# 引物设计

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# 介绍

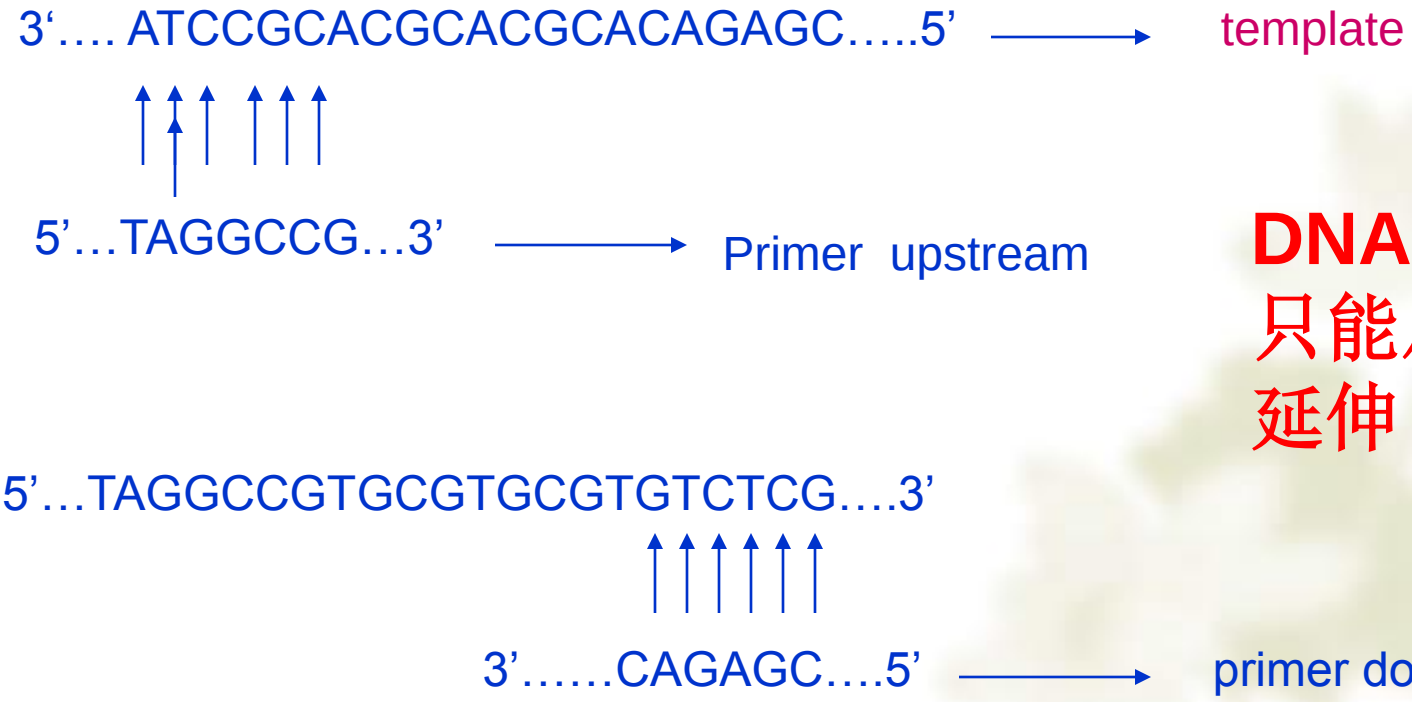
引物设计的原则

Primer5介绍

Oligo6介绍

# 上游引物和下游引物

- 什么是上游引物，什么又是下游引物？




**DNA聚合酶只能从5‘向3’延伸**

谁先延伸就是上游引物，后延伸的就是下游引物

# 参考值

- 引物长度（primer length）
- 产物长度（product length）
- 序列Tm值(melting temperature)
- ΔG值(internal stability)、
- 二聚体，发夹结构（Dimer and hairpin）
- 错误引发位点（false priming site）
- 引物及产物GC 含量（composition）
- 3‘末端稳定性（3’ End Stability）

