

# *In vitro* nucleic acid amplification systems

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*In vitro* amplification systems mainly constitute the polymerase chain reaction (PCR) that permits the amplification of defined sequences of DNA; the ligation chain reaction (LCR), based on cycles of oligonucleotide-targeted ligation to monitor amplified segments for the presence of mutations and self-sustained sequence replication (3SR), an isothermal transcription-based amplification of single-stranded RNA. We critically review their basic methodologies and uses both as diagnostic techniques and research tools. Various aspects of PCR, including overestimation and underestimation of PCR data, have been mentioned. Amplification of nucleic acids has been applied in many areas of biological research permitting studies that were not possible before.

THE concept of making large quantities of DNA has its origin since the discovery of DNA polymerase by Kornberg<sup>1</sup>. However, only in 1984 did Kary Mullis<sup>2</sup> harness the Klenow fragment of *E. coli* DNA polymerase I to extend annealed primers in the polymerase chain reaction (PCR). PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences. It uses two oligonucleotide primers which hybridize to opposite strands and flank the region of interest in the target DNA. Multiple cycles of independent steps carried out at defined temperatures and result in the accumulation of a specific fragment of DNA whose termini are defined by the 5' end of the primers. The steps involved are denaturation (94–98°C), primer annealing (37–65°C) and extension of the annealed primers (at about 72°C) by a thermostable DNA polymerase (Taq polymerase). Because the primer extension products synthesized in one cycle can serve as a template in the next, the copy number of target DNA gets approximately doubled after every cycle. The length of the products generated during PCR is equal to the sum of the lengths of two primers plus the distance of the target DNA between the primers. The process has been diagrammatically represented in Figure 1.

The PCR technique has been utilized for a number of diverse applications such as detection of mutations, rearrangements or deletions within genes, quantitation of gene expression, cDNA amplification (where RNA serves as template through reverse transcription), isolation and cloning of new genes, identification of specific micro-organisms in clinical samples, in forensic sciences and paternity testing etc. Applications of PCR

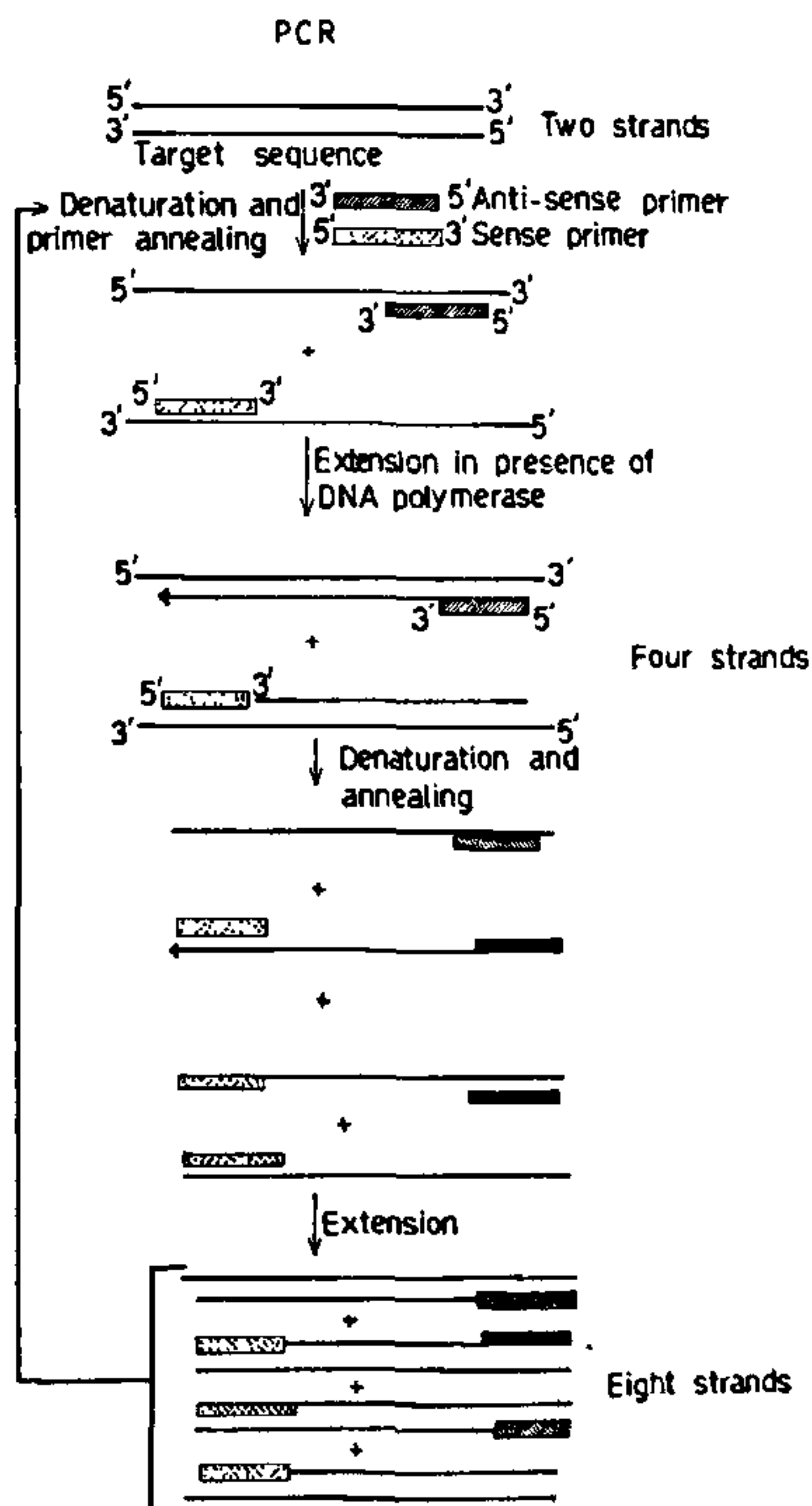


Figure 1. Schematic diagram of PCR amplification depicting various steps involved in different cycles of PCR. In the first cycle two strands of target DNA are denatured followed by primer annealing. Antisense primer (shaded one) gets annealed to one strand and the sense primer (dotted one) gets annealed to another strand in opposite orientation. In the presence of thermostable DNA polymerase the annealed primers get extended resulting in four strands. In the first cycle there is one molecule of DNA (two strands), in the second cycle two molecules of DNA (four strands) and in the third cycle there are four molecules of DNA (eight strands) resulting in exponential amplification.

technology have revolutionized the analysis of DNA samples obtained from prenatal (few hour old embryos) to thousands of years after death, from tissue biopsies, smears, hair roots, blood spots, paraffin-embedded

tissue sections, whole blood samples and from single cells<sup>1</sup>.

