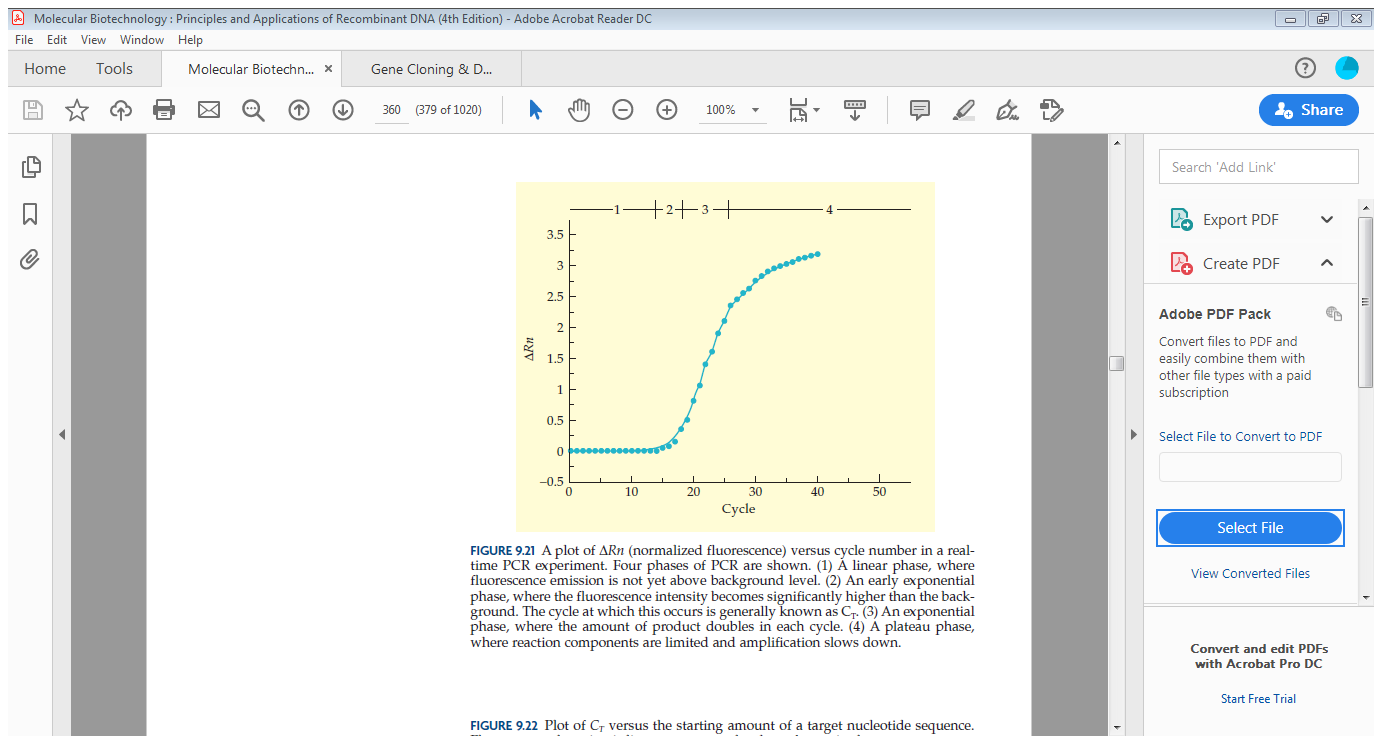
In the real-time PCR, the amplification during each PCR cycle is monitored in a real-time manner.

