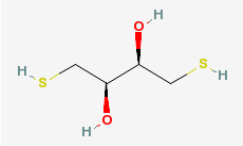
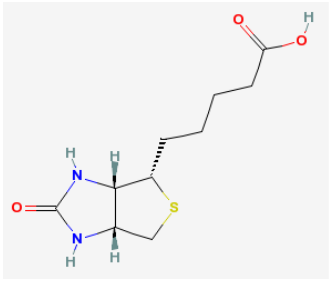
Replication

Molecules, genes and proteins



Dithiothreitol (DTT) IUPAC Name: 1,4-bis-sulfanylbutane-2,3-diol. A reagent commonly used in biochemical studies as a protective agent to prevent the oxidation of SH (thiol) groups and for reducing disulphides to dithiols.



Biotin A water-soluble, enzyme co-factor present in minute amounts in every living cell. It occurs mainly bound to proteins or polypeptides and is abundant in liver, kidney, pancreas, yeast, and milk.

BirA The BirA gene codes for a bifunctional protein that exhibits biotin ligase activity (enzymatic function) and also acts as the DNA binding transcriptional repressor of the biotin operon (regulatory activity). The enzymatic functions include the synthesis of the enzyme-bound biotinyl-5'-adenylate (bio-5'-AMP), and the transfer of the biotin from the adenylate to a lysine residue of the biotin carboxyl carrier protein (BCCP) of the acetyl-CoA-carboxylase. The transfer reaction results in the active form of acetyl-CoA-carboxylase. Alternatively, as a regulator, the enzyme-bound to bio-5'-AMP represses transcription of the biotin biosynthetic operon by binding to the biotin operator sequence.

	Prokaryotes	Eucaryotes
Major initiator protein	DnaA	ORC (origin recognition complex)
DNA helicase (ATP dependent)	DnaB	Antigene T MCM (mini-chro mainten; yeast)
DNA primase	DnaG	DNA pol α
Helicase loader / inhibitor when in solution	DnaC	Cdc6, Cdt1
Ligase		
Topoisomerase II	Gyrase / Topoisomerase II	Topoisomerase II
SSBs	SSB	RPA
DNA polymerase	Pol III α: DNA synthesis Pol III ε: 3'-5' proofreading Pol III γδ: complex binds template Pol III τ: causes dimer formation Pol III β: clamps enzyme to DNA Pol III θ: ?	
Replication rate	500-1000 nt/s	50 nt/s
Okazaki fragments	1000-2000 nt	100-200 nt
Replication origins	OriC = ColE1 ori (E. coli)	ARS (yeast, auton. replic. sequ.)
Replication termination site	ter (E. coli)	

Models, theories and mechanisms

2-4 rule The 2-4 rule is a simple thermodynamic model to calculate the melting point of an oligonucleotide of less than 20 nucleotides. The melting temperature in °C of a duplex (e.g. DNA tag/antitag system) is assumed to be equal to twice the number of A-T base pairs plus four times the number of G-C base pairs.

Replicon A unit of genetic material which behaves autonomously during replication of DNA. In bacteria, a whole chromosome is a replicon. In eukaryotes, chromosomes are divided into hundreds of replicons. Each replicon contains an origin of replication (binding site for RNA polymerase), a termination site and elements that regulate replication.

Replisome The DNA-replicating structure at the replication fork. Minimal requirements are:

