
**Product Profile**

Custom Oligo Synthesis, antisense oligos, RNA oligos, chimeric oligos,
Fluorescent dye labeled oligos, Molecular Beacons, TaqMan Probes
Locked Nucleic Acids (LNA), siRNA, Aptamers

# Fluorescent Molecular Probes

For research use only. Not for use in diagnostic procedures for clinical purposes.

## Oligo Types & Modifications

**Molecular Beacons**
**TaqMan® Probes**
**Aptamers**
**RNA Probes**
**Fluorophores & Quenchers**
**Propyne dC and dU labeled Oligos**
**Phosphorothioate Oligos**
**2'-5' linked Oligos**
**Methylated Oligos**

## Applications

**Real Time Quantitative PCR Analysis (QPCR) Probes**
**Fluorescent Genotyping**
**siRNA Gene Knockout Validation**
**Allelic Discrimination**
**Antisense Targeting**
**SNP Detection**
**Aptamers Detection Probes**

Gene Link™

# Fluorescent Molecular Probes

The use of fluorescent dyes in molecular biology has rapidly transformed from just single dye-labeled primers for fragment analysis to the use of dual-labeled oligos, containing dyes and dark quenchers, as probes for quantitative analysis. Fluorescence based detection offers a safe and sensitive method for quantitative detection. This also means that molecular biologists have to understand new terms like donors, acceptors, quenchers, FRET, Stokes shift etc. The molecular basis of some of the probe designs are simple and elegant, and thus have led to an exponential use of molecular probes, and consequently, furthering new developments. Because all of these methods are fluorescence-based, it is essential to understand the basic concepts of fluorescence.

Gene Link offers synthesis of all different forms of molecular probes. We provide technical service in the design of novel probes and have synthesized numerous combinations of dyes, quenchers, RNA, phosphorothioate, 2'O methyl and chimeric probes.

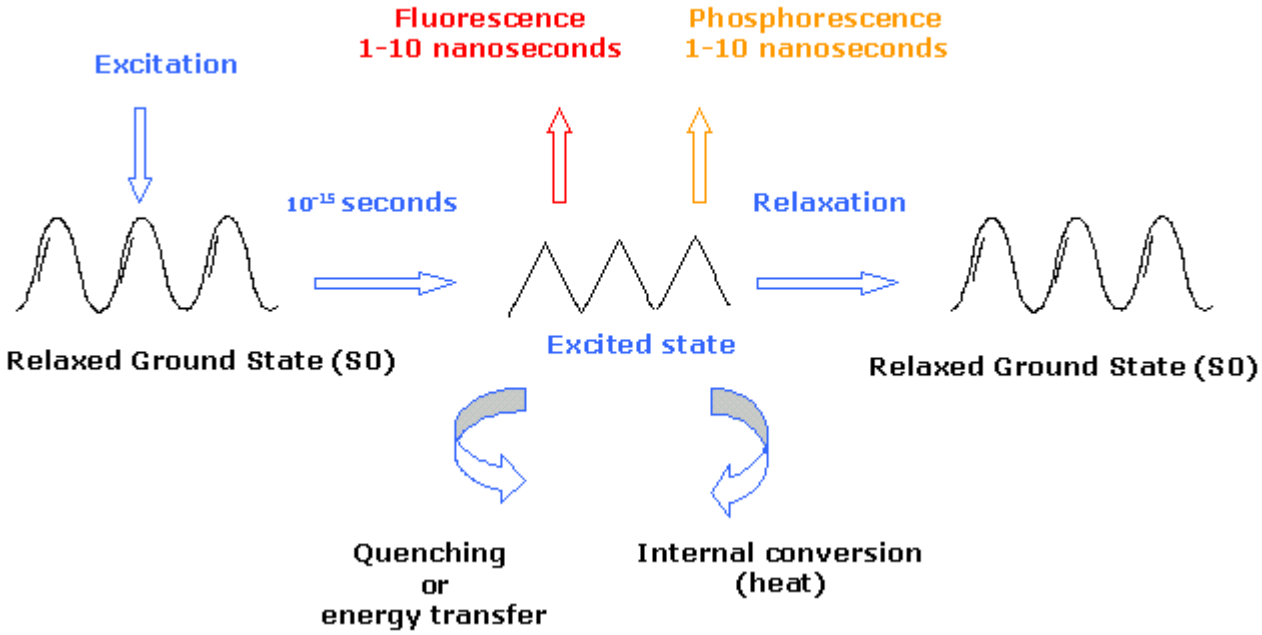
## Luminescence, Phosphorescence & Fluorescence Defined

Molecules absorb energy as photons of light are shined on them. The property of photon absorption depends on the particular atomic configurations of the molecules in question, with said absorption leading to an excited energy state. The energy that is absorbed can be translated into rotational, vibrational or electronic modes. The exact fate of the energy depends on the wavelength of the incoming light; the longer the wavelength, the lower the energy. The vibrational and rotational energy levels are closer together than the electronic levels. Thus, changes in the electron configuration within these levels are often associated with the absorption of infrared radiation and subsequent release of energy as heat. However, light in the visible and UV regions of the spectrum have enough energy to cause changes in the electronic states of a molecule without a relative elevation of temperature.

- Luminescence — The release of energy as light at low temperature is termed luminescence. The molecules emitting light are relatively cool. It is in contrast to light bulbs, fire etc. Luminescence is a general term not limited to the duration of the emitted light.

- Phosphorescence — Delayed luminescence after absorption of energy. The wavelength of the released light energy is at a different (and lower) wavelength and it continues for an extended period of time, even after excitation has ceased.

- Fluorescence — Instantaneous luminescence after absorption of energy. The fluorescence is usually at a different (and lower) wavelength and it ceases almost at once when excitation has ceased.