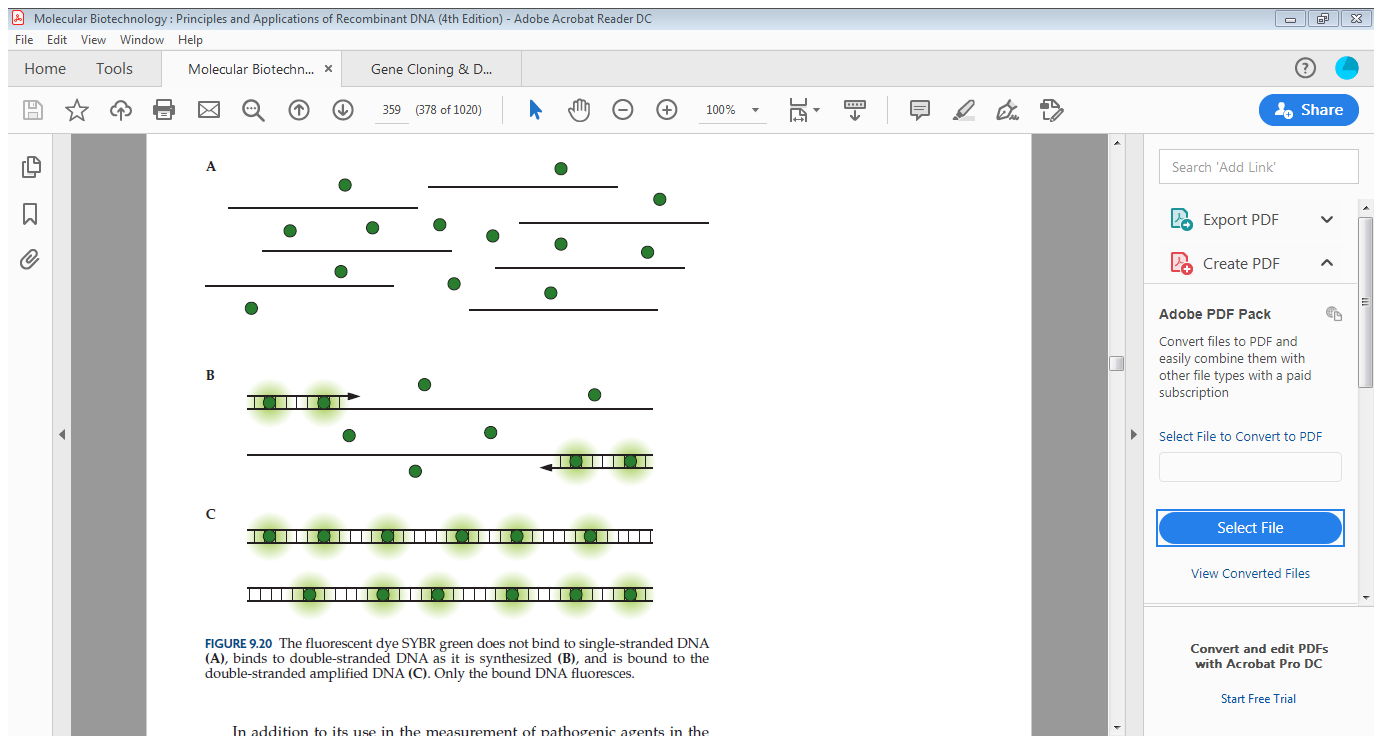
There are two ways of following product synthesis in real time:

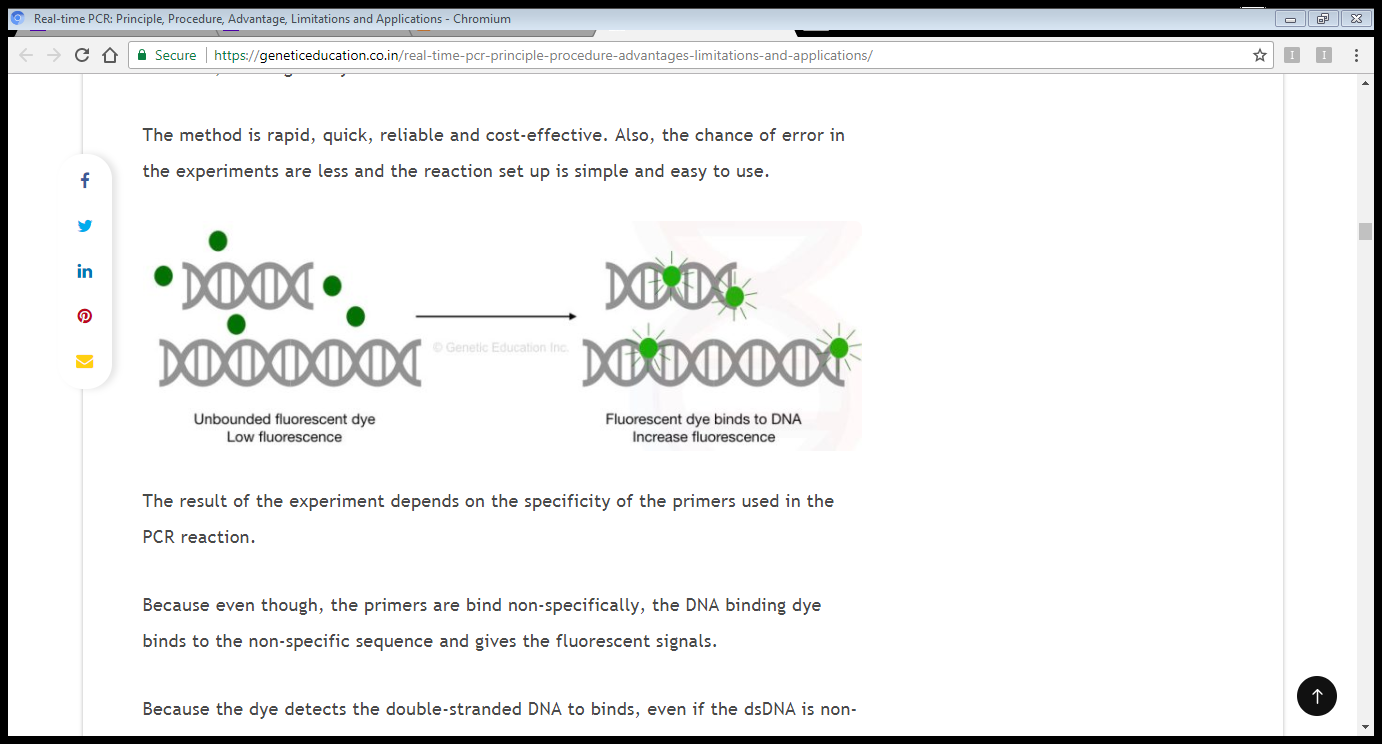
1. A dye that gives a fluorescent signal when it binds to double-stranded DNA can be included in the PCR mixture. This method measures the total amount of double-stranded DNA in the PCR at any particular time, which may over-estimate the actual amount of the product because sometimes the primers anneal to one another in various non-specific ways, increasing the amount of double-stranded DNA that is present.
2. A short oligonucleotide called a **reporter probe**, which gives a fluorescent signal when it hybridizes to the PCR product, can be used. Because the probe only hybridizes to the PCR product, this method is less prone to inaccuracies caused by primer-primer annealing. Each probe molecule has pair of labels.

Real-time PCR may be described as occurring in four phases. In the first, or linear, phase (Fig. 9.21, phase 1), which generally takes about 10 to 15 cycles, fluorescence emission at each cycle has not yet risen above the background level. In the early exponential phase, the amount of fluorescence reaches a threshold at which it is significantly higher than the background. The cycle at which this occurs is known as the threshold cycle (CT, or CP, depending upon the manufacturer of the PCR equipment). The CT value is inversely correlated with the amount of target DNA in the original sample. During the exponential

phase, the amount of product doubles in each cycle under ideal conditions, while in the plateau stage , the reaction components become limited and measurements of the fluorescence intensity are no longer useful.