Initiator protein	Initiator proteins (DnaA) bind to binding sites (DnaA boxes) forming large nucleoprotein complexes that lead to the unwinding of A + T rich regions at the origin. This open complex formation is inhibited by (SeqA) that restrains negative supercoils of the (OriC plasmid).
Helicase	Unwinds the parental template DNA. (DnaA) directs the (DnaB) helicase / (DnaC) complex to the open complex. Binding of the helicase to the replication origin generates the prepriming complex.
Primase	Synthesizes RNA primers; in eucaryotes with a short length of DNA
Polymerase	Polymerisation acitivity 5'-3' (incorporation of dNTPs) Exonuclease activity 3'-5' (proofreading, not all pol) Exonuclease activity 5'-3' (repair functions, only a few pol)
SSB	Protects ssDNA (ssRNA) from degradation by nucleases
Topoisomerase type I	Relaxation of negatively supercoiled DNA turns and, in eucaryotes, positively supercoiled DNA.
Topoisomerase type II	De-catenation of chromosomes. Relaxation of positive supercoiled DNA turns (ATP independent). Gyrase (prokaryotes) introduces as well negative superhelical turns (ATP dependent). In eucaryotes, as well relaxation of negatively supercoiled DNA turns.
Termination proteins	The E. coli genome shows six termination sequences to which Tus proteins bind. Tus proteins allow a replication fork to pass when the fork approaches from one direction but not when it approaches from the other direction, because the DnaB helicase that is moving the fork forwards can disrupt the Tus only if approaching from the right side.
Ligase	Ligase I: replication; ligase II: repair

Replication initiation Initiation of DNA replication in eukaryotes is an intriguing cascade of protein interactions. The sequence of these interactions is controlled by cyclin-dependent kinases (cdk), as well as by ubiquitin-dependent proteolysis in the proteasome.
Replication of genomic DNA is limited to a single round per cell cycle by a licensing factor, which binds to origins of replication in M phase and is released after the origins have fired in S phase. One component of licensing factor is a complex of six MCM proteins which bind to the origin recognition complex (ORC). The MCM genes were originally identified in budding yeast, where they are required for minichromosome maintenance. As predicted by the licensing model, most MCMs are released from chromatin during S phase and reassociate at the end of mitosis. MCMs have a conserved DNA-dependent ATPase domain shared with DNA helicases. They also bind with high affinity to core histone H3-H4 dimers, indicating a possible chromatin-remodeling function. Mammalian cells have at least 10^6 copies of the MCMs per nucleus, which is at least an order of magnitude greater than the number of replication origins.

RNA primers In eucaryotes, RNA primers are made at intervals spaced about 200 nucleotides (prokaryotes: 2000 nt) on the lagging strand, and each RNA primer is approximately 10 nucleotides long. This primer is erased by a special DNA repair enzyme (an RNAseH) that recognizes an RNA strand in an RNA / DNA helix and fragments it; this leaves gaps that are filled in by DNA polymerase and DNA ligase.

Primosome In prokaryotes, the primase molecule is linked directly to a DNA helicase to form a unit on the lagging strand called a primosome.

Mismatch proofreading The strand-distinction mechanism used by the mismatch proofreading system in E. coli depends on the methylation of selected A residues in the DNA. Methyl groups are added to all A residues in the sequence GATC, but not until some time after the A has been incorporated into a newly synthesized DNA chain.
In eucaryotes, the mechanism for distinguishing the newly synthesized strand from the parental template strand does **not** depend on DNA methylation. Indeed, some eucaryotes (yeast, Drosophila) do not methylate any of their DNA. Newly synthesized DNA strands are known to be preferentially **nicked**, and experiments revealed that such nicks (also called single-strand breaks) provide the signal that directs the mismatch proofreading system to the appropriate strand in a eucaryotic cell.

Strand-directed Mismatch repair MutS (E. coli; in eucaryotes there are more enzymes, other names) binds specifically to a mismatched base pair, while MutL scans the nearby DNA for a nick. Once a nick is found, MutL triggers the degradation of the nicked strand all the way back through the mismatch.
In bacteria there is an additional protein in the complex, MutH, that nicks unmethylated GATC sequences. This function is not present in eucaryotes.
Mutations in MutS and MutL homologues lead to HNPCC (hereditary non-polyposis colon cancer).