- 引物的长度一般为15-30bp，常用的是18-27bp。

- 因为过长会导致其延伸温度大于74℃，即Taq 酶的最适温度。

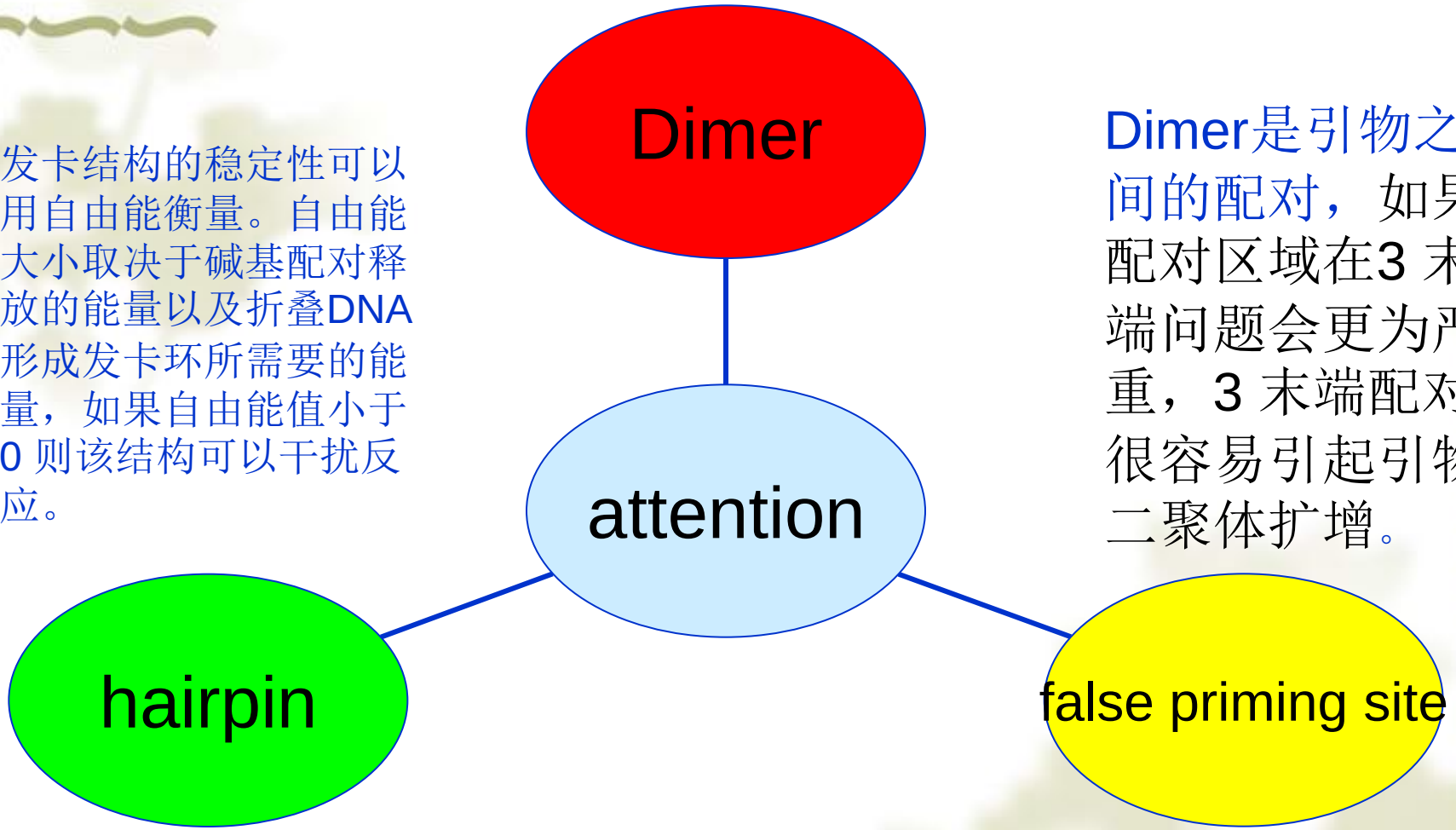
# Tm值与GC含量

- GC含量大，Tm值大，解链温度高。
- 对于PCR反应来说GC含量在40%—60%，一般50%左右比较合适。 Tm值控制在50-70之间
- 有一些模板本身的GC 含量偏低或偏高，导致引物的GC含量不能在上述范围内，这时应尽量使上下游引物的GC 含量以及Tm 值保持接近。

# ΔG值

- 反映了引物与模板结合的强弱程度
- 一般情况下，引物的ΔG值最好呈正弦曲线形状，即5’端和中间ΔG值较高，而3’端ΔG值相对较低，且不要超过9（ΔG值为负值，这里取绝对值），如此则有利于正确引发反应而可防止错误引发。

| ΔG / 3’ | -2 | -6 | -8 | -10 |
|---|---|---|---|---|
| 产量 | 100% | 40% | 20% | 0 |