With widespread and varied applications of PCR, a battery of research reports have accumulated describing the details of various modifications and experimental procedures for diversified uses of PCR, almost with the same frequency and ease as the amplification of products in the basic chain reaction protocol. In this review, we report the current status of PCR technique, some of the critical tips to be kept in mind before setting up PCR reaction and related techniques developed from the basic principle of PCR.

### Primers

Success or failure of amplification reactions basically depend on the correct sequence of the primers. The mistakes here will result in either no amplification or nonspecific amplification. There are no set rules which will ensure the synthesis of an effective primer pair. Primers should have a random composition of bases and the G:C content similar to that of the entire fragment being amplified, ideally 50% or higher G:C content (higher the G:C, better the annealing). Sequences with significant secondary structures, particularly at the 3' end of the primer, should be avoided. Primer sequences should be in reverse orientation to each other and the ideal length of a primer is about 20–30 bases. There should not be any complementarity between primers, particularly at 3' end so that the incidence of 'primer-dimer' can be reduced<sup>4</sup>. A number of computer programs are now available to facilitate the primer designing.

The optimal concentration of primers to be used in the reaction mixture ranges between 0.1 and 0.5  $\mu\text{M}$ . Higher primer concentration should be avoided as this may promote mispriming and accumulation of nonspecific products. The calculated  $T_m$  (melting temperature) for a given set of primer pair (amplimers) should be balanced. A thumb-rule for the calculation of  $T_m$  is 2°C for every A or T and 4°C for every G or C. The temperature and length of time required for primer annealing depends upon the base composition, length and concentration of the amplification primers. Annealing temperature should be 5–10°C below  $T_m$  of the amplimers. By increasing the annealing temperature, mispriming can be minimized, which will result in reduced extension of non-specific sequences. Therefore, stringent annealing temperatures, especially during the first few cycles, will help in increasing the specificity of the primer pair used in the reaction mixture.

### PCR buffer

Changes in PCR reaction buffer conditions usually affect the quantum of amplification. Particularly, the

concentration of  $\text{MgCl}_2$  can have a profound effect on the specificity and yield of amplification. The presence of EDTA or other chelators in primer stocks or in template DNA may disturb the apparent  $\text{Mg}^{2+}$  concentration. Generally, excess of  $\text{MgCl}_2$  in PCR reaction results in the accumulation of non-specific products. Therefore, the titration of  $\text{Mg}^{2+}$  concentration in the range of 1.5 to 4 mM is highly desirable. Some recent protocols have recommended the use of 10% dimethyl sulphoxide (DMSO) to reduce secondary structures of target DNA. Use of 0.1% triton-X 100 has also been shown to have stabilizing effect on several enzymes used in PCR reaction and helps in better yield.

### Deoxynucleotide triphosphates (dNTPs)

A primary stock of 10 mM of each of the four dNTPs is prepared and used in a final concentration of 200  $\mu\text{M}$ . Approximately 50% of dNTPs are left even after 50 cycles of amplification. The dNTPs appear to quantitatively bind with  $\text{Mg}^{2+}$ . The amount of dNTPs present in a reaction will determine the amount of free  $\text{Mg}^{2+}$  available for optimal enzyme activity. Stock dNTP solutions should be neutralized with Tris base to pH 7.0 and their concentrations should be determined spectrophotometrically. However, this is cumbersome. A number of biotechnology companies have come up with ready-to-use dNTP solutions which are stabilized and have higher shelf half-life and are easy to use.

### Enzymes

Thermostable DNA polymerase is the enzyme required for the amplification reaction and is now manufactured by different companies with different trade names. Different DNA polymerase preparations have been reported to amplify with different efficiency (i.e. yield of product per cycle) and fidelity (error-free incorporation of nucleotides) depending on reaction conditions. Each error once initiated is amplified along with the original wild type sequences, increasing the fraction of polymerase-induced mutant sequence linearly with the number of amplification cycles. It is advisable to titrate each batch of enzyme for the amount required for optimal amplification.

Taq DNA polymerase, the commonly used enzyme in PCR reaction, has been isolated from *Thermus aquaticus*, which has 5' to 3' polymerase activity but lacks a 3' to 5' exonuclease activity. Amplitaq marketed by Perkin Elmer Cetus Corporation, USA is the recombinant form of Taq polymerase gene cloned in *E. coli*. It has an extension rate of 2000 to 4000 bases per minute at 70–80°C. Vent<sup>TM</sup> DNA polymerase from New England Biolabs is a high-fidelity recombinant thermophilic DNA polymerase from *Thermococcus litoralis*.



**Table 1.** Comparison of thermostable DNA polymerase isolated from various sources and marketed by different companies<sup>5</sup>

Enzymes	Error rate (%)	Calculated half-lives at 95°C (hours)
Taq	20	1.6
Vent	8	6.7
Pyrococcus (Pfu)	1.6	23

The error rate of Vent<sup>TM</sup> DNA polymerase is 5–15-fold less than that of Taq DNA polymerase. This high fidelity is derived in part from an integral 3'–5' proof-reading exonuclease activity. Greater than 90% of the polymerase activity is retained even after one hour incubation at 95°C. Recently, the Pfu enzyme, isolated from the hyperthermophilic marine archaebacterium *Pyrococcus furiosus*, has been introduced to the market and seems to be the best for its thermostability and fidelity among all the enzyme preparations available. A comparison of fidelity or misincorporation and thermostability is given in Table 1.

