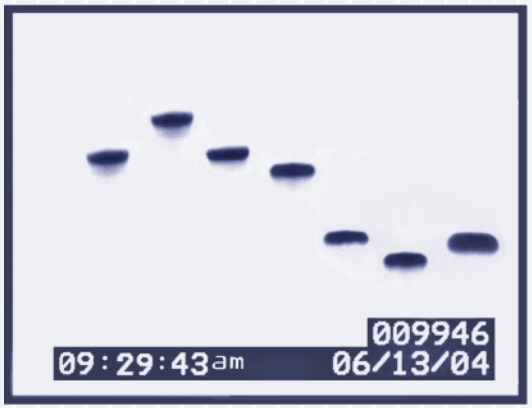
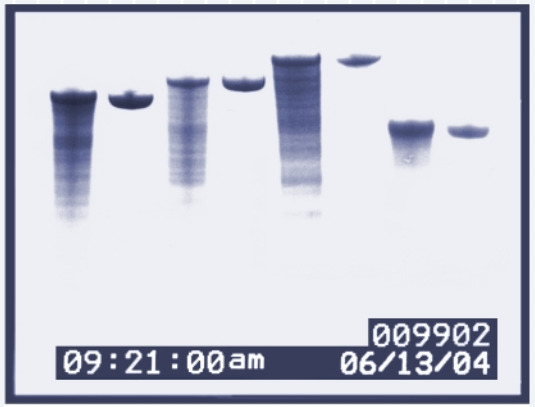
Quality • Consistency • Confidence

Customer Name: Alyson Rodgers **Order Number:** 136039

Customer Number: 10532AJ1 **Date:** June 13, 2004

Lane	Oligo Name	Sequence (5'–3')	Size	MW	TM	nmols	µg	A_{260} Units
1.	Primer 1	CATCCTGCAGGGCTAGCTCATAGAGCTTGCGCGTCAATTAGGATACCTAGG	51	15,715	74.8	48.7	765.3	26.61
2.	Primer 2	GGTGCTCTAGATCAGGAGCTTGCGCAGTCCCCGTGGGGATACCTAGTCACGTACTACTATGTCA	64	19,719	77.9	47.8	941.9	32.11
3.	Primer 3	CATCCTGCAGGGCTAGCTCATAGAGCTTGCGCGTCAATTAGAGCTTGG	48	14,800	74.6	45.5	672.7	23.10
4.	Primer 4	CTCAAGCAGGAAATCGGGAGCGGCACTTCGTACGGCGCGTCC	42	12,950	77.0	48.9	633.6	21.92
5.	Primer 5	CGGAATTCGGTCACAGGCTTGGTCA	25	7,698	63.9	48.2	370.7	12.79
6.	Primer 6	GGTCTGTCTGGGATCCCA	18	5,507	56.6	52.6	289.8	9.49
7.	Primer 7	AAGAGAAAGGTAGGAAGCAC	20	6,257	53.4	40.2	251.5	10.37
8.	Primer 8	CCAACCTCCTGTCCACCAACTTTCTTTCGTTGGATGTCCATCTGCGGCGTTTATGTTGGTTCTCCTGTAGGACTGGAA	78	23,869	77.7	9.3	222.7	7.26
9.	Primer 9	TGGTCAGAATTCTAGCCTTTCGTGACGAAATTTTAACATAAAAGAAAGGCTTCTTGATATATTATCAAGAAACCTTTCTTTTCTATTAAATTTACA	96	29,513	70.2	8.5	252.0	9.14
10.	Primer 10	AATTCTCAGTACTGTGTTTCAGCAGAAGGAGTCTTACATGTGATGGGGTGTTACAACTGAAAAGTCAAAAGAAGTTTGTATTACCATTTTCAATAGCAGTATAAAAGGTTCTCTTTGGATTCCAGTTGTTGCTGCTTTACTACTCTTTCTAGTGCTTAGCT	161	49,736	76.2	6.2	309.4	10.87
11.	Primer 11	CTCAAGCAGGAAATCGAGCGGCACTTCGTACGTAAATGCCAA	42	12,925	71.1	28.6	369.8	13.53





NOTES

Oligos 1-7 are crude unpurified. Oligos 8-11 are gel purified. Gel lanes for oligos 8-11 correspond to crude followed by gel purified.