发卡结构的稳定性可以用自由能衡量。自由能大小取决于碱基配对释放的能量以及折叠DNA形成发卡环所需要的能量，如果自由能值小于0 则该结构可以干扰反应。

Dimer是引物之间的配对，如果配对区域在3 末端问题会更为严重，3 末端配对很容易引起引物二聚体扩增。

引物二聚体及发夹结构的能量一般不要超过

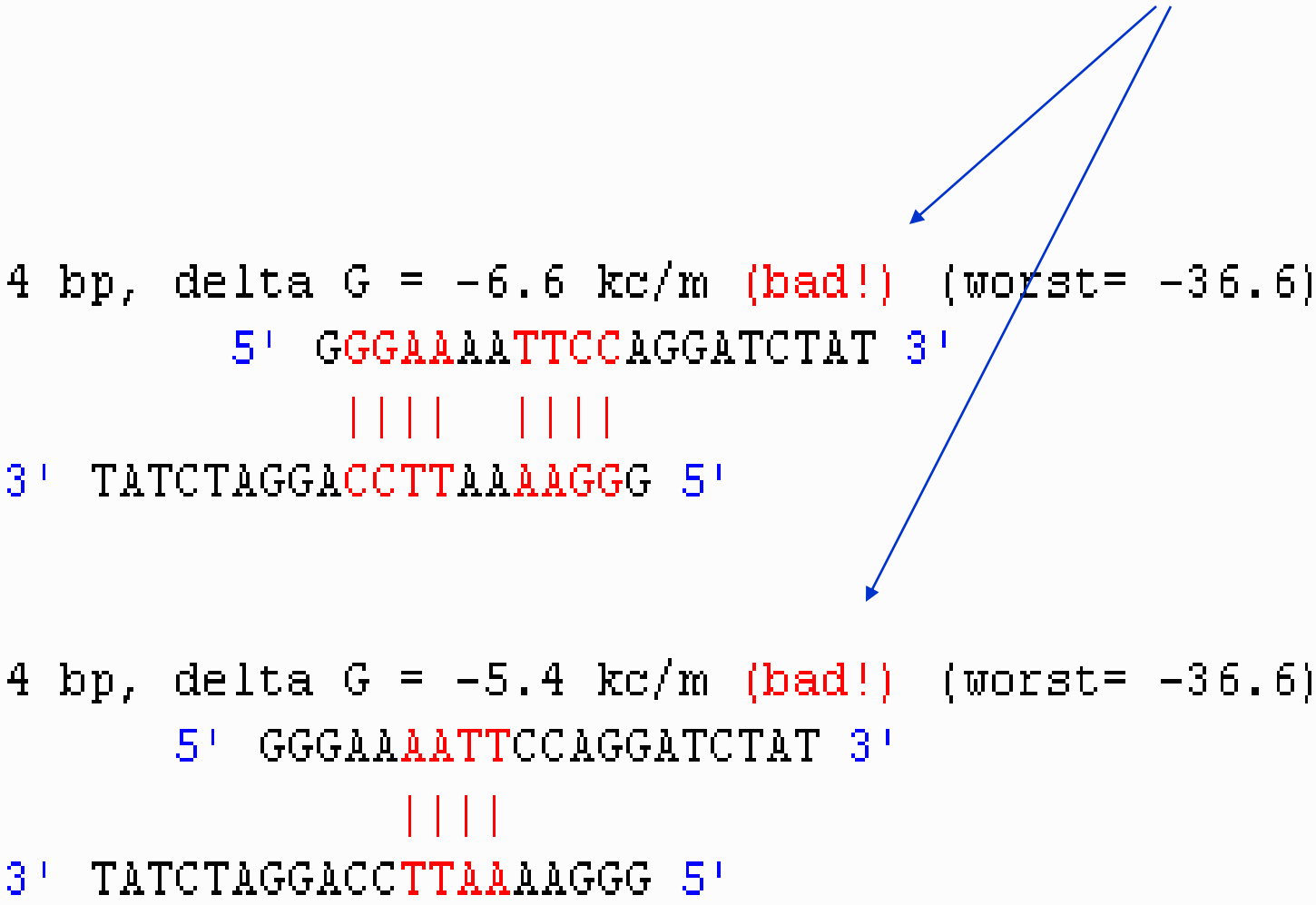
4.5

# Hairpin

Oligo, 3 bp (Loop=4), delta G = -0.1 kc/m


Oligo, 2 bp (Loop=3), delta G = 2.1 kc/m

# Self-Dimer


4 bp, delta G = -6.6 kc/m (bad!) (worst= -36.6)
5' GGGAAAATTCCAGGATCTAT 3'
3' TATCTAGGACCTTAAAAGGG 5'
4 bp, delta G = -5.4 kc/m (bad!) (worst= -36.6)
5' GGGAAAATTCCAGGATCTAT 3'
3' TATCTAGGACCTTAAAAGGG 5'

# 3‘末端稳定性

- 一条理想的引物应该有一个稳定性较强的5 末端和相对稳定性较弱的3 末端。如果引物3 稳定性强，有可能在即使5 末端不配对的情况下造成错配，形成非特异性扩增条带（secondary bands）