Origin of replication Location on a DNA molecule at which duplication of the DNA begins. The E. coli origin of replication (oriC) is approximately 245 bp in length. It contains three copies of a 13-nucleotide repeat motif and five copies of a 9-nucleotide repeat. Three of the 9-nucleotide repeats are regarded as major sites for DnaA attachment; the other two repeats are minor sites. Attachment of DnaA proteins to *oriC* results in melting of the helix within the AT-rich 13-nucleotide sequences.

DNA methylation DNA methylation in E. coli occurs at GATC sequences, 11 of which are found in the origin of replication. Methylation is catalysed by the Dam (DNA adenine methyltransferase) protein.

Nucleosomes Both of the newly synthesized DNA helices behind a replication fork inherit old histones. The addition of new histones to the newly synthesized DNA is aided by chromatin assembly factors (CAFs), which are proteins that associate with replication forks and package the newly synthesized DNA as soon as it emerges from the replication machinery.

Base excision repair (BER) The major repair pathway, base excision repair, involves a battery of enzymes called DNA glycosylases, each of which can recognize a specific type of altered base in DNA and catalyse its hydrolytic removal. It is thought that DNA glycosylases travel along DNA using base-flipping to evaluate the status of each base pair. Once a damaged base is recognized, the DNA glycosylase reaction creates a deoxyribose sugar that lacks its base. This “missing tooth” is recognized by an enzyme called AP endonuclease (apurinic or apurimidinic site endonuclease and phosphodiesterase), which cuts the phosphodiester backbone, and the damage is then removed and repaired.

Depurination Depurination, which is by far the most frequent type of damage suffered by DNA, also leaves a deoxyribose sugar with a missing base. Depurinations are directly repaired beginning with AP endonuclease, following the base excision repair pathway.

Nucleotide excision repair (NER) The second major repair pathway is called mucleotide excision repair. This mechanism can repair the damage caused by almost any large change in the structure of the DNA double helix. Such “bulky lesions” include those created by the covalent reaction of DNA bases with large hydrocarbons, as well as the various pyrimidine dimmers caused by sunlight. In this pathway, a large multienzyme complex scans the DNA for a distortion in the double helix. Once a bulky lesion has been found, the phosphodiester backbone of the abnormal strand is cleaved on both sides of the distortion, and an oligonucleotide containing the lesion is peeled away from the DNA double helix by a DNA helicase enzyme. The large gap produced in the DNA helix is then repaired by DNA polymerase and DNA ligase.

Nonhomologous end-joining In nonhomologous end-joining the broken ends are juxtaposed and rejoined by DNA ligation, generally with the loss of one or more nucleotides at the site of joining.

Homologous end-joining In homologous end-joining, general recombination mechanisms are called into play that transfer nucleotide sequence information from the intact DNA double helix to the site of the double-strand break in the broken helix. This type of reaction requires special recombination proteins that recognize areas of DNA sequence matching between the two chromosomes and bring them together. A DNA replication process then uses the undamaged chromosome as the template for transferring genetic information to the broken chromosome, repairing it with no change in the DNA sequence.

Important methods

Protein affinity chromatography Method that can be use to isolate and identify proteins that interact physically. To capture interacting proteins, a target protein is attached to polymer beads that are packed into a column. Cellular proteins are washed through the column and those proteins that interact with the target adhere to the affinity matrix. Elutriation and identification.

Co-immunoprecipitation An antibody is used to recognize a specific target protein. Affinity reagents that bind to the antibody and are coupled to a solid matrix are then used to drag the complex out of solution to the bottom of a test tube. If this protein is associated tightly enough with another protein when it is captured by the antibody, the partner precipitates as well.
Co-immunoprecipitation techniques require having a highly specific antibody against a known cellular protein target, which is not always available. One way to overcome this requirement is to use recombinant DNA techniques to add an epitope tag or to fuse the target protein to a well-characterized marker protein, such as the small enzyme glutathione S-transferase (GST). Commercially available antibodies directed against the epitope tag or the marker protein can then be used to precipitate the whole fusion protein, including any cellular proteins associated with the protein of interest. If the protein is fused to GST, antibodies may not be needed at all: the hybrid and its binding partners can be readily selected on beads coated with glutathione.