Mobility of an oligonucleotide is dependent upon the size and base composition. Oligos of the same size may not share the same mobility patterns based on the following migration rate C>A>T>G. A stretch G's and GC's induces strong secondary structure that travels as higher mobility fragments.

Gene Link™

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Custom Oligo Specifications

Gene Link custom oligonucleotides are supplied desalted and lyophilized. They are ready to use after appropriate reconstitution. Dry oligonucleotides are stable at room temperature for an extended period of time.

Storage & Reconstitution

The oligonucleotide should preferably be frozen upon receipt. TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) is recommended for dissolving the oligonucleotides. After reconstitution store the stock solution at -80°C or -20°C.

Gel Photo Documentation

An actual gel picture of the synthesized custom oligonucleotide is supplied. A major single band represents high purity of the crude oligonucleotide.

Purity & Usage

The crude, desalted oligonucleotide supplied is suitable for all amplification and sequencing protocols. Gel purification is advised for all oligos used for cloning applications and for oligos longer than 50 mer.

Biophysical Data

Each oligo after desalting is quantified by recording A_{260}. Exact nmols and µg is determined by the extinction coefficient and molecular weight of the oligo.

Oligo Scale of Synthesis and Typical Yield of Unmodified Oligos*

	Crude Desalted		RPC Purified***		Gel Purified	
	20 mer oligo**		30 mer oligo**		50 mer oligo**	
Scale	**A_{260} Units**	**nmols**	**A_{260} Units**	**nmols**	**A_{260} Units**	**nmols**
50 nmol	8-10	30+	4-5	12+	NR* [1-2]	NR* [2-4]
200 nmol	20-25	80+	8-12	24+	4-6	8+
1 µmol	100-120	400+	40-50	30+	20-25	40+
Purity & Yield	Purity is more than 80% depending on oligo sequence and structure.		Purity 85% to 95% depending on oligo sequence and structure. Not recommended for oligos longer than 35 mer.		Purity 98% to ~100% depending on oligo sequence and structure. Yield will gradually decrease as length of oligo increases.	

*The yield of modified oligos varies based on modification.

**Yield of 30 µg/A_{260} unit for oligos is calculated for an ~equimolar base composition. Long stretches of a single base or homopolymers will have variable yields. Example for homopolymeric 50 mer: A(50) = ~20/A_{260} Unit; G(50) = ~28/A_{260} Unit; T(50) = ~35/A_{260} Unit and C(50) = ~39/A_{260} Unit.

***RPC is reverse phase purification using a cartridge; a substitute for HPLC.

NR* Not Recommended.

Primer Design

Successful use of oligos as primers for amplification and sequencing starts with functional primer design followed by optimized PCR amplification conditions.

Fortunately, both PCR and sequencing reactions are inherently 'robust' and have been observed to tolerate wide variations in quality of primers when using unique templates. The same 'tolerance' can also lead to false priming, poor results and frustrating time loss with templates of higher complexity.

Primer specificity alone does not guarantee an optimum amplification yield. Numerous computer applications are available for primer search and design. Most of these applications do not consider the effect of hairpin structures which tend to be quite stable thermodynamically. General guidelines for primer design are given below followed by a brief account of stable hairpin structure formation and non-Watson-Crick base pairing induced by a stretch of G's and G's interspersed with A's or C's (1-3).

General Guidelines

1. **Specificity:** Select an 18 to 24 mer stretch with perfect specificity.
2. **Base Composition:** Preferably maintain GC content below 60% with no stretches of more than 3G's or 4 runs of the same base.
3. **Tm:** Select primer Tm within a few degrees of the pair.
4. **Cross Homologies:** Perform NCBI blast to determine extent of cross homologies.
5. **Secondary Structure:** Perform computer assisted analysis to view formation of stable dimers, loops and hairpins.