- 引物3’端的序列要比5’端重要。引物3’端的碱基一般不用A（3’端碱基序列最好是G、C、CG、GC），因为A在错误引发位点的引发效率相对比较高

# 补充:GC clamp

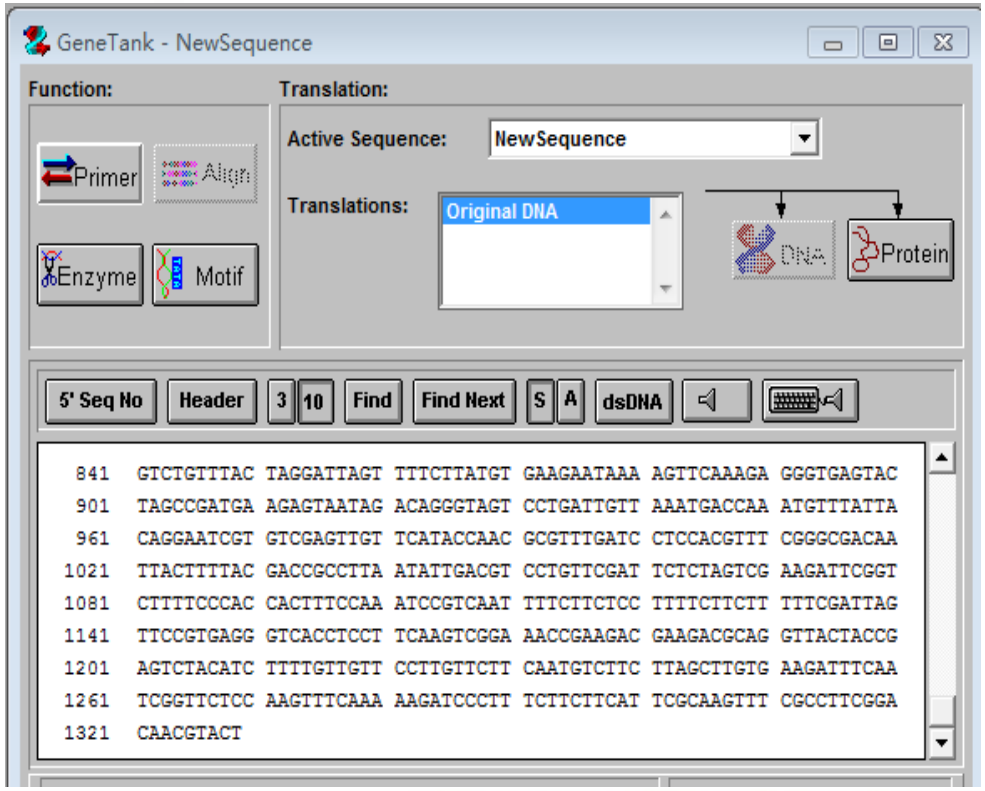
- GC钳
- 引物与目的位点的有效结合需要有稳定的5末端。这一段有较强稳定性的5 末端称为GC钳。

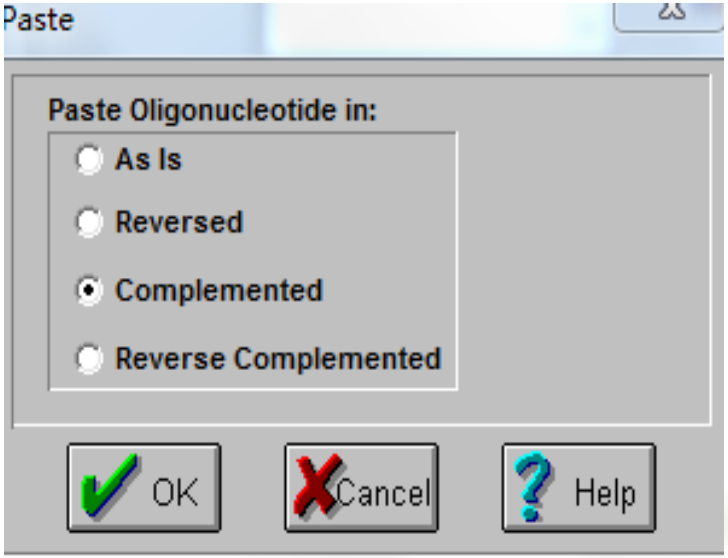
# 添加酶切位点

- 1.上下游引物修饰的序列选用不同限制酶的识别序列
- 2.酶切位点前加入保护碱基，大多数限制酶对裸露的酶切位点不能切断。
- 3.保护碱基加入后要考虑是否形成新的酶切位点。