### Analysis of PCR-amplified products

The basic principle of PCR technology is to generate a large amount of a particular stretch of DNA for a conclusive analysis, which includes the following commonly used techniques.

#### Detection of amplification

Simple and sensitive detection methods are required to use the amplification technology in the clinical or research environment. Classical detection methodologies involve the analysis of DNA in agarose gels or in oligo-hybridization format<sup>6</sup>. In agarose gel analysis, the addition of ethidium bromide to the gel itself and in the running buffer, in our observation, improves resolution rather than staining of the gel after electrophoresis. In oligo-hybridization usually a gamma <sup>32</sup>P-labelled unique stretch of nucleotides from the amplified product sequence is used to confirm the authenticity of amplification. However, since these methods use radioactive probes to achieve sensitive detection, their drawbacks are the instability and the hazards generally associated with radioactivity. A rapid and sensitive method for detecting HIV-1 DNA sequences amplified by PCR has been developed by Suzuki *et al.*<sup>7</sup> which uses solution phase hybridization for rapid annealing between digoxigenin-labelled targets and biotinylated capture probes. Hybrids containing biotin are then captured onto streptavidin-coated microwells and all other PCR components are washed away, including spurious amplification products. The presence of the digoxigenin-

labelled amplified target is then detected by anti-digoxigenin alkaline phosphatase conjugates using the chemiluminiscent substrate PPD. This approach maintains high specificity by nucleic acid-dependent capture, and high sensitivity by efficient solution hybridization. This method is very rapid (2 h) and capable of detecting as low as 10 HIV particles.

#### Direct cloning and sequencing

Sequencing of PCR-amplified DNA was first done by cloning the DNA into an M13 sequencing vector and sequence data generated by analysing a number of clones. However, recently developed techniques allow the sequencing of amplified fragments without the need of M13 cloning. This represents a considerable reduction in the time required to obtain the same information by conventional techniques. In particular, it is advantageous for viral nucleic acids because direct amplification, cloning and sequencing do not require cultivation of viruses *in vitro*. Infectious organisms which are often difficult to propagate and are also risky may be studied directly from clinical materials. Sequence data obtained from this approach will more accurately reflect the viral sequences actually present in clinical samples.

Several methods have come up for direct cloning of amplified products in recent days. Restriction enzyme sites can be introduced at the 5' end of each primer used in PCR reaction. After the required number of cycles of amplification the product is usually purified with glass milk to remove dNTPs (competitive inhibitors of ATP in the ligation reaction) and excess primers which interfere in subsequent reactions. The purified product is then used for restriction digestion followed by ligation to the digested vector. However, low cloning frequencies are sometimes encountered, presumably due to incomplete digestion or Taq polymerase carryover<sup>8</sup>. To avoid all these, Lorenz<sup>9</sup> has devised a protocol of Klenow-kinase-ligase for reliable cloning in which concatamers are prepared before restriction digestion. Blunt end cloning is also done when enzyme sites are not present in the primers. Sometimes the glass milk purification followed by proteinase K digestion is also helpful in improving the cloning efficiency.

Direct sequencing of PCR-amplified material has two major advantages over conventional cloning of PCR fragments into plasmids. First, it is not dependent on live organisms and can readily be standardized. Second, it is more reliable and can be achieved much faster as the sequence of only a single clone needs to be determined for each sample in contrast to sequencing of several clones generated through cloning of PCR product to avoid misincorporated nucleotides introduced by DNA polymerase during PCR or *in vitro* recombination. Several standard protocols are now



available for direct sequencing of PCR products. These methods rely on isolation of specific PCR products from agarose gels, their purification to remove left-over primers and dNTPs (glass milk or ultrafiltration<sup>10</sup> by Amicon filters) and subsequent sequencing of double-stranded DNA products by commercial sequencing kits. The extra dNTPs left from the PCR pose a major problem by altering the ratio of dNTPs to ddNTPs in the sequencing reaction (optimally the ratio should be 80  $\mu$ M : 8  $\mu$ M or 10:1). The second problem in direct sequencing of PCR products concerns the concentration of primers in the PCR. If end-labelled primers rather than incorporation of labelled <sup>35</sup>S dATP are used in sequencing, the presence of left-over cold primers from PCR could dilute out the signals by competing with labelled primers during the sequencing reactions. Gel purification of PCR products by glass milk is sufficient to remove the enzyme, dNTPs and primers. However, for purification with glass milk, it is necessary to remove mineral oil completely from the PCR reaction mixture, otherwise the yield of DNA drops significantly<sup>11</sup>. Asymmetric PCR using different amounts of two primers in order to produce an excess of one strand is sometimes practised for direct sequencing<sup>12</sup>.

Recently, few kits have been introduced into the market for thermal cycle sequencing of amplified products and we have found these easy to use, efficient and beneficial in many ways. Only a small amount of template (a few nanograms) is required in these methods as compared to a standard reaction as the labelled product undergoes linear amplification. 'Pre-reaction' denaturation of double-stranded template and annealing steps have been eliminated. Specifically, the use of highly thermostable DNA sequencing enzymes allows sequencing at high temperatures, an advantage for obtaining DNA sequence information from DNA templates which have high degrees of secondary structure that may pose problems during low-temperature sequencing. However, each of the above mentioned protocols has its own advantages and limitations which are to be kept in mind before the selection of a method for a particular application.

### Contamination in PCR reaction

Due to exquisite sensitivity of PCR, any contamination also gets multiplied and results in artefacts. The contamination of amplification reaction with products of a previous PCR reaction (carry over) leads to false positive results. Nucleic acid contamination is not uncommon, possibly from a target-rich sample to the negative samples or in the PCR reagents if a particular organism is being studied for a long time and the same reagents are used for the amplification of a different fragment. This can be avoided by taking care of the precautions listed in Table 2.