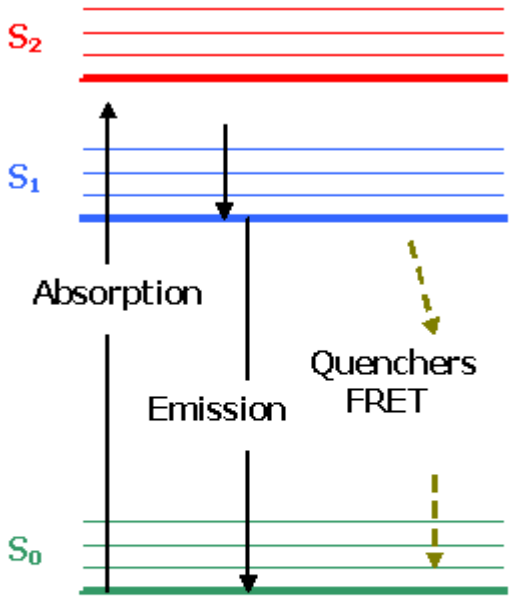



**Excitation and Emission**

The excitation level of molecules varies at different wavelength. Molecules exposed to a beam of light usually absorb much more energy at one particular wavelength than others. This specific wavelength is termed the Excitation Maximum. By contrast, the Emission Maximum is the wavelength at which the maximum amount of light is released after a previous light absorption event. The molecule stays in the excited state for a finite time, usually <1-10 nanoseconds in the case of fluorescence and returns to the relaxed state with emission of energy. The lifetime is measured in nanoseconds, for example, the lifetime of Cy3 is <0.3ns and Cy5 is 1.0ns. Excitation and Emission is a cyclic process and consequently can be repeated for a molecule unless damaged by the energy absorption, at which point the process ceases for that molecule, an effect termed as photobleaching.

We will now focus our discussion by referring to fluorescent dyes used for molecular probes and primer design. The popular ones in use are Fluorescein, 6-FAM (6-fluorescein amidite), HEX (hexachloro fluorescein), TET (tetrachloro fluorescein), all other fluorescein derivatives, Cy series dyes, the AF dyes and rhodamine derivatives. The dyes are selected based on their excitation and emission wavelengths, rate of photobleaching, quenching efficiency and various other biophysical factors. All dyes when excited undergo a conformational change based on their interaction with other molecules, bases and dyes in close proximity. The emission is thus bound by these variables of the microenvironment interaction. Thus, the amount of energy emitted will vary, and not all the energy absorbed during excitation is released as fluorescence.

The excitation time usually is in picoseconds to nanoseconds; similarly, the emission time is in a nanoseconds time frame. The energy used for excitation elevates the dye from a stable and relaxed ground state (S0) to an excited state (S1, S2, etc). If the excitation is to S2 (a vibrational state), the transition from S2 to S1 (an electronic state) is without any release of light energy, just heat energy. The light energy of the photons subsequently emitted from the excited S1 state on the electrons' return to the relaxed, ground S0 state is thus less than that of the photons initially absorbed to excite the electrons to S2. As a result, the Excitation and Emission Maxima will occur at different wavelengths, with the latter always being lower than the former. The difference in wavelength between the Excitation and Emission Maxima is termed the Stokes shift. The Stokes shift represents the energy lost as heat while the molecule was in the excited state.




Absorption of a photon and excitation to $S_1$ or $S_2$. Radiationless energy loss and return to $S_1$. Return to $S_0$ from $S_1$ with emission of fluorescence or by energy transfer to quenchers or other acceptor dye (FRET).

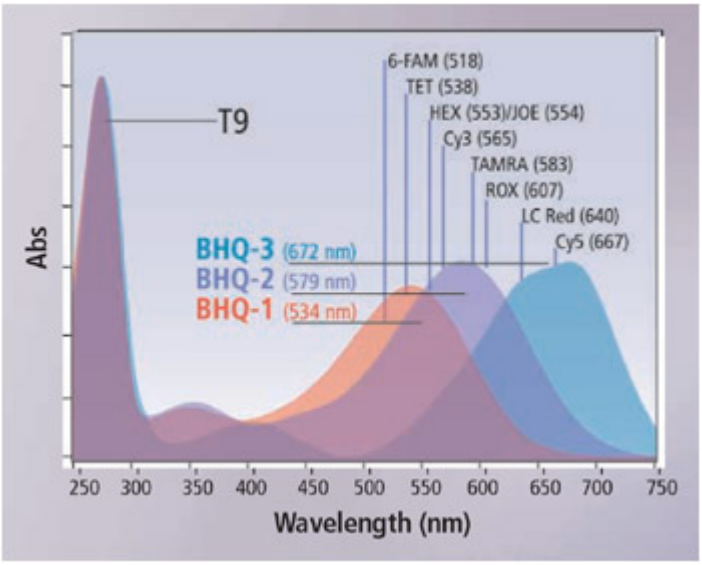
**Quenching**

Reduction in the expected fluorescence emission intensity is termed quenching. Generally, it would be an impediment if the emission intensity were reduced. The phenomenon of quenching forms the basis of the mode of action of certain molecular probes; the designed and controlled fluorescence based on hybridization to the target sequence.

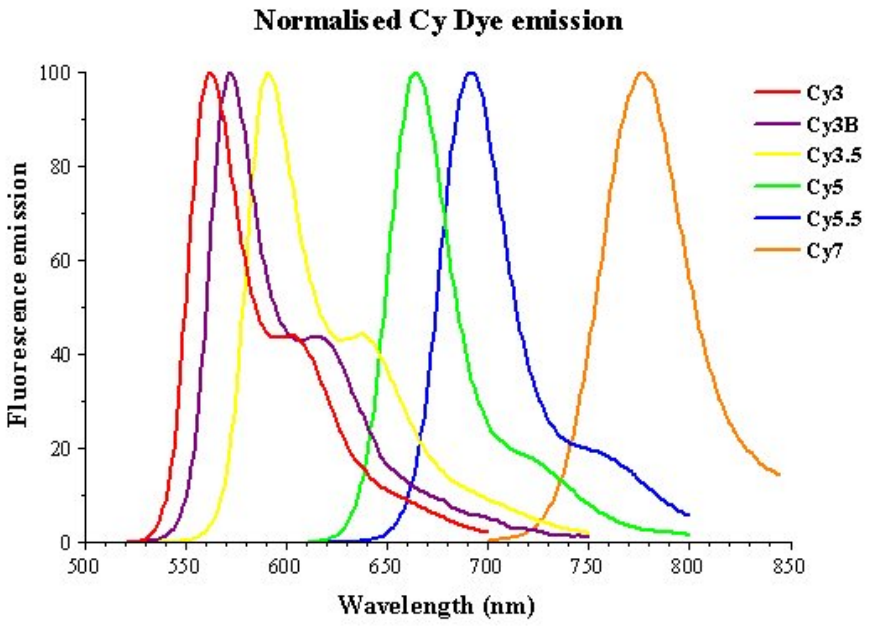
Natural quenching occurs due to 'fading' after repeated cycles of excitation and relaxation. The decrease in the ability of further excitation of a proportion of molecules is termed as photobleaching. Photobleaching is due to structural damage of molecules due to absorption of high energy photons. Some dyes are much more sensitive than other to photobleaching, for example, fluorescein photo bleaches very easily. Often the rate of decomposition is proportional to the intensity of illumination. So, a simple practical way to overcome this is to reduce the incident radiation. It is sometimes possible to introduce antioxidants, such as phenylalanine or azide, to reduce bleaching. Quenching is also observed when the concentration of the dye is too high and the overall brightness decreases. This is 'self quenching'. It is observed that multiple labeling of an oligo with the same dye does not always lead to an increase in fluorescence.