Yeast two-hybrid system A reporter gene is used to detect the physical interaction of a pair of proteins inside a yeast cell nucleus. This system has been designed so that when a target protein binds to another protein in the cell, their interaction brings together two halves of a transcriptional activator, which is then able to switch on the expression of the reporter gene.
The technique takes advantage of the modular nature of gene activator proteins. These proteins both bind to DNA and activate transcription – activities that are often performed by two separate protein domains. Using recombinant DNA techniques, the DNA sequence that codes for a target protein is fused with DNA that encodes the DNA-binding domain of a gene activator protein. When introduced into yeast, the corresponding fusion protein binds to the regulatory region of a reporter gene, where it serves as “bait” to fish for proteins that interact with the target protein inside a yeast cell. To prepare a set of potential binding partners, DNA encoding the activation domain of a gene activator protein is ligated to a large mixture of DNA fragments from a cDNA library. Members of this collection of potential “preys” are introduced individually into yeast cells containing the “bait”. If the cell has received a DNA clone that expresses a prey partner for the bait protein, the two halves of a transcriptional activator are united, switching on the reporter gene. Cells that express this reporter are selected and grown, and the gene (or gene fragment) encoding the prey protein is retrieved and identified through nucleotide sequencing.

Reverse two-hybrid system This technique can be used to identify mutations – or chemical compounds – that are able to disrupt specific protein – protein interactions. In this case the reporter gene can be replaced by a gene that kills cells in which the bait and prey proteins interact. Only those cells in which the proteins no longer bind can survive.

Phage Display This powerful method for detecting protein-protein interactions involves introducing genes into a virus that infects the E. coli bacterium (e.g. phage M13). In this case the DNA encoding the protein of interest (or a smaller peptide fragment of this protein) is fused with a gene encoding one of the proteins that forms the viral coat (gp8 for M13). When this virus infects E. coli, it replicates, producing phage particles that display the hybrid protein on the outside of their coats. This bacteriophage can then be used to fish for binding partners in a large pool of potential target proteins.
However, the most powerful use of this phage display method allows one to screen large collections of proteins or peptides for binding to selected targets. This approach requires first generating a library of fusion proteins, much like the prey library in the two-hybrid system. This collection of phage is then screened for binding to a purified protein of interest. For example, the phage library can be passed through an affinity column containing an immobilized target protein. Viruses that display a protein or peptide that binds tightly to the target are captured on the column and can be eluted with excess target protein. Those phage containing a DNA fragment that encodes an interacting protein or peptide are collected and allowed to replicate in E. coli. The DNA from each phage can then be recovered and its nucleotide sequence determined to identify the protein or peptide partner that bound to the target protein.

Surface plasmon resonance (SPR) One particularly useful method for monitoring the dynamics of protein association is called surface plasmon resonance (SPR). SPR detects binding interactions by monitoring the reflection of a beam of light off the interface between an aqueous solution of potential binding molecules and a biosensor surface carrying immobilized bait protein. The bait protein is attached to a very thin layer of metal that coats one side of a glass prism. A light beam is passed through the prism. At the resonance angle some of the energy from the light interacts with the cloud of electrons in the metal film, generating a plasmon – an oscillation of the electrons at right angles to the plane of the film, bouncing up and down between its upper and lower surfaces like a weight on a spring. The plasmon, in turn, generates an electrical field that extends a short distance – about the wavelength of the light – above and below the metal surface. Any change in the composition of the environment within the range of the electrical field causes a measurable change in the resonance angle.
To measure binding, a solution containing proteins (or other molecules) that might interact with the immobilized bait protein is allowed to flow past the biosensor surface. The changes in the resonance angle are monitored in real time and reflect the kinetics of the association – or dissociation – of molecules with the bait protein. The association rate (k_{on}) is measured as the molecules interact, and the dissociation rate (k_{off}) is determined as buffer washes the bound molecules from the sensor surface ($K_{binding} = k_{off} / k_{on}$).
In addition to determining the kinetics, SPR can be used to determine the number of molecules that are bound in each complex: the magnitude of the SPR signal change is proportional to the mass of the immobilized complex.