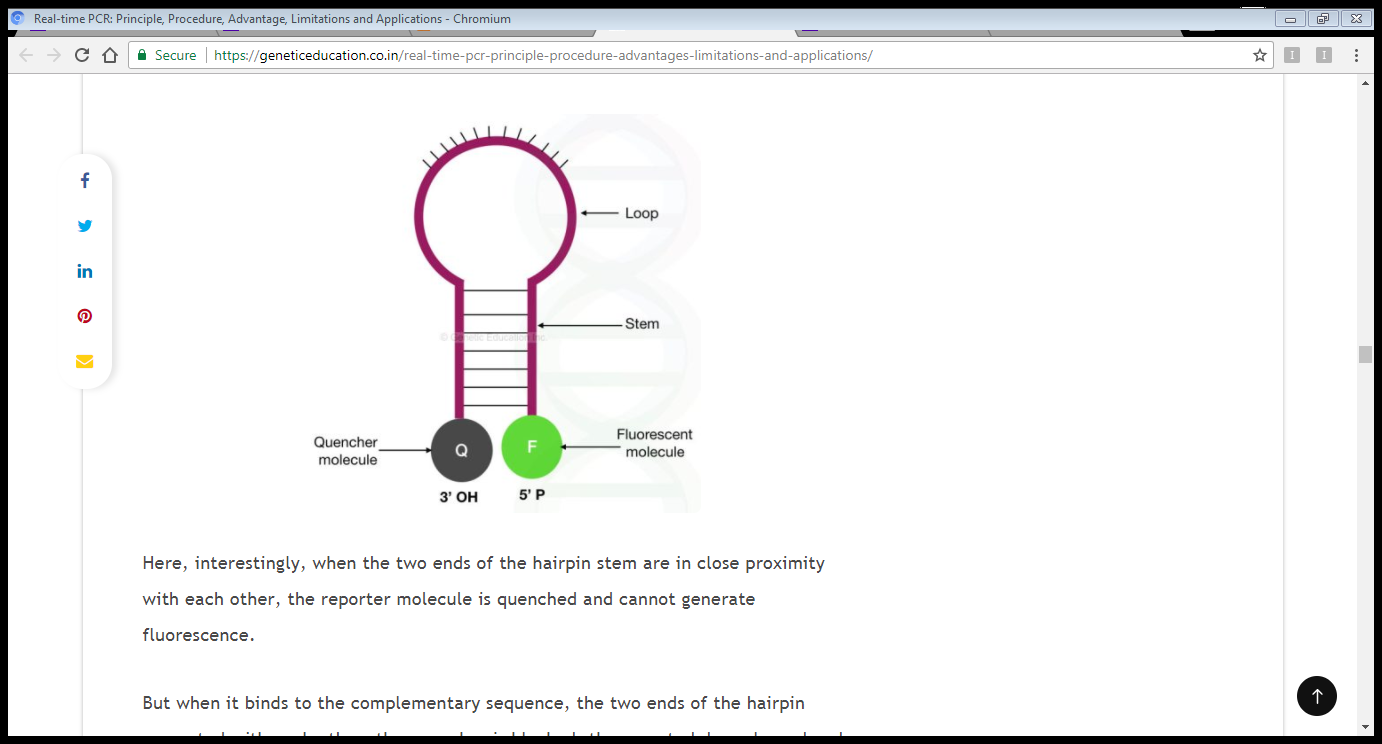




Fluorescent DYES used: SYBER GREEN

Probe based detection method

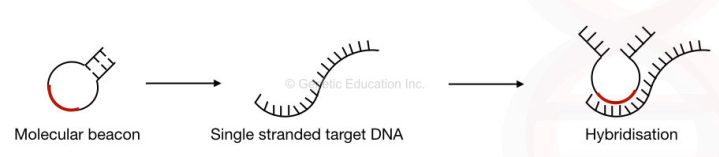
In this case the probe is a hairpin loop-like structure of the oligonucleotides which has complementary sequences on both the ends. The central loop is complementary to the target sequences. One end of the hairpin loop has the quencher dye and one end has the reporter fluorescent dye.