Placing a molecule that absorbs light in close proximity to the fluorophore can induce quenching. Quenching is distance-dependent, quite similar to Fluorescence Resonance Energy Transfer (FRET), and it can be assumed that the energy transfer typically occurs over a distance of 1-10 nm. The distance of 10 nm is equivalent to approximately 30 nucleotides with each nucleotide being 3.4 Å (0.34 nm). The placement of dyes to achieve FRET thus will thus be dependent on the distance they are apart from each other with either nucleotides or spacers. The quenching effect is exhibited by fluorescent as well non-fluorescent molecules. A non-fluorescent ("dark") quencher is the basis of the design of Molecular Beacons. This molecule could be non-fluorescent and acts as an energy sink, and is termed a quencher. In other instances, this molecule could itself be a dye with overlapping absorption and emission spectra; in such cases, energy is transferred from one to the other without any emission of light energy. This is termed as resonance energy transfer. Generally, the term

'quencher' is used for non-fluorescent molecules in probe design, and 'double dye' or 'dual dye' used for probes with two dyes having spectral overlap.




| Quencher Spectral Data | | |
|---|---|---|
| **Quencher** | **Absorbance max (nm)** | **Quenching Range (nm)** |
| Dabcyl | 453 | 380-520 nm |
| BHQ-1 | 534 | 480-580 nm |
| BHQ-2 | 579 | 559-650 nm |
| BBQ-650 | 650 | 550-750 nm |
| BHQ-3 | 672 | 620-730 nm |




**Fluorophore Spectral Data & Quencher Selection Guide**

| Fluorophore Name | Excitation Max, nm | Emission Max, nm | Extinction coefficient* | Color** | Quencher |
|---|---|---|---|---|---|
| AFDye-350 NHS | 346 | 445 | 19,000 | Blue | Dabcyl λ (max) = 453 nm Range = 380-530 nm |
| AFDye-405 NHS | 402 | 424 | 33,000 | | |
| PBlue-455 NHS | 410 | 455 | 46,000 | | |
| MBlue-460 NHS | 362 | 459 | 20,000 | Blue-Green | |
| AFDye-488 NHS | 494 | 517 | 73,000 | | BHQ-1 λ **(max)** = 534 nm Range = 480-580 nm |
| **FAM** | **495** | **520** | **75,850** | | |
| **TET** | **521** | **536** | **99,000** | | |
| AFDye-430 NHS | 430 | 539 | 15,000 | Yellow-Green | |
| Cal Fluor Gold 540 | 552 | 543 | 81,100 | Yellow | |
| **JOE** | **520** | **548** | **75,000** | | |
| **Yakima Yellow** | **531** | **549** | **83,800** | | |
| AFDye-532 NHS | 530 | 555 | 81,000 | | |
| **HEX** | **535** | **556** | **98,000** | | |
| **Cal Orange 560** | **537** | **558** | **81,000** | | BHQ-2 λ (max) = 579 nm Range = 550-650 nm |
| **Cy3** | **550** | **570** | **150,000** | | |
| AFDye-555 NHS | 555 | 572 | 155,000 | | |
| **TAMRA** | **555** | **576** | **65,000** | | |
| **CAL Fluor Red 590** | **569** | **591** | **79,000** | | |
| Redmond Red | 579 | 595 | 52,300 | | |
| **Cy3.5** | **581** | **596** | **150,000** | Yellow-Orange | |
| ROX NHS | 575 | 602 | 82,000 | Orange | |
| AFDye-568 NHS | 578 | 602 | 88,000 | | |
| **Cal Red 610** | **590** | **610** | **108,000** | | |
| TXRed-616 NHS | 589 | 616 | 69,000 | | |
| AFDye-594 NHS | 590 | 617 | 92,000 | Orange-Red | |
| CAL Fluor Red 635 | 616 | 637 | 112,000 | Red | BBQ-650 λ (max) = 650nm Range = 550-750 nm |
| LC Red 640 NHS | 625 | 640 | 110,000 | | |
| AFDye-647 NHS | 649 | 671 | 270,000 | | |
| **Cy5** | **649** | **670** | **250,000** | | |
| **Cy5.5** | **675** | **694** | **190,000** | | |
| AFDye-680 NHS | 678 | 701 | 185,000 | Near-IR region. Human vision is insensitive to light beyond ~650 nm; it is not possible to view near-IR fluorescent dyes. | |
| Cy7 NHS | 750 | 773 | 199,000 | | |
| IR 750 NHS | 756 | 776 | 260,000 | | |
| Cy7.5 NHS | 788 | 808 | 223000 | | |

* Extinction coefficient at λ (max) in cm–1M–1. ** Typical emission color seen through the eyepiece of a conventional fluorescence microscope with appropriate filters.