RNA interference (RNAi / siRNA) RNA interference is found in a wide variety of organisms, including single-celled fungi, plants, worms, mice, and probably humans. In plants, RNA interference protects cells against RNA viruses. In other types of organisms, it is thought to protect against the proliferation of transposable elements that replicate via RNA intermediates.
The presence of free, dsRNA triggers RNAi by attracting a protein complex containing an RNA nuclease and an RNA helicase. This protein complex cleaves the dsRNA into small (approximately 23 nucleotide pair) fragments which remain associated with the enzyme. The bound RNA fragments then direct the enzyme complex to other RNA molecules that have complementary nucleotide sequences, and the enzymes degrades these as well. These other molecules can be either single- or double-stranded (as long as they have a complementary strand). In this way, the experimental introduction of a dsRNA molecule can be used by scientists to inactivate specific cellular mRNAs.
Each time it cleaves a new RNA, the enzyme complex is regenerated with a short RNA molecule, so that an original dsRNA molecule can act catalytically to destroy many complementary RNAs. In addition, the short dsRNA cleavage products themselves can be replicated by additional cellular enzymes, providing an even greater amplification of RNA interference activity. This, for example, permits progeny cells to continue carrying out RNAi activity that was provoked in the parent cells. In addition, the RNAi activity can be spread by the transfer of RNA fragments from cell to cell. This is particularly important in plants (whose cells are linked by plasmodesmata), because it allows an entire plant to become resistant to an RNA virus after only a few of its cells have been infected.

Nested PCR Nested PCR means that two pairs of PCR primers are used for a single locus. The first pair amplifies the locus as seen in any PCR experiment. The second pair of primers (nested primers) bind within the first PCR product and produce a second PCR product that will be shorter than the first one. The logic behind this strategy is that if the wrong locus was amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers. Nested PCR therefore offers higher specificity as well as higher sensibility due to double amplification.

Protocol: PCR amplification of cDNA segments by 2 stage nested PCR

Template:	50ng cDNA	5µl of 1st round product
Primer:	each 0.5µM	each 0.3µM
dNTPs:	each 0.2mM	each 0.2mM
PCR Buffer:	5µl [10X, 100mM Tris-HCl (pH 8.4), 500mM KCl]	5µl [same]
	Mg^{2+} 1.5mM	Mg^{2+} 1.5mM
	Sigma Taq Polymerase: 0.025U/ul	Sigma Taq Polymerase: 0.025U/ul
STANDARD AMPLIFICATION		
	1st round	2nd round
size:	1192 bp	271 bp
Initial incubation:	94°C for 4 min	95°C for 2 min
Denaturation:	94°C for 45 s	95°C for 45 s
Annealing:	58°C for 45 s	60°C for 45 s
Polymerization:	72°C for 45 s	72°C for 30 s
PCR Cycles:	36	36

Telomerase PCR ELISA The Telomerase PCR ELISA Kit provides a way to perform a highly sensitive photometric enzyme immunoassay for the detection of telomerase activity, using non-radioactive ELISA techniques. The assay can be separated into the following steps:

- ***Elongation / amplification***

Telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin labelled synthetic P1-TS-primer. Then, these elongation products are amplified by PCR with the primers P1-TS and P2, generating PCR products with the telomerase-specific 6-nucleotide increments. The Telomerase PCR ELISA contains all compounds required for the telomerase reaction and PCR in a ready-to-use reaction buffer. Additionally, optimised primer sequences eliminate the need for hot start PCR or separation of the primers by a wax barrier and avoids amplification artefacts, such as primer dimers.