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**Here, interestingly, when the two ends of the hairpin stem are in close proximity with each other, the reporter molecule is quenched and cannot generate fluorescence.**

**But when it binds to the complementary sequence, the two ends of the hairpin separated with each other, the quencher is blocked, the reported dye released and emits the fluorescence.**

The emission is recorded by the detector. See the figure below,



The molecular beacon probes are highly sequenced specific and are the best choice for sensitive reactions.

If the probe (molecular beacon) cannot find its complementary sequence, it remains in the hairpin loop form and prevents non-specific bindings.

**Advantages of Real-time PCR**:

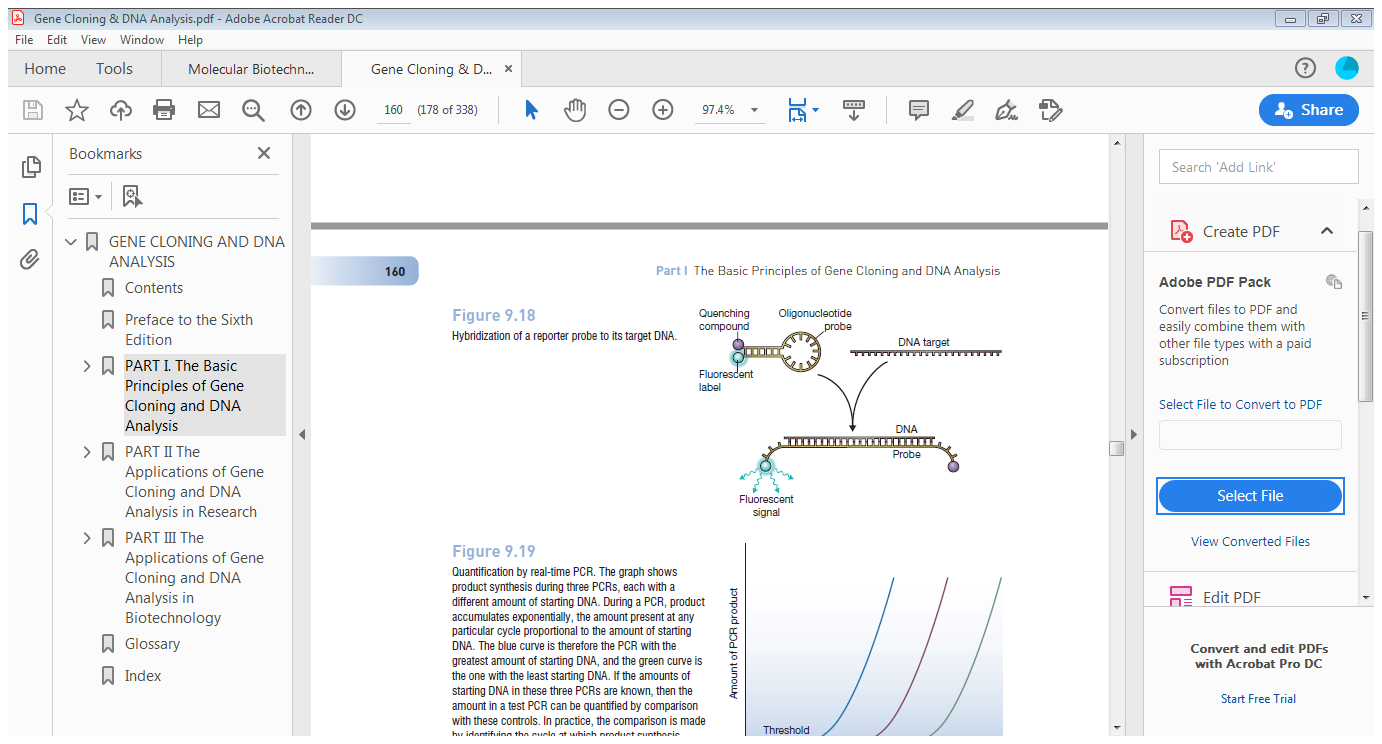
*The method is cost-effective.*

The conventional PCR method is costly than the qPCR due to the use of so many other chemicals and agarose gel electrophoresis.