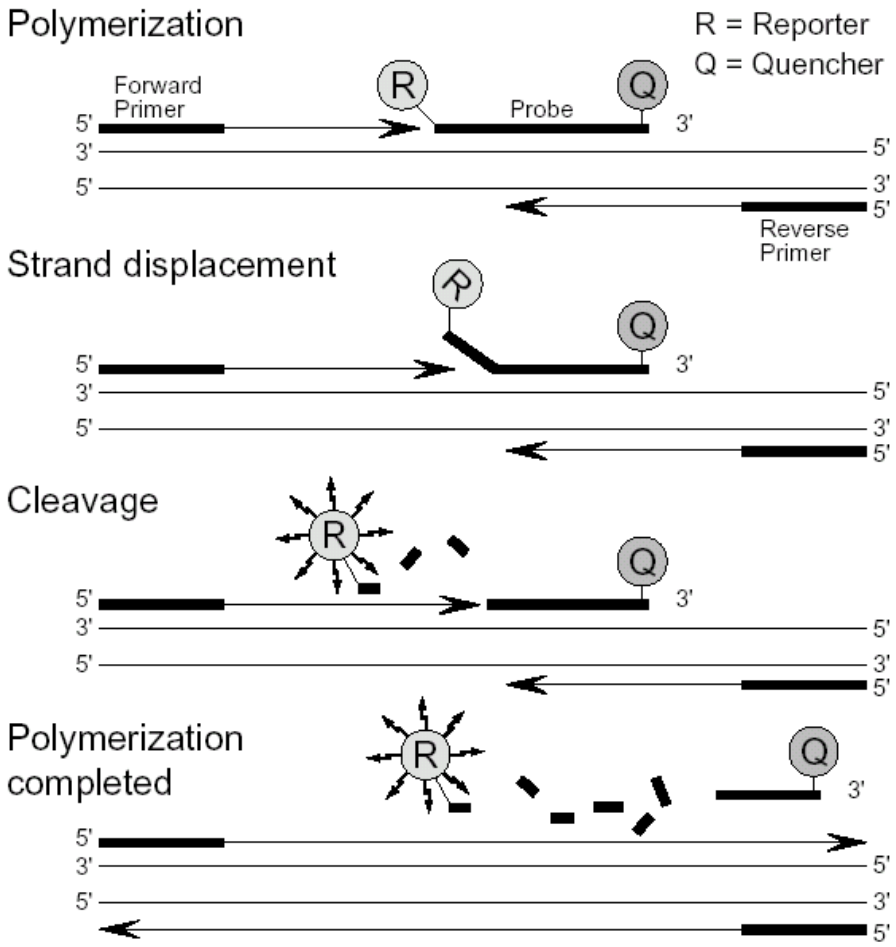
## Fluorescence Resonance Energy Transfer (FRET)

Resonance energy transfer, often known as fluorescence resonance energy transfer (FRET) or Förster energy transfer. It is the radiation-less transfer of excitation energy from a donor to an acceptor. An important consequence of this transfer is that there is no emission of light by the donor. The acceptor may or may not be fluorescent. FRET is a distance-dependent interaction where the energy transfer occurs typically over a distance of 1-10 nm. The distance dependent nature of FRET is highlighted by the fact that it is proportional to the inverse sixth power of the intermolecular separation. The fact that FRET typically occurs in the 1-10 nm region means that these separation distances are comparable with the dimensions of biological macromolecules. This means that FRET can be a valuable tool in studying proximity events in biological systems. One helical turn is ~3.4nm and comprises 10 nucleotides. A nucleotide is thus ~0.34 nm or 3.4Å (1 Å= 0.1nm). Thus, to observe FRET and quenching the donor and acceptor should not be placed more than 30 nucleotides away.
FRET varies based on the degree of spectral overlap of the donor and acceptor. That is to say, the degree to which the emission band of the donor and the absorption band of the acceptor overlap. This is called the "spectral overlap" or sometimes the "Förster overlap integral". This describes the amount of overlap where resonance can occur, i.e. where the donor and acceptor have the same frequencies.

There are many other applications and consequences arising from these equations. For example, if the donor and acceptor are the same molecular species it is still possible to observe FRET. This is called homo-transfer and could be thought of as energy migration. The obvious conclusion from this is that the observed fluorescence would not be changed. However, at high concentration of dye it is possible to observe concentration quenching. In this case the transfer of the energy does not result in emission, and the explanation for this is that the transfer is occurring at less than a critical distance and some of the dyes are acting as energy sinks.

## TaqMan

TaqMan (also known as Fluorogenic 5’ nuclease assay) probes contain two dyes, a reporter dye (e.g. 6-FAM) at the 5’ end and a 3’ acceptor dye, usually TAMRA. Recent designs substitute the 3’ TAMRA fluorescent acceptor quencher dye with non-fluorescent quencher, e.g. Black Hole Quencher. The proximity of the quencher to the reporter in an intact probe allows the quencher to suppress, or “quench” the fluorescence signal of the reporter dye through FRET. If the target of interest is present, these probes specifically anneal between the forward and reverse primer sites. During the reaction, the 5’ to 3’ nucleolytic activity of Taq polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are displaced from the target, separating the reporter dye from the quencher dye and thus resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Because increase in fluorescence signal is detected only if the target sequence is complementary to the probe, nonspecific amplification is not detected.


Polymerization
R = Reporter
Q = Quencher
Forward Primer
R
Probe
Q
5'
3'
3'
5'
5'
3'
5'
3'
Reverse Primer
Strand displacement
R
Q
5'
3'
3'
5'
5'
3'
5'
3'
Cleavage
R
Q
5'
3'
3'
5'
5'
3'
5'
3'
Polymerization completed
R
Q
3'
5'
3'
5'
5'
3'
5'
3'

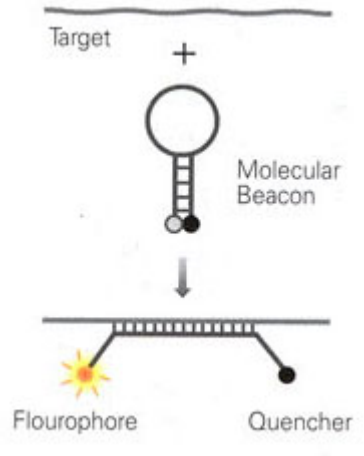
## Design of TaqMan Primers and Probes

Some guidelines for TaqMan probes and primers selection are as follows:

- G-C content between 20% and 80%.
- Avoid runs of an identical nucleotide, especially guanine.
- Avoid G to be on the 5' end.
- Probes and primers should contain more C than G.
- Melting temperature ($T_m$) should be 68-70°C for probes and 58-60°C for primers.
- The five nucleotides at the 3' end of each primer should have no more than two Gs and/or Cs.
- Give precedence to better probes over primers.
- Probe should be as close to 5' primer as possible without overlapping.