Table 2. Recommended laboratory design for PCR work<sup>13, 14</sup>

Separate working area with instruments allocated exclusively for Pre-PCR, PCR and Post-PCR work.

#### (a) Pre-PCR work

- 1 Nucleic acid extraction exclusively at one place
- 2 Handling of plasmid with inserts of target sequence in a particular isolated area
3. Use screw-capped disposable plastic wares for PCR buffer stock preparation
- 4 Use dedicated chemicals for PCR work including water
- 5 Keep all reagents in aliquots

#### (b) PCR-work

- 1 Use positive displacement pipettes with barriers to avoid pipette barrel contamination
- 2 Always prepare a PCR reaction cocktail and aliquot to each tube to avoid variation
- 3 Set up proper negative controls (template negative as well as DNA negative sample like negative sera) at the beginning of reaction
- 4 Set up proper positive controls at the end of reaction and avoid when a reaction is already standardized
5. Care should be taken in opening the tube and open one tube at a time.
- 6 Keep thermocyclers away from template contact.

#### (c) Post-PCR

1. Confirm the repeatability of the assay
- 2 Store/discard amplified positive controls properly.
- 3 Ensure interpretation of results by hybridization

Aerosol and amplicon (amplified product) contact are major sources of false positives and arise due to repeated handling and time taken for standardization of a PCR reaction in laboratory<sup>15</sup>. A number of approaches with the basic principle of interference in ability of amplicons to serve as template have been developed which minimize the amplicon build-up. Two amplicon sterilization methods, one enzymatic and the other photochemical, have been developed. The principle behind the enzymatic sterilization<sup>16</sup> is the incorporation of uracil in place of thymidine in amplicon present in PCR reaction mixture and its subsequent cleavage by bacterial uracil-N-glycosylase immediately before the amplification, which renders it inactive. This strategy still allows the template DNA (without uracil) to serve as a target for amplification. The photochemical inactivation<sup>17</sup> is more complicated and requires the addition of isopsoralen to PCR tube, which is photo-activated after amplification, forming cyclobutane adducts with pyrimidine bases in PCR product and preventing subsequent template function. However, neither of these methods is a quick solution to amplicon build-up. Therefore, inactivation of PCR mixture without enzyme and template by short wavelength ultraviolet irradiation prior to amplification may help laboratories carrying out PCR routinely<sup>18</sup>.

In contrast, few investigators have commented on a somewhat less frequent but equally problematic phenomenon, namely the false negative PCR results. The main



source of the problem is poor quality of the reagents, plastic wares and gloves (used to reduce amplicon carryover). Therefore, the use of highly purified reagents and disposable gloves are recommended to minimize these problems<sup>19</sup>.

## Allied techniques of PCR

### *Nested PCR*

The detection of single-step PCR product is usually achieved by a labelled probe. For simplicity, many workers choose the nested PCR method over a single-step PCR to increase sensitivity and specificity even without handling of radioactive materials. The specificity of this test is achieved by using the second set of internal primers in adjacent to G:C-rich regions in the target sequence, eliminating the misprimed amplification products of PCR with first set of primers. The internal primers are virtually equivalent to a hybridization reaction and any non-specific priming is eliminated by accepting only samples with correct bands of the precise size on the gel. The only disadvantage of this two-step PCR is that it is not as rapid or convenient as the single-step PCR. However, this technique is now in routine use for detection of causative agents of infectious diseases like hepatitis C and HIV infection<sup>20</sup>.

### *RT-PCR*

Adaptations of PCR technology have provided a breakthrough for the detection and analysis of RNA molecules. The first published report stating the use of PCR to amplify RNA sequences through cDNA came from Veres *et al.*<sup>21</sup> They studied the mouse ornithine transcarbamylase gene for point mutation. Since then a number of modifications have been incorporated to make the process simple, rapid and sensitive for detection of RNA. But all RNA-PCR protocols always require two separate major reactions, a reverse transcription (RT) step, followed by a PCR amplification. In the early two-stage protocols<sup>22</sup> the reverse transcription and the subsequent hydrolysis of the RNA or removal of the RT primers were technically complicated and required a lot of time. Because of similarity of the buffer conditions for reverse transcriptase and Taq DNA polymerase, the two reactions were performed sequentially in subsequently developed protocols. It results in minimal loss of material and the reactions can take place within a single tube<sup>23</sup>. In Perkin Elmer's recent protocol for RNA-PCR using thermostable reverse transcriptase, only one enzyme is used for both RT and PCR with different ionic requirements. Particularly in diseases where the viral agent cannot be propagated such as in hepatitis C virus infection or to detect active stage of mycobacterium infection, RT-PCR has long way to go<sup>24</sup>.

### *Anchored PCR*

Anchored PCR or one-sided PCR is defined as rapid amplification of cDNA ends (RACE) for amplification of nucleic acid sequences from messenger RNA template between a defined internal site and either the 3' or 5' end of the mRNA<sup>25</sup>. The 5' RACE is well suited for rare messages with very little known sequence information. Buchman *et al.*<sup>26</sup> designed a 3' RACE system to convert mRNA sequences of interest to cDNA with the help of oligo-dT, which may then be amplified using PCR. In both procedures mRNAs are first copied using reverse transcriptase and the specific cDNA products are then amplified. For 5' RACE an intermediate terminal deoxynucleotidyl transferase (TdT) tailing is required prior to amplification. RACE has been used for amplification, cloning, sequencing<sup>27</sup>, probe preparation<sup>28</sup> and to generate full-length cDNAs<sup>29</sup>.