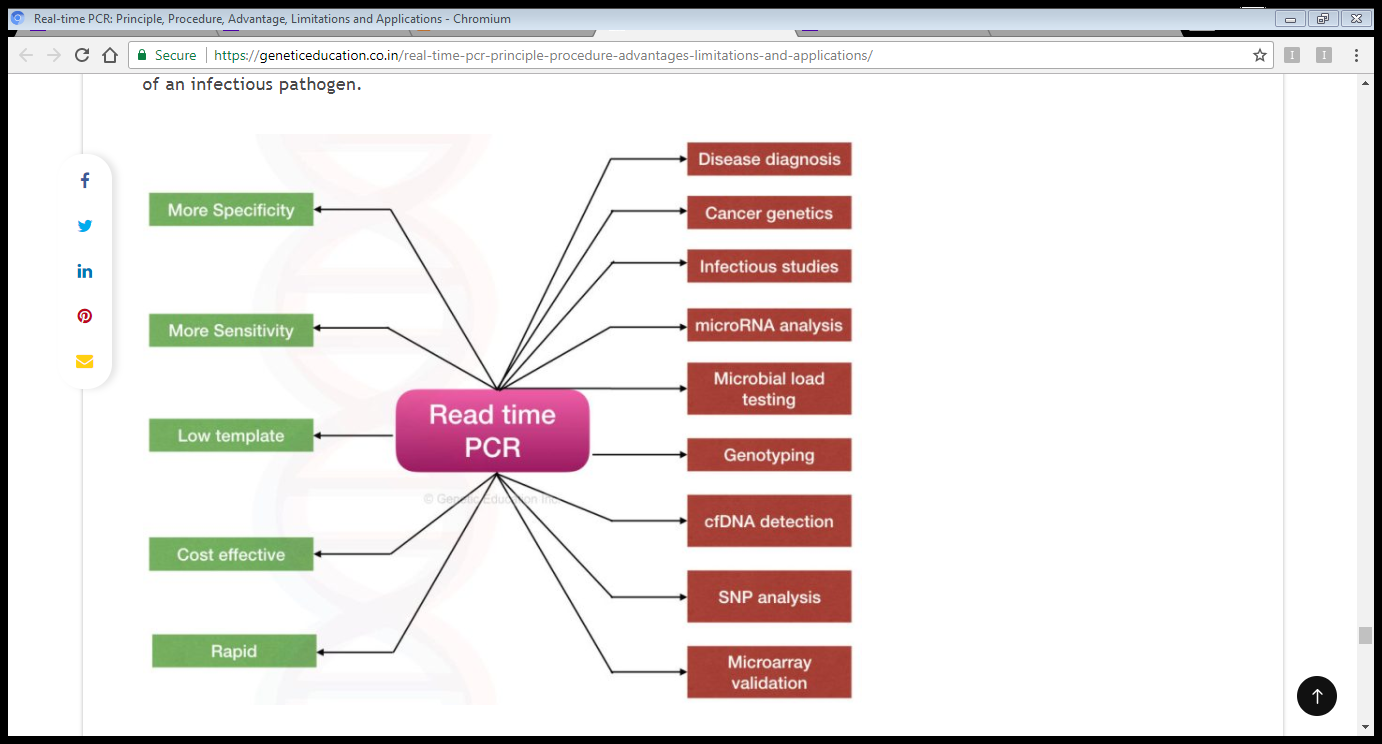
*It is time-efficient.*

The average time consumed by the PCR reaction along with the agarose gel electrophoresis and data interpretation is approximately 4 to 4.5 hours.