## Molecular Beacons

Molecular beacons are oligonucleotide probes that can report the presence of specific nucleic acids in homogenous solutions (Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization, Nature Biotechnology 1996; 14: 303-308.) They are useful in situations where it is either not possible or desirable to isolate the probe-target hybrids from an excess of the hybridization probes, such as in real time monitoring of polymerase chain reactions in sealed tubes or in detection of RNAs within living cells. Molecular beacons are hairpin shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid (Figure 1). They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Since the quencher moiety is a non-fluorescent chromophore and emits the energy that it receives from the fluorophore as heat, the probe is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence.




Operation of molecular beacons: On their own, these molecules are non-fluorescent, because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence.

In order to detect multiple targets in the same solution, molecular beacons can be made in many different colors utilizing a broad range of fluorophores (Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination, Nature Biotechnology 1998; 16: 49-53.) DABCYL, a non-fluorescent chromophore, serves as the universal quencher for any fluorophore in molecular beacons. Owing to their stem, the recognition of targets by molecular beacons is so specific that single-nucleotide differences can be readily detected.

**Molecular Beacon Example Sequence**

```
Fluorophore at 5' end; 5'-GCGAGCTAGGAAACACCAAAGATGATATTTGCTCGC -3'-Quencher

5' GCGAGCTAGGAAACACCA
   ||||||            ]
3' CGCTCGTTTATAGTAGAA
STEM AT 1 IS 6 BP LONG. LOOP = 24.
```

The vertical lines at the 5' and 3' ends identify the arm sequences that are complementary.

The length of the probe sequence (10-40 nt) is chosen in such a way that the probe target hybrid is stable in the conditions of the assay. The stem sequence (5-7 nt) is chosen to ensure that the two arms hybridize to each other but not to the probe sequence. Folding of the designed sequence with the help of a computer program can indicate whether the intended stem-and-loop conformation will occur. The computer program can also predict the melting temperature of the stem.

### Signal to background ratio

**1**. Determine the fluorescence ($F_{buffer}$) of 200 µL of molecular beacon buffer solution using 491 nm as the excitation wavelength and 515 as the emission wavelength. If the fluorophore is not fluorescein, chose wavelengths that are optimal for the fluorophore in the molecular beacon.
**2**. Add 10 µL of 1 µM molecular beacon to this solution. Record the new level of fluorescence ($F_{close}$)
**3**. Add a two-fold molar excess of the oligonucleotide target and monitor the rise in fluorescence until it reaches a stable level ($F_{open}$).
**4**. Calculate the signal to background ratio as $(F_{open}-F_{buffer})/(F_{close}-F_{buffer})$.

### Thermal denaturation profiles

**1**. Prepare two tubes containing 50 µL of 200 nM molecular beacon dissolved in 3.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.0 and add the oligo target to one of the tubes at a final concentration of 400 nM.
**2**. Determine the fluorescence of each solution as a function of temperature using a thermal cycler with the capacity to monitor fluorescence. Decrease the temperature of these tubes from 80 °C to 10 °C in 1 °C steps, with each hold lasting one min, while monitoring the fluorescence during each hold.

### Real time monitoring of polymerase chain reactions

Utilize molecular beacons that are complementary to a sequence in the middle of the expected amplified target fragment. The length of their arm sequences should be chosen so that a stem is formed at the annealing temperature of the polymerase chain reaction. The length of the loop sequence should be chosen so that the probe-target hybrid is stable at the annealing temperature. Whether a molecular beacon exhibits these designed features is determined by obtaining thermal denaturation profiles. The molecular beacons with appropriate thermal denaturation characteristics are included in each reaction at a concentration similar to the concentration of the primers. During the denaturation step, the molecular beacons assume a random coil configuration and fluoresce. As the temperature is lowered to allow annealing of the primers, stem hybrids form rapidly, preventing fluorescence. However, at the annealing temperature, molecular beacons also bind to the amplified target fragment and generate fluorescence. When the temperature is raised to allow primer extension, the molecular beacons dissociate from their targets and do not interfere with polymerization. A new hybridization takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplified target fragment.

### Procedure

**1**. Set up six 50 µL reactions so that each contains a different number of targets, 0.34 µM molecular beacon, 1 µM of each primer, 2.5 units of Taq polymerase, 0.25 mM of each deoxyribonucleotide, 3.5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.0.
**2**. Program the thermal cycler to incubate the tubes at 95 °C for 10 min to activate Amplitaq Gold DNA polymerase (omit this step if not using hot start DNA polymerase), followed by 40 cycles of 30 sec at 95 °C, 60 sec at 50 °C and 30 sec at 72 °C. Monitor fluorescence during the 50 °C annealing steps.

**Troubleshooting**

- The assay medium may contain insufficient salt. There should be at least 1 mM $MgCl_2$ in the solution, in order to ensure that the stem hybrid forms.
- The molecular beacon may fold into alternate conformations that result in a sub-population that is not quenched well. Change the stem sequence (and probe sequence, if necessary) to eliminate that possibility.
- Incomplete restoration of fluorescence at low temperatures. If the stem of a molecular beacon is too strong, at low temperatures it may remain closed while the probe is bound to the target. This may happen inadvertently if the probe sequence can participate in the formation of a hairpin that results in a stem longer and stronger than originally designed. Change the sequence at the edges of the probe and the stem sequence to avoid this problem.

**Molecular Beacon Design**

The PCR primers themselves should have been optimized in a regular PCR to see that it performs well. Assuming the melting temperature of the primers are ~55 degrees.