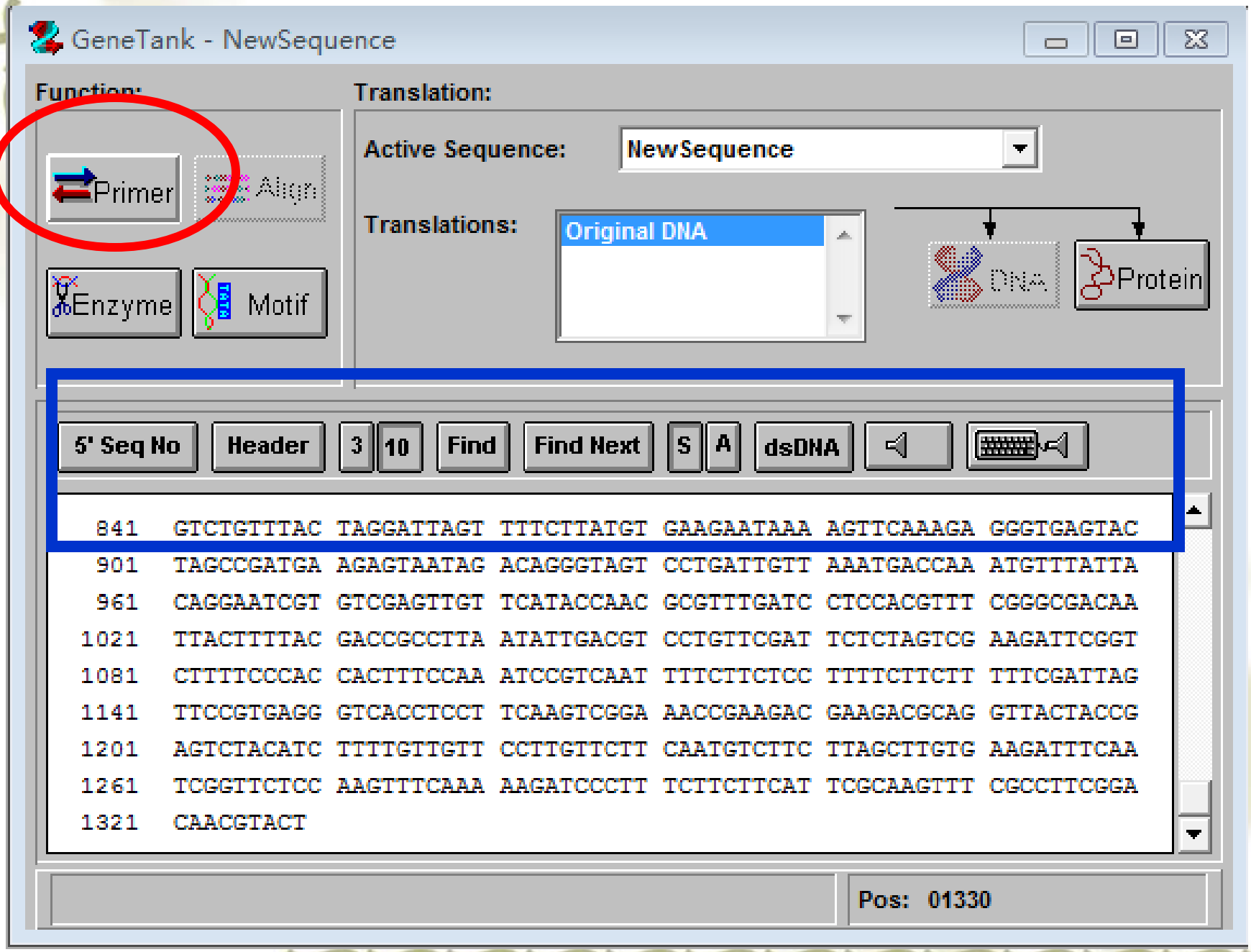
# Primer 5

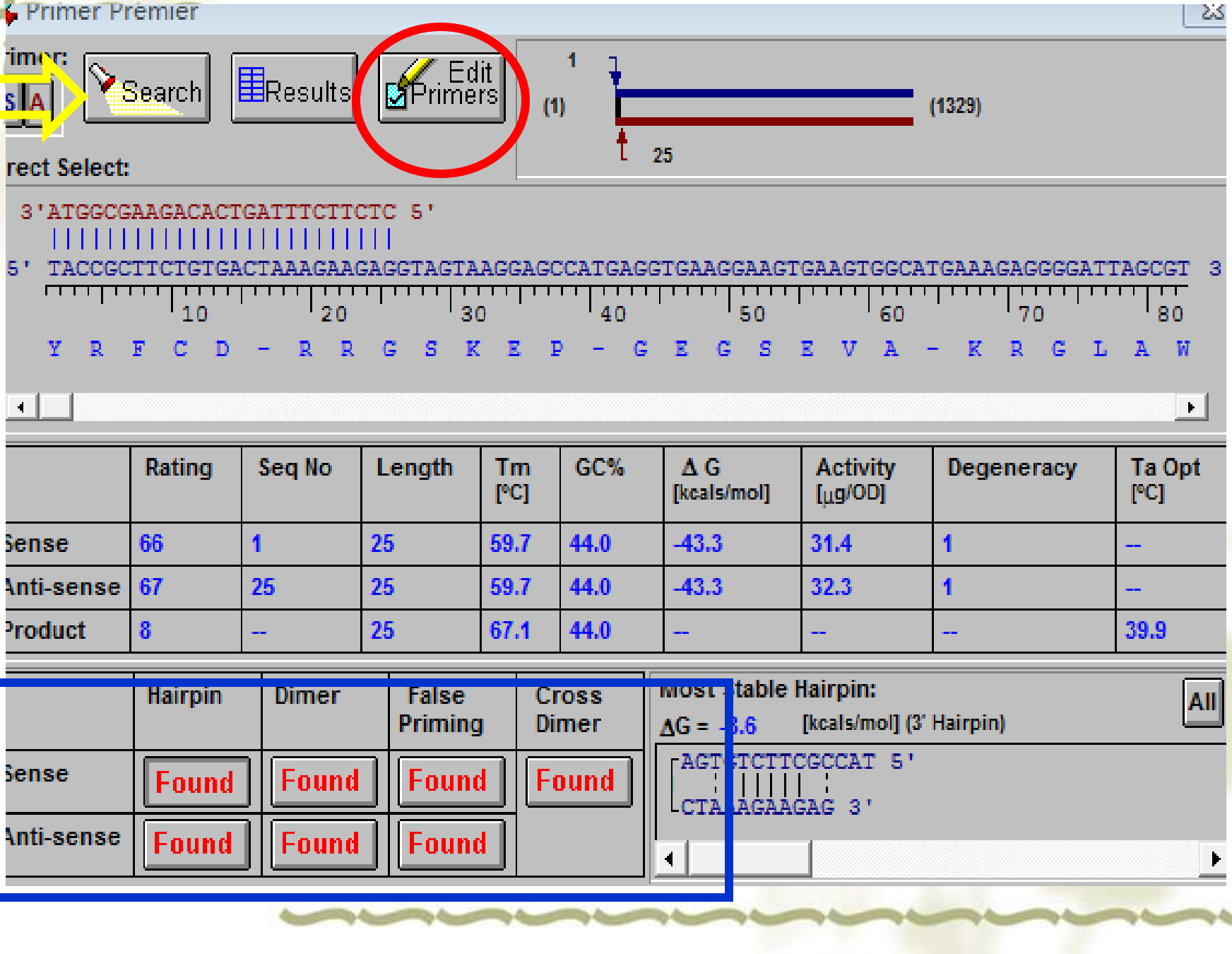
- File → new seq

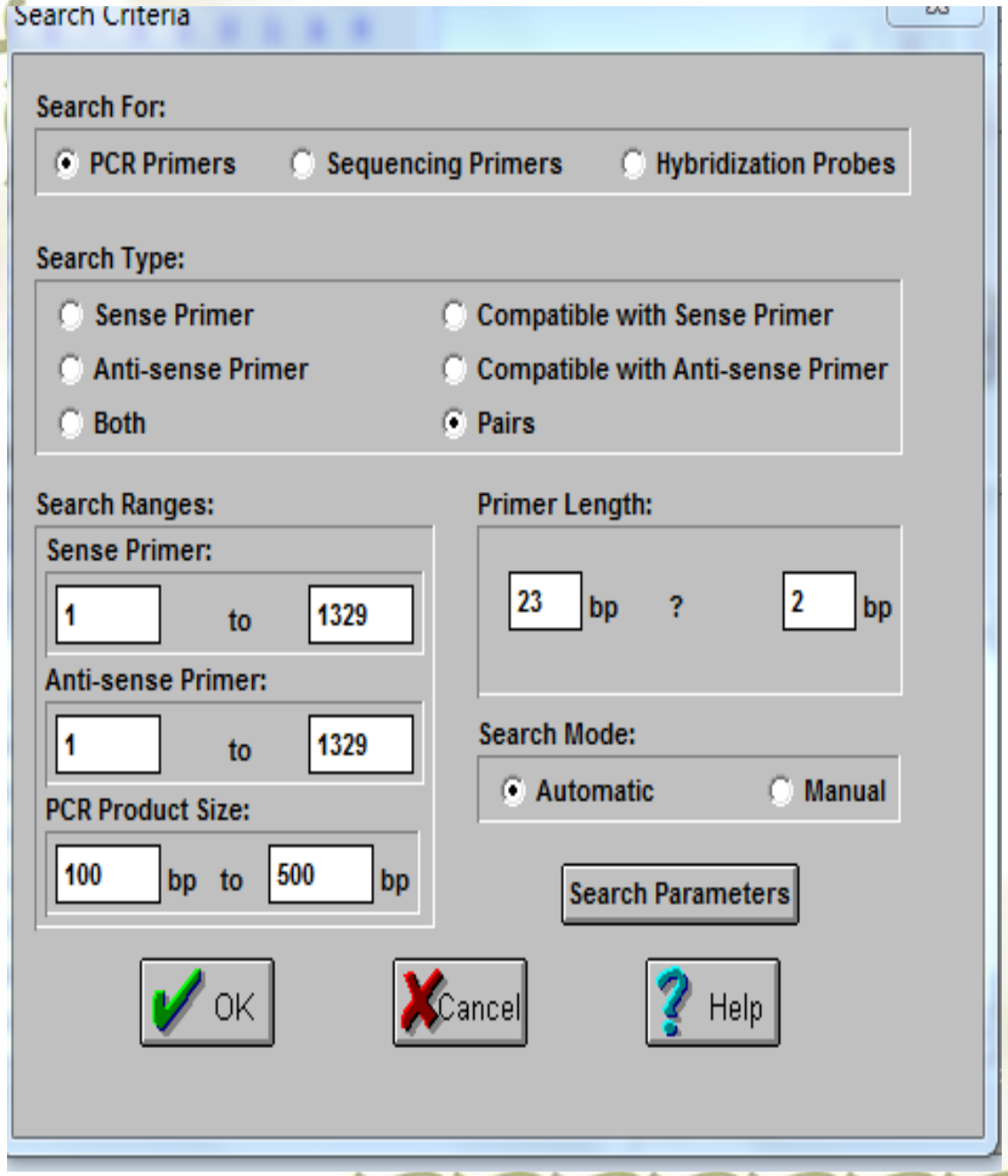



点击ctrl+v将序列粘贴上去

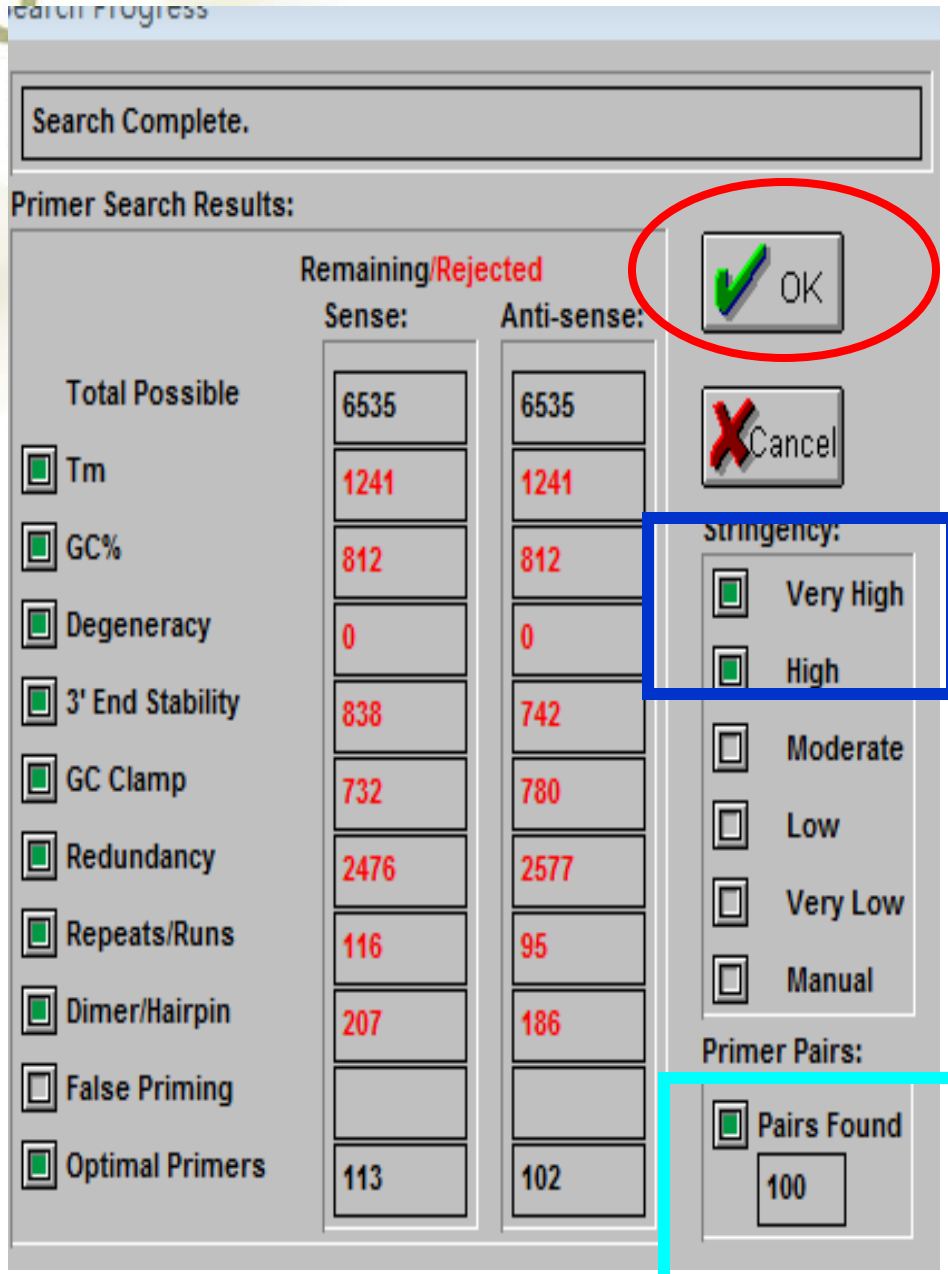

GeneTank - NewSequence
Function:
Translation:
Primer
Align
Enzyme
Motif
Active Sequence:
NewSequence
Translations:
Original DNA
DNA
Protein
5' Seq No
Header
3
10
Find
Find Next
S
A
dsDNA
841 GTCTGTTTAC TAGGATTAGT TTTCTTATGT GAAGAATAAA AGTTCAAAGA GGGTGAGTAC
901 TAGCCGATGA AGAGTAATAG ACAGGGTAGT CCTGATTGTT AAATGACCAA ATGTTTATTA
961 CAGGAATCGT GTCGAGTTGT TCATACCAAC GCGTTTGATC CTCCACGTTT CGGGCGACAA
1021 TTACTTTTAC GACCGCCTTA ATATTGACGT CCTGTTCGAT TCTCTAGTCG AAGATTCGGT
1081 CTTTTCCCAC CACTTTCCAA ATCCGTCAAT TTTCTTCTCC TTTTCTTCTT TTTCGATTAG
1141 TTCCGTGAGG GTCACCTCCT TCAAGTCGGA AACCGAAGAC GAAGACGCAG GTTACTACCG
1201 AGTCTACATC TTTTGTTGTT CCTTGTTCTT CAATGTCTTC TTAGCTTGTG AAGATTTCAA
1261 TCGGTTCTCC AAGTTTCAAA AAGATCCCTT TCTTCTTCAT TCGCAAGTTT CGCCTTCGGA
1321 CAACGTACT
Pos: 01330