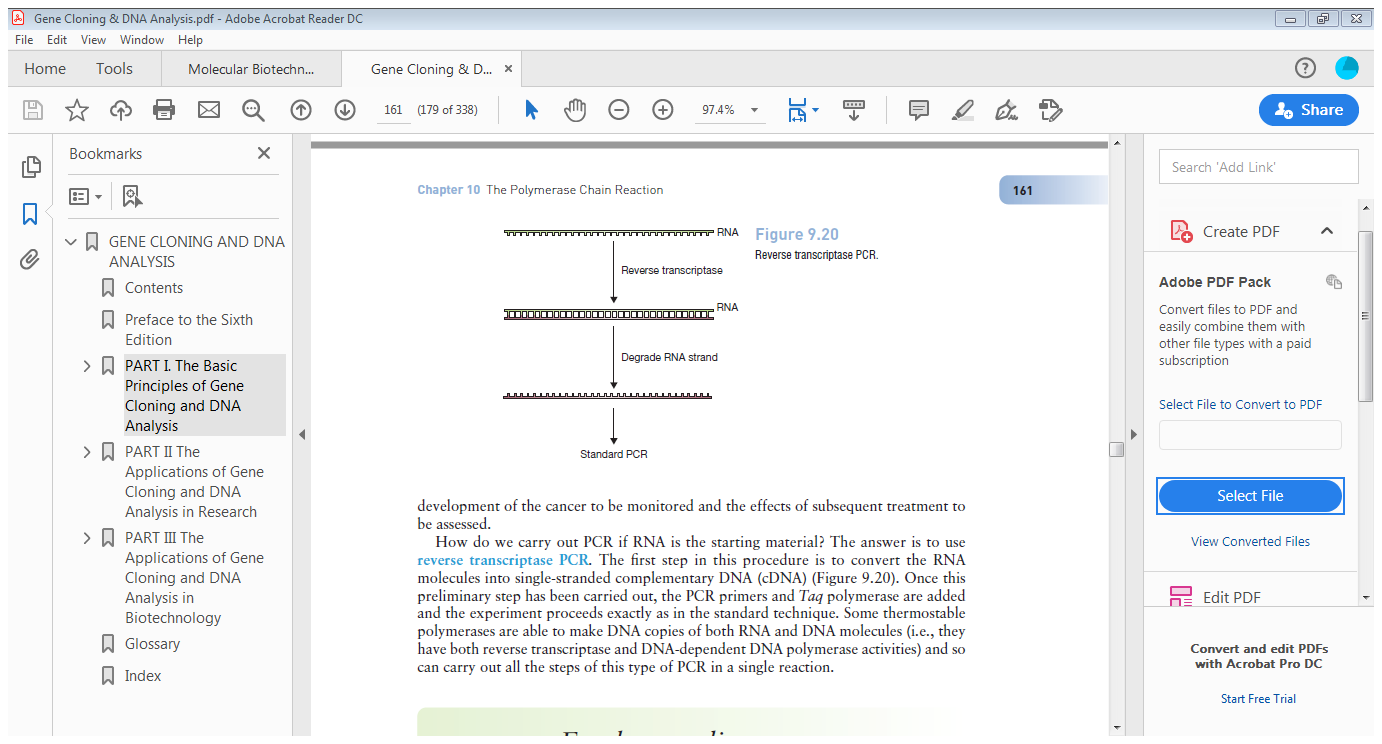
Contrary, real-time qPCR gives results in ultra-fast time. The average duration of the qPCR reaction is around 30 minutes to 2 hours.

*More sensitivity and specificity*.- The quantitative real-time PCR method is more sensitive, specific and efficient. Though the probes and primers are highly equence-specific, if any non-specific bindings occurred, it is monitored immediately during the reaction. Also, the main reaction or the quantification of our template cannot be influenced by the non-specific bindings.

The overall assay required less amount of the template material. It required 1000 folds less template DNA or RNA for the reaction to occur as compared with the conventional PCR.



Real-time PCR can also quantify RNA



References

1. <https://geneticeducation.co.in/>
2. T.A. BROWN, Gene Cloning and DNA analysis, Sixth Edition, Wiley-Blackwell
3. Bernard.R. Glick, Jack J Pasternak, Cheryl L. Patten, Molecular Biology, Fourth Edition,