There are two independent features to control in the design of the MB probe. The stem and the target loop sequence. Design the probe sequence and see that there is minimal secondary structures, loop formation and dimers and the TM is ~5 degree higher than the PCR primer annealing temp. For a good guideline keep it at ~60 degrees. Add the stem sequence of 5-7 bp. The TM of the stem itself will be ~ 60-70 degrees. You are done!.

**General guidelines for MB design at Gene Link are as follows:**

**1**. Design regular 18-24mer PCR primers for amplification with a TM around $55^0$C. The optimal amplified fragment should be between 100-300 bp. Perform PCR, optimize conditions. Should get good clean amplification product visible on ethidium bromide stained gels.
**2**. Design target probe sequence with a TM ~8-10 degrees higher than the PCR primers annealing temp. Example 60-65 degrees. The probe should be designed near the center of the amplified fragment avoiding stretches of strong secondary structure. [Taqman probes are designed ~ 5-10 bases near the primer of the same strand]
**3**. Add the stem 5- 7 bp stem sequence with a GC content of 70-80%. Avoid a G at the 5' end next to the fluorophore. G's seem to have a quenching effect. Hairpin Stem TM should be 7-10 degrees higher than the PCR annealing temperature. Example 65-70 degrees.
Caution: See that by adding the stem you have not created secondary structures with the loop sequence. Try variation of stem sequence to avoid secondary structure with the loop sequence.

The Hairpin stem TM is based on free energy stabilization and folding, the following is a good guideline.

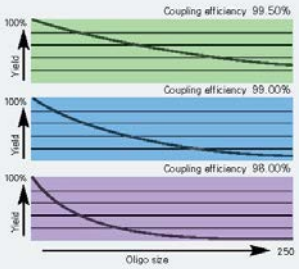
GC rich stem
5 bp = $55^0$C- $60^0$C
6 bp = $60^0$C-$65^0$C
7 bp = $65^0$C-$70^0$C

# Yield

Chemical synthesis of oligo starts from the 3' direction and proceeds to the 5'. All automated synthesis use a solid support that is attached with the first base (nucleoside), for fluorescent probes the 3' usually is the quencher moiety that is attached to the solid support. The scale of synthesis determines the nanomole (nmol) of first base/quencher or fluorophore. The overall crude nmol yield of full-length oligo depends on the average coupling efficiency of each base and on the number of couplings, as the oligo length increases the overall yield of full length product decreases. At Gene Link we maintain a coupling efficiency of 98-99%. Yields of full-length fluorescent probes vary considerably based on the type of fluorophore and quencher. Most fluorescent dyes that are used directly for coupling on automated DNA synthesizers have a coupling efficiency above 85%, whereas the dyes that are conjugated based on NHS chemistry (succinimidyl ester) is a post synthesis conjugation to amino labeled probes. Gene Link minimum yield guarantees are based on the synthesis, type of dye and purification of your oligo.

**Coupling Efficiency and Full Length Oligo Yield**

| Oligo Size | 99.50% | 99.00% | 98.00% |
|---|---|---|---|
| 20 | 90.916 | 82.617 | 68.123 |
| 40 | 82.243 | 67.573 | 45.48 |
| 60 | 74.398 | 55.268 | 30.363 |
| 80 | 67.301 | 45.204 | 20.27 |
| 100 | 60.881 | 36.973 | 13.533 |
| 120 | 55.074 | 30.24 | 9.034 |
| 140 | 49.821 | 24.734 | 6.031 |
| 160 | 45.068 | 20.23 | 4.027 |
| 180 | 40.769 | 16.546 | 2.688 |
| 200 | 36.88 | 13.533 | 1.795 |
| 220 | 33.36 | 11.07 | 1.19 |
| 240 | 30.18 | 9.05 | 0.8 |
| 250 | 28.7 | 8.19 | 0.65 |




PCR and sequencing reactions are very robust and can tolerate up to 50% failure/truncated sequence oligos. However, you are clearly taking a chance by using long oligos synthesized at anything below 99.5% coupling efficiency.

Gene Link offers synthesis of all different forms of molecular primers and probes. We provide technical service in the design of novel probes and synthesize numerous combinations of dyes, quenchers, RNA, phosphorothioate, 2'O methyl, ultra-modified and chimeric probes.

The table below lists the yields for common dyes for use in TaqMan probes with Tamra or BHQ at 3' end and Molecular Beacons with universal fluorescence quencher dabcyl or BHQ at 3' end. Gene Link considers gel purification to be the best method of purification and essential for optimum performance of fluorescent dye labeled oligonucleotides.

| **Fluorescent Probes PAGE Purified Yield*** | | | |
|---|---|---|---|
| **Dye/Quencher** | **50 nmol scale Yield** | **200 nmol scale Yield** | **1 µmol scale Yield** |
| | PAGE Purified* | PAGE Purified* | PAGE Purified* |
| 5' 6-Fam/3'-Tamra, BHQ1 or Dabcyl | 10 nmol | 25 nmol | 60 nmol |
| 5'-Hex, Tet, Cy3 or Cy5/BHQ2 | 5 nmol | 15 nmol | 40 nmol |
| 5' -Cy3.5, Cy5.5,/quencher | 5 nmol | 12 nmol | 25 nmol |
| AFDyes and other NHS Dyes/quencher | 2 nmol | 5 nmol | 16 nmol |
| *Approximate yield for dye and quencher listed. Lower yield with additional modifications. ** Purified yield is for gel purified (PAGE, polyacrylamide gel electrophoresis). Inquire for higher scale of synthesis | | | |

# Reconstitution, Use & Stability of Fluorescent Probes

All Gene Link custom oligo products including, molecular probes, RNA and siRNA includes a datasheet that contains the exact nmols, µg, $A_{260}$ units(OD Units) and other physical data. This data is important for reconstituting the product. All fluorescent probes are shipped in amber tubes to prevent exposure to light and minimize photobleaching. Gene Link guarantees the stability of oligos for 1 year and fluorescent molecular probes for 6 months if reconstituted and stored appropriately as detailed below.

In our experience unmodified oligos are stable for numerous years if reconstituted and stored properly. Avoid multiple freeze thaws; do not exceed 6-10 freeze thaw cycles. If the same oligo is intended to be used repeatedly then it is prudent to make a numerous aliquots of the stock solution and frozen.