Hairpin Loop Formation and Primer Design*

Sequence	5'-CAGCGCACTACAGGCATGACGT-3' 22 mer; dG = -47.5; Tm(NN) = 61.6°C	5'-GTCCGCACGTACGGACAT-3' 18 mer; dG = -38.4; Tm(NN): 57.0°C	5'-GTCAGCCGCACGTACGGACAT-3' 21 mer; dG: -46.3; Tm(NN): 61.70°C	5'-AGTAACGCACTACGGACTTACGAC-3' 24 mer; dG = -47.1; Tm(NN) = 58.8°C
***Dimers**	5' CAGCGCACTACAGGCATGACGT 3' |||| 3' TGCAGTACGGACATCACGCGAC 5' STACK AT 3 IS 4 BP LONG. dG = -4.8; Tm = -58.4°C	5' GTCCGCACGTACGGACAT 3' + |||||| + 3' TACAGGCATGCACGCCTG 5' STACK AT 8 IS 6 BP LONG. dG = -5.7; Tm = -42.4°C	5' GTCAGCCGCACGTACGGACAT 3' + |||||| + 3' TACAGGCATGCACGCCGACTG 5' STACK AT 11 IS 6 BP LONG. dG = -4.65; Tm = -28.2°C	5' AGTAACGCACTACGGACTTACGAC 3' |||| + ++ + ++++ 3' CAGCATTCAGGCATCACGCAATGA 5' STACK AT 2 IS 4 BP LONG. dG=-2.05; Tm=-47.3°C
Hairpin Loops	None	5' GTCCGCAC |||||] 3' TACAGGCATG STEM AT 1 IS 5 BP LONG. LOOP=6. dG = -5.3; Tm = 87.3°C	5' GTCAGCCGCAC |||] 3' TACAGGCATG STEM AT 6 IS 3 BP LONG. LOOP=6 dG = -2.4; Tm = 68.9°C	5' AGTAACGCACT ||||] 3' CAGCATTCAGGCA STEM AT 2 IS 4 BP LONG. LOOP=12. dG = 0.8; Tm = 13.8°C

*Secondary structure results are truncated to show the most stable structures. All thermodynamic values including Tm and secondary structures calculated and displayed solely indicate the relative stability of the secondary structures. They should only be used to compare the relative stability of the structures. dG value unit is kcal/mol.

Visit www.genelink.com/tools/gl-SOD.asp to design oligos or click on the 'Analyze' button while on the online oligo ordering page.

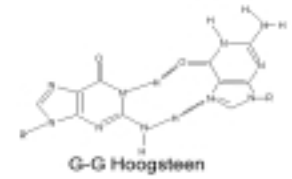
Hairpin Structures

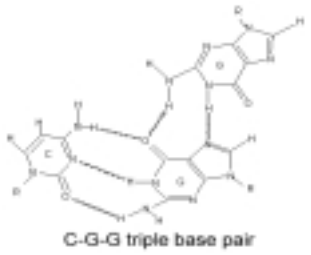
One essential element of efficient primer design is to minimize internal secondary structure, especially hairpin loops which tend to be deceptively stable at standard annealing temperatures. Hairpins are stable with as few as 4 bases stacked in the stem and a loop size of 4 to 6 bases. The stability declines as the loop size increases. The stem and loop size are related proportionately such that longer stem sizes can tolerate longer loop sizes (4).

As a general rule, avoid hairpins with more than 3 bases in the stem. Stable hairpin loop formation drastically reduces the primer concentration available for hybridization to the target sequence.

Base Composition

Higher GC content stabilizes hybridization, but a string of G's and C's can exhibit internal Hoogsteen base pairing, non-Watson-Crick base pairing and should be avoided (3,4). Although this anomalous behavior is difficult to predict, these structures can disrupt stable primer binding. In general, avoid runs of more than three consecutive G's in primers. Also, examine potential primers for self-complementary and hairpin structures. Nuclear Magnetic Resonance (NMR) studies have shown that a stable hairpin can form with just four G-C basepairs in the stem and just three bases in the loop (5).


G-G Hoogsteen


C-G-G triple base pair

References

1. Michael Zuker (2003) *Nucleic Acids Res.*, 31, 3406–3415.
2. SantaLucia, J. (1998) *Proc. Nat. Acad. Sci. USA* 95, 1460.
3. Sarochi, M-T., Courtois, Y., Guschlbauer, W. 1970. *Eur. J. Biochem.* 14: 411.
4. Gene Link, Inc. internal data.
5. Summer, M.F., Byrd R.A., Gallo, K.A., Samson, C.J., Zon, G., Egan, W. 1985. *Nucleic Acids Res.* 13: 6375.