### *Asymmetric PCR*

There are inherent difficulties in preparing double-stranded template DNA for sequencing, particularly to PCR-amplified fragments, because of the rapid reassociation of short linear template strands. This can be avoided by modifying the PCR such that single-stranded DNA of chosen strand is produced. This modified type of PCR utilizes an unequal or asymmetric concentration of two primers and has been termed asymmetric PCR<sup>30</sup>. During the initial 15–25 cycles, most of the double-stranded products are generated exponentially. As the low concentration primer gets depleted, further cycles generate an excess of one of the two strands, depending upon which of the primers is limiting. The single-strand product accumulates linearly and is complementary to the limiting primer. Typical primer ratios for asymmetric PCR are between 50:1 and 100:1. This single-stranded product can be sequenced either by using the limiting primer or by some internal primer to increase the degree of specificity.

### *Inverse PCR*

Inverse PCR extends the utility of PCR in allowing the geometric amplification of an unknown DNA sequence that flanks a region of known sequence (Figure 2). DNA containing the known sequence is first digested by a suitable restriction enzyme to produce a fragment suitable for PCR amplification. The ends of the fragment are then allowed to self-ligate and form a circular molecule. Progressive PCR amplification of the unknown region in the circles becomes possible using primers homologous to the ends of known region but oriented with their 3' ends towards the unknown region

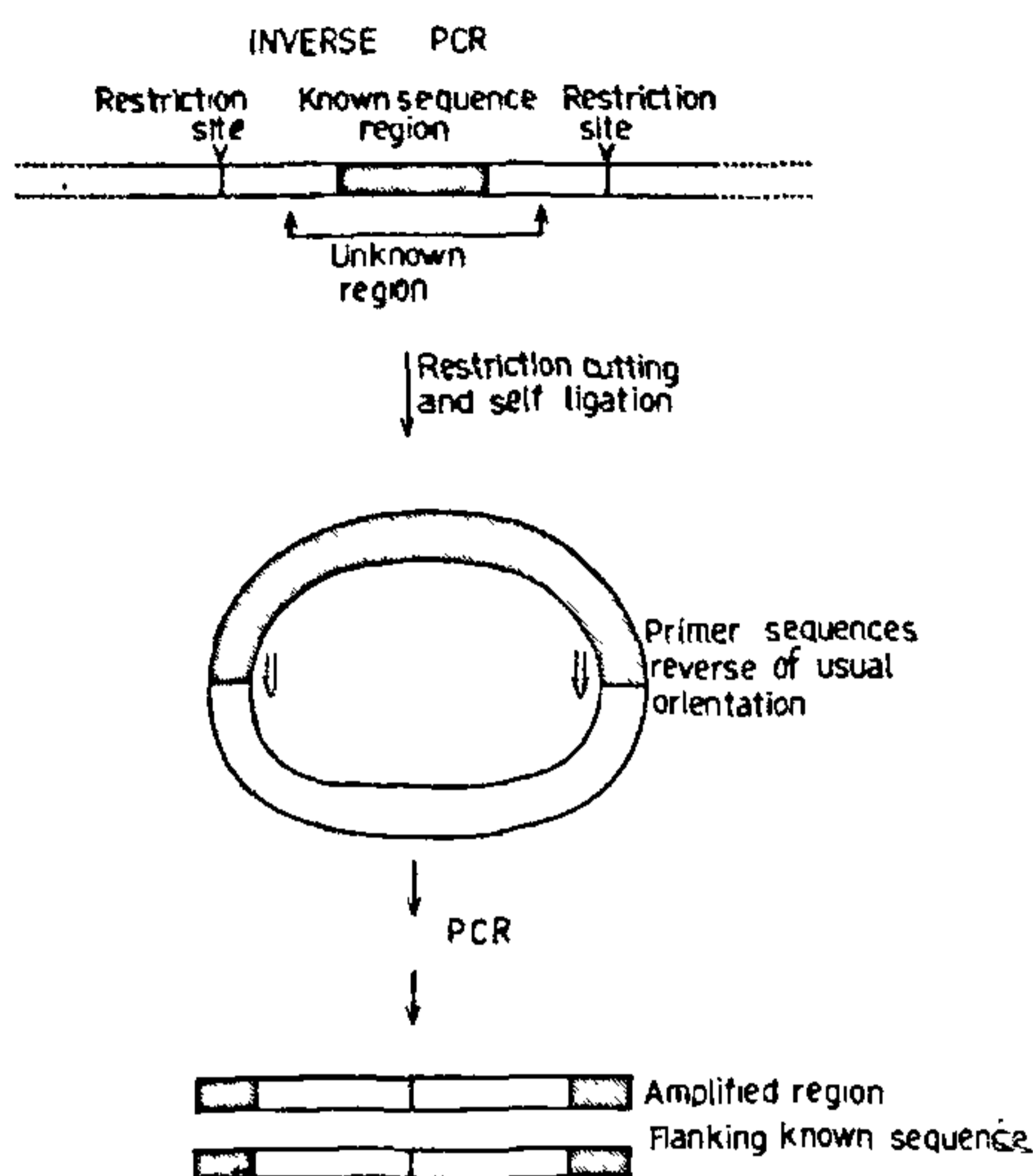


Figure 2. Amplification of unknown DNA flanking a known sequence can be amplified by inverse PCR. In a linear molecule of DNA if the sequence of a gene or segment of a gene is known, the known DNA sequence along with the flanking DNA is cleaved with a restriction enzyme. The digested ends of the DNA are ligated to form a circle. PCR primers are selected to direct synthesis from the known sequence away from each other and around the unknown sequence in rest of the circle. Amplified DNA molecule is linear with the known sequences at either end.

Inverse PCR has many important applications in the study of transposable elements, oncogenes and retroviruses that can integrate or transpose in the genome<sup>31</sup>. The only disadvantage with this technique is that enough sequence information is needed to know the restriction sites which can enable the selection of suitable restriction enzymes to generate appropriate fragments for PCR. It may be desirable to run pilot experiments to get the desired fragments.

### PCR-related techniques

A number of modifications, adaptations and enhancements in basic PCR protocols have been added primarily to make the technique more versatile and meet the challenge of answering numerous biological questions, to which it has been successfully applied. Some of these modified techniques are described below.