**Reconstitution & Storage**
Gene Link oligos are supplied lyophilized. These are stable at room temperature for an extended period of time. The oligonucleotide should preferably be frozen upon receipt. TE buffer (10mM Tris, 1mM EDTA, pH 7.5) is recommended for dissolving the oligonucleotides; EDTA inhibits the activity of the nucleases.

| **Preferred TE Buffer Reconstitution & Storage pH for Fluorescent Probes** | |
|---|---|
| 6-FAM, HEX, TET, ROX, and TAMRA | TE Buffer pH 7.5 or 8.0 |
| Cy3, Cy3.5, Cy5, and Cy5.5 | TE Buffer pH 7.0 or 7.5 |
| Cy dyes rapidly degrade in acidic pH | |

Further dilution can be made in TE buffer. After reconstitution store the stock solution at -80°C or -20°C. Fluorescently labeled oligos should be stored in light-free conditions.

**Preparation of Stock Solution of 100 pmols/µl [100µM]**
Gene Link provides the exact amount of nmols of each oligo supplied on the tube and on the Oligo Report. Multiply the 'nmol' amount by 10 to arrive at the volume of TE to be added.
**Example:** 45.10nmols x 10 = 451 µL
Dissolve the oligo in 451 µL to get 100pmols/µl stock solution.
Use as required.
Dilute 10 fold to prepare a 10pmols/µl [10µM]. Use as required.

For optimal long-term storage, it is recommended that the oligonucleotides should be stored dry at -20°C in the dark. If numerous experiments are planned using the same oligonucleotide, prepare aliquots, dry them and store the aliquots at -20°C.

**Stability**
Gene Link unmodified oligos are stable for 1 year and fluorescent probes for 6 months if reconstituted and stored appropriately as recommended by Gene Link. The stability is usually several years and can be increased several folds by instituting proper handling conditions, avoiding exposure to light and multiple freeze thaws.

# Gene Link Molecular Beacon Melt Curve Protocol

1. Prepare Molecular Beacon stock solution at 100 pmols/μL [ 100 μM (micromolar) ] in 1 X PCR Buffer. Gene Link provides the exact amount of nmoles of each oligo supplied on the tube and on the Oligo Report. Multiply the 'nmol' amount by 10 to arrive at the volume of solvent to be added.

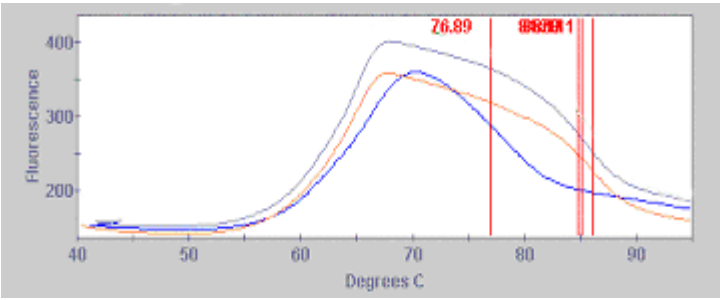
2. Prepare Molecular Beacon working solution at 5 pmols/μL [ 5 μM (micromolar) ] in 1 X PCR Buffer.

3. Set up two 25 μl reactions, one with probe alone, one with target + probe as follows.

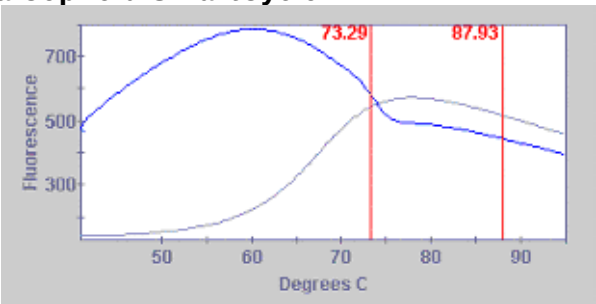
| Molecular Beacon Probe Alone | Molecular Beacon Probe plus Target |
|---|---|
| 2.5 μL10X PCR buffer | 2.5 μL 10x PCR buffer |
| 3 μL 25 mM MgCl2 | 3 μL 25 mM MgCl2 |
| 1 μL 5 pmol/ul probe [0.2 pmol/μL final or 200 nM] | 1 μL 5 pmol/ul probe [0.2 pmol/μL final or 200 nM] |
| | 3 μL 5 pmol/ul target [0.6 pmol/μL final or 600 nM] |
| 18.5 μ $H_2O$ | 15.5 μL $H_2O$ |

The protocol for Molecular Beacon Melt Curve ramps from 40 to 95 degrees C at 0.2 degrees/second