Gene Link™

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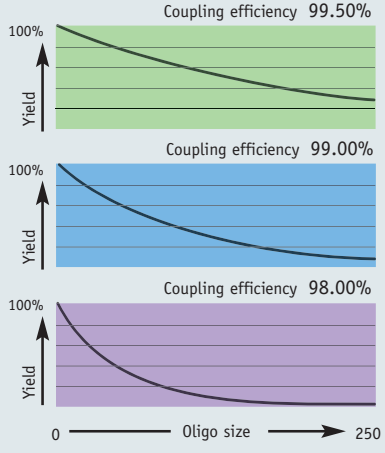
Synthesis of Long Oligos

Coupling Efficiency

Chemical DNA synthesis comprises of multiple reactions to complete a cycle of the appropriate base coupling. Thus the use of reagents of exacting specifications, state of the art instruments and optimized software driven protocols are necessary to maintain the highest possible coupling efficiency. This clearly becomes enormously important when synthesizing a long oligo. Gene Link specializes in long oligos. Our description of long oligos is 180mer to 250mer. PCR and sequencing reactions are very robust and can tolerate up to 50% failure/truncated sequence oligos. Coupling efficiency of 99.5% and 98% seems very good but on closer examination the yield is almost half for a 40mer! Please see the coupling efficiency table and graph.

Coupling Efficiency and Full Length Oligo Yield

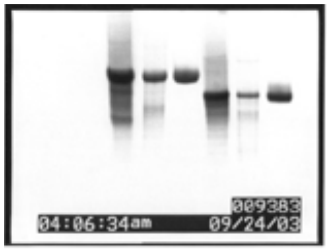
Oligo Size	99.50%	99.00%	98.00%
20	90.916	82.617	68.123
40	82.243	67.573	45.48
60	74.398	55.268	30.363
80	67.301	45.204	20.27
100	60.881	36.973	13.533
120	55.074	30.24	9.034
140	49.821	24.734	6.031
160	45.068	20.23	4.027
180	40.769	16.546	2.688
200	36.88	13.533	1.795
220	33.36	11.07	1.19
240	30.18	9.05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



Purification

Crude oligo is the total yield after chemical synthesis; this contains the full-length product as well as all truncated n-1 sequences. For example, at 99% coupling efficiency the crude yield of a 70mer is ~50% full length and ~50% truncated shorter sequences. Gel purification is strongly recommended for all oligos above 50mer. All Gene Link oligos shorter than 40mer usually do not require any further purification if the application is for PCR or sequencing without downstream cloning of the product. Gel purification involves electrophoresis of the entire crude product on a preparative polyacrylamide gel followed by excision and purification of the full-length oligo.

HPLC is not capable of consistently resolving oligos above 40mer and thus is not a recommended for purification of long oligos.



Unpurified, RPC and Gel Purified Oligos

Polyacrylamide gel electrophoresis of crude, reverse phase cartridge (RPC) and gel purified oligos.

Approximately 15µg of crude unpurified oligo was loaded to show the truncated failure sequences.

Approximately 8µg of purified oligos were loaded.

Lanes 1–3 is a 68mer oligo.
Lanes 4–6 is a 56mer oligo.

Lanes 1&4: crude unpurified.
Lanes 2&5: RPC purified.
Lanes 3&6: gel purified.

Sequence Accuracy

Statistically sequence accuracy is guaranteed in a portion of the full length product. Despite the effort to maintain a coupling efficiency above 99%, it still leaves the unavoidable failure rate of less than 1%. This ~1% failure rate is cumulative; meaning at every step of each cycle; including miniscule but still probable, error of deletions and insertions of erroneous bases. For long oligos this becomes more pronounced and exaggerated on occasion due to amplification during PCR. Mathematical and statistical analysis based on Avogadro's number (6.022×10^{23} mol^{-1}) and synthesis of a long oligo at 1 micromolar scale (6.022×10^{17} mol^{-1}) at 99% coupling efficiency will still yield quite a few million copies of the exact sequence.

It is imperative to pick several colonies of cloned inserts and confirm by sequencing. It is observed that on occasions due to high GC content or long stretches of bases, relatively more colonies have to be screened by sequencing to find the clone of the correct sequence.

Purity and Yield

Purity is generally between 98% to ~100% depending on oligo sequence and structure. Yield will gradually decrease as length of oligo increases. Palindromes, hairpins, high GC content oligos and oligos containing stretches of 3 or more G's induce strong secondary structure and base stacking. These are not completely denatured and travel as a broad band on polyacrylamide gels thus decreasing purity and yield.

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