#### *Coupled amplification and sequencing (CAS)*

It is possible to generate simultaneous sequence ladders for each strand of a double-stranded template in PCR if

ddNTPs are introduced during amplification. Ruano and Kidd<sup>32</sup> explored this possibility to develop CAS of a 300 base-pair fragment directly from the human genomic DNA. It is a biphasic method where phase I selects and amplifies a single target from the genomic DNA, which is then sequenced as well as further amplified in phase II using the aliquots of phase I reaction mixture after the addition of end-labelled primers and ddNTPs. Synthesis of truncated products proceeds from both ends of double-stranded DNA amplified by PCR. Therefore, both strands of double-stranded template are sequenced at the same time. Electrophoresis of the composite CAS products, followed by electroblotting of sequencing gels and hybridization to strand-specific probes, should render CAS amenable to significant and widespread uses in large-scale sequencing projects.

#### *Ligase chain reaction (LCR)*

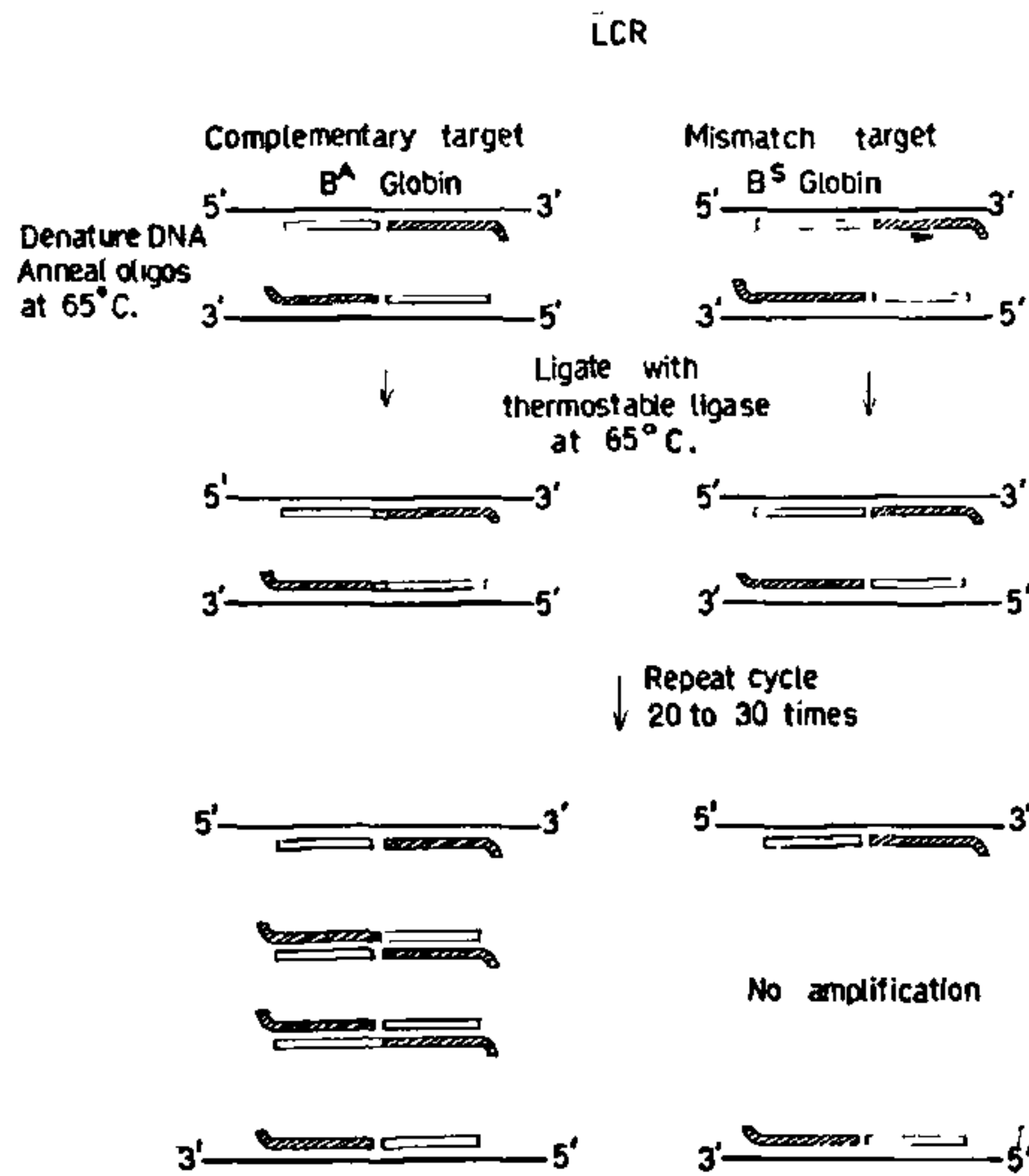
Cloning of a thermostable ligase enabled the designing of LCR which can discriminate single base mutation along with amplification. This method could detect 200 target molecules and could distinguish between normal ( $B^0$ ) and sickle ( $B^s$ ) B-globin genotypes from as little as 10  $\mu$ l blood samples from sickle cell anaemia patients<sup>33</sup>. In contrast to conventional PCR, LCR utilizes a set of four oligonucleotides for detection. In a typical LCR reaction the oligonucleotides hybridize adjacent to one another on each of the denatured target strands in such a manner that a nick is formed (Figure 3). This nick is sealed by a thermostable DNA ligase<sup>34</sup> and each of the ligated products along with the original target serves as template in subsequent rounds of denaturation, annealing and ligation. LCR amplification aims to discriminate accurately between different alleles. Simultaneous increase in allele-specific discrimination avoiding the target-independent ligation can be achieved when a short gap is formed after annealing of the oligonucleotides to the template. The gap is filled in by a thermostable DNA polymerase and the resulting nick is ligated<sup>35</sup> in a process termed GAP-LCR (G-LCR).