**Screen capture of graphs from a Cepheid SmartCycler**




Multiple Molecular Beacon Probe Alone Melt Curve



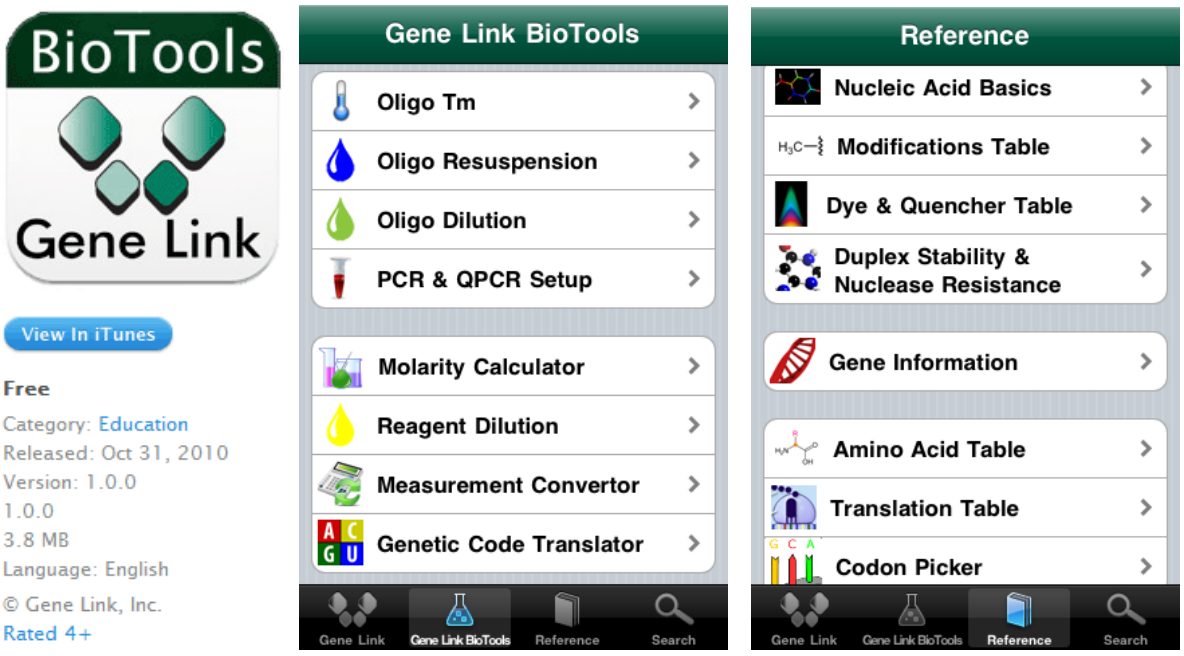

Molecular Beacon Probe plus Target and Probe Alone Melt Curve

Notes:
-$MgCl_2$ needs to be higher for MB reactions than for regular PCR as it helps to stabilize the stem structure of the probe during the high ramp rate. Final concentration of $MgCl_2$ should be between 2.5 and 4 mM. Here we use 3 mM final. This is the same range of concentration used in an actual amplification reaction.
-Final concentration of probe should be 200-600 nM. Here we use 200 nM. This is also the same concentration range used for the real time reaction.
-For a melt curve it is important to saturate the probe with target. Use 2-3 X the *Molar* amount of target. Here we use 3X target for a final concentration of 600 nM. For real time monitoring, 500 ng genomic DNA, diluted 10X to various concentrations can be used as a starting point.

QPCR
Once you have your melt curve you want to select an annealing temperature for your real time PCR where the probe alone is completely closed (shows no fluorescence), and the probe+target is completely open (shows maximal fluorescence). This temperature should be about 5-8 degrees below the Tm of the probe/target hybrid (red vertical line on melt curve of probe+target). It is important to test your primers at the annealing T to ensure that you will have strong, clean amplification at this temperature.

Signal-to-noise (S:N) ratios is calculated by dividing the fluorescence signal of a 25-mer in the presence of a two to five-fold excess of an exactly complementary target sequence by the fluorescence intensity of the probe alone.

## BioTools application from Gene Link for iPhone / iPod / iPad
## BioTools: An Array of Genetic Tools




The BioTools app also has advanced modules for setup of Polymerase Chain Reaction (PCR) and Quantitative Real Time PCR (Q-PCR).

The main focus of this app is to have a handy source of calculation modules and quick reference sections for designing and executing experiments involving PCR and Q-PCR.

| BioTools | Reference |
|---|---|
| 1. Oligo Tm: A robust oligo melting temperature calculation module using three methods; it also calculates other physical attributes.<br>2. Oligo Resuspension<br>5. Oligo Dilution<br>6. PCR & QPCR: Convenient calculator for multiple reaction setup for PCR, TaqMan QPCR and Molecular Beacon QPCR setup. Includes stock solution information and cycling profiles<br>7. Molarity Calculator<br>8. Reagent Dilution<br>9. Measurement Convertor: A convenient selection of calculators to convert length, area, mass, temperature and volume units.<br>10. Genetic Code Translator: Enter DNA sequence to see coding pattern. | A selection of topics, relevant to life scientists for quick access to basic information. This section includes the following sections and sub sections.<br><br>1. Nucleic Acid Basics<br>2. Modifications Table: A list of common modifications with molecular structure and basic properties.<br>3. Dye & Quencher Table: A convenient list of fluorophores and quencher matching the emission max.<br>4. Duplex Stability & Nuclease Resistance<br>5. Gene Information: Simply enter the accession number and retrieve detailed gene information from NCBI database,<br>6. Amino Acid Table: Molecular structure and detailed physical properties of all amino acids.<br>7. Translation Table: Genetic code for all amino acids.<br>8. Codon Picker: Select codon sequence and see the corresponding amino acid and detailed information. |

## Document Warranty and Liability

Information in this document is subject to change without notice. This document and all information presented in this document are written as a guide. Gene Link, Inc. does not warrant this document to be free of errors and assumes no responsibility for any errors that may appear in this document.
Gene Link disclaims all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall Gene Link be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

### Website

As the receipt of information on the Internet is highly dependent upon factors, including without limitations to, the user's computer, browser, operation system, etc., information may be perceived incorrectly. Therefore, Gene Link does not warrant or guarantee that the information contained on its website www.genelink.com is error free.

## Product Warranty and Liability

**Warranty:** Gene Link makes no warranty of any kind, specifically disclaims and excludes all other warranties of any kind or nature, directly or indirectly, express or implied, including, without limitation, as to the suitability, productivity, durability, fitness for a particular purpose or use, merchantability, condition, or any other matter with respect to Gene Link products. Gene Link products are for research purposes only including custom products. There is no warranty or claim of its performance for any specific research application. All Gene Link products are guaranteed to meet or exceed the specifications stated. Each Gene Link product is shipped with documentation stating specifications and other technical information. If the product fails to meet the stated specifications the sole remedy is prompt replacement by Gene Link or within 30 days of purchase a refund of the purchased price.

**Liability**: Under no circumstances shall Gene Link be liable for any damages directly or indirectly related to Gene Link's products and services. Whether direct, incidental, foreseeable, consequential, or special (including but not limited to loss of use, revenue or profit), whether based upon warranty, contract, tort (including negligence) or strict liability arising in connection with the sale or the failure of Gene Link products to perform in accordance with the stated specifications.

**Research Use Only**. Not for use in diagnostic or clinical procedures.

Notice to Purchaser: The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact support@genelink.com.



Gene Link, Inc.
Email: support@genelink.com
www.genelink.com