- ***Detection by ELISA***

An aliquot of the PCR product is denatured and hybridised to a digoxigenin-(DIG-) labelled, telomeric repeat specific detection probe. The resulting product is immobilized via the biotin labelled primer to a streptavidin-coated microtiter plate. The detection probe and the hybridisation conditions have been optimised to obtain the highest specificity and sensitivity. The immobilized PCR product is then detected with an antibody against digoxigenin that is conjugated to peroxidase (anti-DIGPOD). Finally, the probe is visualized by virtue of peroxidase metabolizing TMB to form a coloured reaction product.

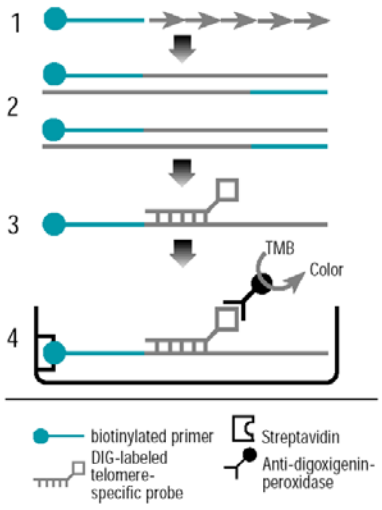


Figure 9 **Detection of telomerase activity with the Telomerase PCR ELISA.**
Step 1. Telomerase, if present, adds multiple 6-nucleotide telomeric repeats to a biotinylated synthetic primer.
Step 2. The telomerase reaction product is amplified by PCR, using a biotinylated primer.
Step 3. After denaturation, the PCR product hybridizes to a digoxigenin-labeled probe specific for the telomeric repeat.
Step 4. The DNA hybrid binds to a streptavidin-coated microtiter plate, and anti-digoxigenin-peroxidase and TMB substrate generate a colored product measurable with a microplate reader.
Note: If desired, the TRAP reaction product from Step 2 can also be detected by the traditional gel electrophoresis method.

Immuno-PCR A method for very sensitive antigen detection by means of specific antibody-DNA conjugates. A streptavidin-protein A chimera that possesses tight and specific binding affinity both for biotin and immunoglobulin G is used to attach a biotinylated DNA specifically to antigen-monoclonal antibody complexes that had been immobilized on microtiter plate wells. Then, a segment of the attached DNA is amplified by PCR. Given the enormous amplification capability and specificity of PCR, this immuno-PCR technology has a sensitivity greater than any existing antigen detection system and, in principle, could be applied to the detection of single antigen molecules.

The detection of nucleic acids can be achieved at levels of a few molecules using the polymerase chain reaction (PCR). In contrast, the analysis of proteins using conventional methods, such as enzyme linked immuno-sorbent assay (ELISA), hardly surpasses sensitivity levels below 1 x 10^{-18} mol of the antigen. By combining the enormous amplification power of PCR with antibody-based immunoassays, Immuno-PCR (I-PCR) allows the detection of proteins at a level of a few hundred molecules.

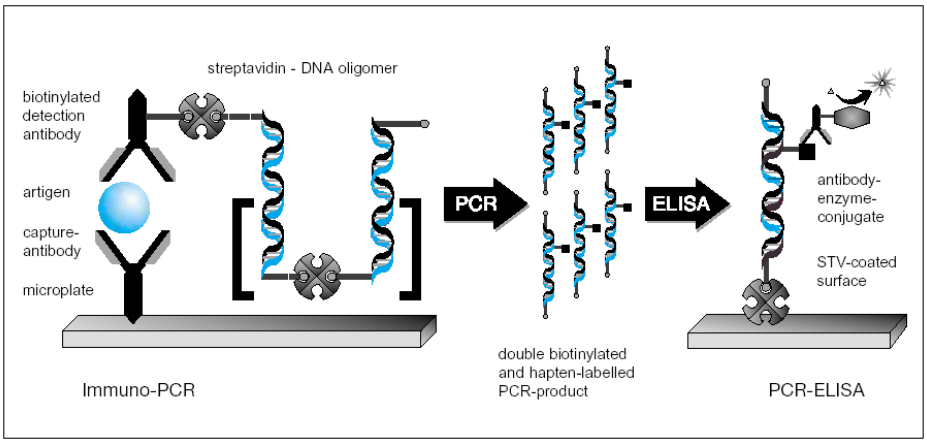
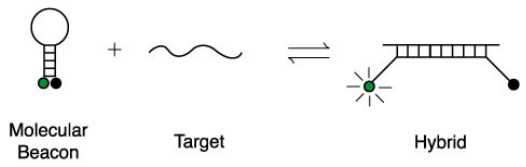