LCR amplification is compatible with radioactive, chemiluminescent, fluorescent or enzymatic reporter groups. The process creates a covalent link between oligonucleotides where use of a capture group on one primer and a reporter group on the adjacent primer give the advantage of allowing automation. Such an automated multiplex PCR/LCR detection assay could rapidly screen large populations for monogenetic disease polymorphisms<sup>36</sup> and determine HLA haplotypes for tissue typing and transplantation.

#### *Self-sustained sequence replication (SSR)*

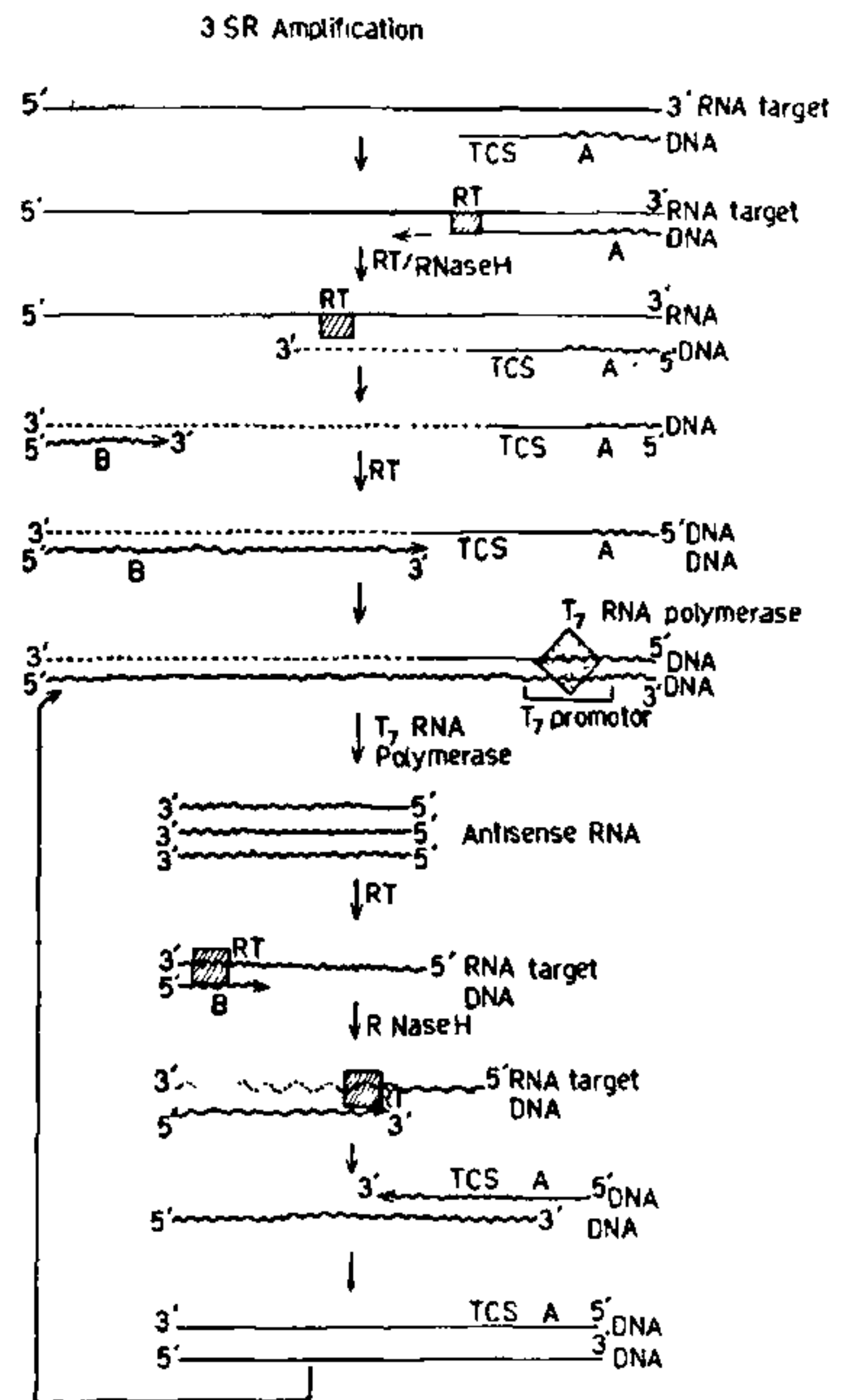
In retroviruses the genetic information is transferred first from RNA to DNA and then back to RNA. With





**Figure 3.** Allele-specific DNA amplification and detection by the ligase chain reaction (LCR). First target DNA is denatured, then four complementary oligonucleotides are annealed to the denatured target strand, lining up end-to-end at a temperature close to their melting point (65°C). The adjacent oligonucleotides that are perfectly complementary to the targets are joined covalently by thermostable ligase (left). The products from one round of ligation become the targets for the next round and thus increase exponentially. Oligonucleotides having a single base mismatch at the junction do not ligate efficiently, hence they are not amplified (right).

this basic mechanism of RNA genome replication, a transcription-based amplification system (TAS) has been designed in which the production of RNA copies of the target sequence provides the principle means of *in vitro* nucleic acid amplification. TAS is a concerted, three-enzyme *in vitro* reaction to carry out an isothermal replication of target nucleic acid sequences, involving the activities of three enzymes, namely avian myeloblastosis virus reverse transcriptase (AMV-RT), *E. coli* RNase H, and T7 RNA polymerase. This reaction is a self-sustained sequence replication (SSSR or 3SR) system. Each cycle of 3SR is composed of two steps. The first is the synthesis of cDNA, producing one copy of a double-stranded DNA template for each of the RNA molecule or DNA target nucleic acid. During the course of this cDNA synthesis step, a sequence recognized by a DNA-dependent RNA polymerase is inserted into the cDNA copy of the target sequence to be amplified. In the second step, amplification of target sequence occurs by the transcription of the cDNA template into multiple copies of RNA<sup>37</sup>. In comparison to PCR protocol, the denaturation step is obviated in 3SR by changing the



**Figure 4.** Schematic representation of strategy involved in 3SR amplification of RNA. 3SR comprises of continuous cycles of reverse transcription and RNA transcription using a double-stranded cDNA intermediate. Oligonucleotide A and B having target complementary sequence (TCS) prime DNA synthesis producing a double-stranded cDNA containing a functional T<sub>7</sub>-promoter. Digestion of RNA in the intermediate RNA-DNA hybrid by RNase H determines the complete cDNA synthesis. Transcription-competent cDNAs are used to produce multiple copies of antisense RNA transcript of the original target. These antisense RNA molecules are immediately converted to T<sub>7</sub>-promoter-containing double-stranded cDNA and again used as transcription templates. This self-sustained sequence amplification continues in cyclic fashion under isothermal conditions (42°C) until it becomes anaemic.

product of amplification process from double-stranded DNA to single-stranded RNA. Addition of 10% DMSO and 15% sorbitol showed 10<sup>6</sup>-fold increased amplification when 3SR is performed without *E. coli* RNase H activity. This increase in amplification is due to the RNase H activity of AMV-RT itself in the presence of these additions, thus obviating the need of *E. coli* RNase H, giving rise to a two-enzyme 3SR system (Figure 4).

Distinguishable advantages of 3SR over PCR are the inherent property of RNA polymerase to produce 10-1000 copies of RNA per copy of DNA template, thereby reducing the required number of cycles of amplification to achieve large increase in the copy number (up to 10<sup>6</sup>

Table 3. Comparison of different amplification systems<sup>40,41</sup>

	PCR	LCR	3SR
No. of primers required	Two primers enclosing the sequence to be amplified	Four primers enclosing point mutation or a gap.	Two primers enclosing target DNA having T7 promoter sequence.
No. of enzymes required	Single enzyme single step addition.	1 (blunt end) or 2 (G-LCR) enzymes, one step addition.	Two/three enzyme as per protocol.
Nature of enzyme	Highly specific thermostable	Highly specific with thermostable ligase	Rapid kinetics
Template	DNA/RNA up to 10 kb	Small length sequence amplified.	RNA or DNA. RNA products difficult to size
Sensitivity	High	High	High
Fidelity	More and varies with enzymes	Theoretically none	Minimum
Automation	No	Yes	
Reaction time	2-4 h	< 2 h	1-2 h
Detection time	1-2 h	< 0.8 h	< 0.8 h
Special requirements	Thermal cycler	Thermal cycler, particularly in gap LCR	Isothermal reaction
Carry over contamination	Control strategy available	No control system	No control system
Product	Easily characterized by agarose gels or conformation by hybridization	Easily characterized by agarose gels or by hybridization	Sandwich hybridization
Efficiency	Slow amplification kinetics, requires more steps to differentiate between RNA and DNA	Low amplification efficiency with lower kinetics	Length limitation in amplification

copies in four cycles). The product of 3SR can be detected directly by hybridization without denaturation because of the single-stranded RNA. Such a single-stranded RNA product can also be used as templates for sequencing. Detection and positive identification of 3SR-generated RNA by oligobeads with a sandwich hybridization protocol provide an added level of specificity. Addition of recognition sequences from the T7 RNA polymerase to the primers of PCR can be lead to production of RNA transcripts from the PCR-amplified DNA<sup>38</sup>. The time required to carry out each cDNA and transcription step in a single cycle of TAS is 24 and 25 minutes respectively. Subsequently, four cycles of TAS can be completed in less than 4 h. The detection of final product by sandwich hybridization requires a further 4 h, thus avoiding the need for autoradiography. This procedure has been applied to the detection of human immuno-deficiency virus type-1 (HIV-1) infected cells, where fewer than one HIV-1 infected CEM cell could be detected in a population of  $10^6$  uninfected CEM cells, with a resultant increase in a  $2-5 \times 10^6$ -fold increase in copy number of original target sequence after four cycles<sup>39</sup>. Some of the advantages

and drawbacks inherent in 3SR, PCR and LCR amplification protocols are summarized in Table 3.

### Conclusion

The current integration of molecular genetics into biomedical and natural sciences has gained momentum by addition of *in vitro* nucleic acid amplification system. This process has been used for numerous studies where it has facilitated the easy isolation of nucleic acid from target material even if it was present in extremely small quantities. A rarest of the rare sequence can serve as template and can be amplified. Scientists without a formal background of molecular biology can now quickly generate sufficient quantities of a target sequence of interest for further studies. However, the successful utilization of PCR amplification technique requires an extensive training and expertise, though the process sounds easy to perform. Great care has to be taken (see Table 2) while working with PCR set-up. Even the smallest amount of contaminated DNA can be amplified leading to ambiguous results. Refinement in



DNA sequencing of amplified products using commercially available kits is making these techniques accessible to many laboratories and facilitating the generation of enough sequence data. In addition to basic PCR techniques, a number of modified techniques have been developed which make it more versatile. Other than the different PCR-related techniques discussed here, the recent breakthroughs like *in situ* PCR, where a tissue slice can be put with a PCR premix droplet for rapid diagnosis or multiplex PCR using a number of primer pairs in single reaction for detection of genetic disorders or co-amplification using specific primer pairs for specific organisms in a single tube reaction, are slowly and slowly being refined. These modifications may revolutionize its application in diagnostics and other health care related areas. The LCR protocols may prove to be unique because of the specific advantage of combining both amplification and mutation detection, that is not immediately found with PCR or 3SR. However, the importance of 3SR amplification protocols proved to be effective in the specific amplification of RNA target molecules which permits the independent amplification of mRNA and not the genomic copies present in the same sample. However, *in vitro* amplification systems with their enormous increased sensitivity in analysis of small amounts of DNA or RNA and superior resolution properties, add to the potential of established or other newly developed methods.

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