Figure 1. Immuno-PCR detection of an antigen with subsequent quantitative analysis of product yields by PCR-ELISA. Capture antibodies are immobilized on MicroWell™ plates to bind the antigen selectively. Sequential coupling of a biotinylated detection antibody, streptavidin, and a biotinylated DNA marker assemble a signal generating immuno-complex. Signal amplification by PCR using a biotinylated primer and a digoxigenin-labelled nucleotide generates doubly labelled amplificate which can be quantified in a PCR-ELISA assay. The PCR products are immobilized on streptavidin-coated plates and analysed by anti-digoxigenin IgG-alkaline phospatase conjugate with either chromogenic or fluorogenic substrates.

Molecular beacons Molecular beacons are single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore is covalently linked to the end of one arm and a quencher is covalently linked to the end of the other arm. Molecular beacons do not fluoresce when they are free in solution. However, when they hybridise to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them to fluoresce brightly.



In the absence of targets, the probe is dark, because the stem places the fluorophore so close to the non-fluorescent quencher that they transiently share electrons, eliminating the ability of the fluorophore to fluoresce. When the probe encounters a target molecule, it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid, restoring fluorescence.
Molecular beacons can be used as amplicon detector probes in diagnostic assays. The number of amplification cycles required to generate detectable fluorescence provides a quantitative measure of the number of target oligonucleotides present.
Molecular beacons easily discriminate target sequences that differ from one another by a single nucleotide substitution. The reason that molecular beacons are so "finicky" is that they can exist in two different stable physical states. In one state, the molecular beacons are hybridised to their targets, and energy is stored in the probe-target helix. In the second state, the molecular beacons are free in solution, and energy is stored in their stem helix. Molecular beacons are designed so that their probe sequence is just long enough for a perfectly complementary probe-target hybrid to be more stable than the stem hybrid.
In summary, molecular beacons have three key properties that enable the design of new and powerful diagnostic assays:
- they only fluoresce when bound to their targets,
- they can be labeled with a fluorophore of any desired color, and
- they are so specific that they easily discriminate single-nucleotide polymorphisms.

Now that a number of new and versatile spectrofluorometric thermal cyclers are available, assays that simultaneously utilize as many as seven differently coloured molecular beacons can be designed. This enables cost-efficient multiplex assays to be developed that identify which member of a panel of potential infectious agents is present in a clinical sample.

TAQMAN technology TaqMan® Probes have become the traditional linear, dual-labelled FRET probes of choice now incorporating a Black Hole Quencher dye and a fluorophore reporter molecule covalently linked to either the 3' or 5' end of a 20 to 30bp oligo. TaqMan probes are routinely used to detect the presence and quantify the amount of specific target sequences by employing the 5'→3' exonuclease activity of Taq polymerase and the subsequent increase in fluorescence detected during repeated amplification cycles. The TaqMan assay offers a sensitive method to determine the presence or absence of specific target sequences.

FRET FRET is a highly distance-dependent ($1/r^6$) interaction between a reporter dye in an excited state and a quencher in its ground state. Energy is transferred from the fluorophore to the quencher without the emission of a photon. In order for efficient FRET quenching to take place:
- the fluorophore and quencher molecules must be close to each other (approx. 10 - 100 Å) and,
- the absorption spectrum of the quencher must overlap with the emission spectrum of the fluorophore.