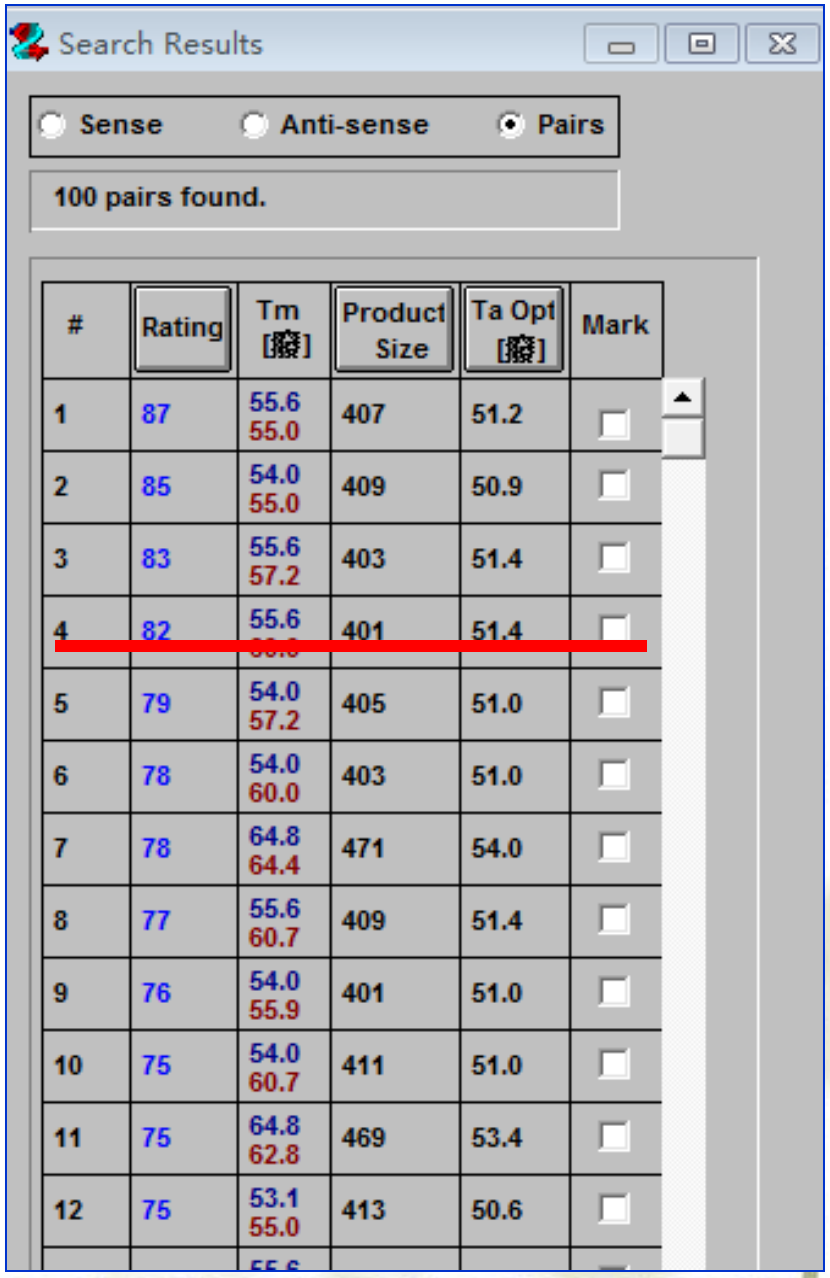

Primer Premier
Search
Results
Edit Primers
1
(1)
(1329)
25
rect Select:
3'ATGGCGAAGACACTGATTTCTTCTC 5'
5' TACCGCTTCTGTGACTAAAGAAGAGGTAGTAAGGAGCCATGAGGTGAAGGAAGTGAAGTGGCATGAAAGAGGGGATTAGCGT 3
10
20
30
40
50
60
70
80
Y R F C D - R R G S K E P - G E G S E V A - K R G L A W
Rating
Seq No
Length
Tm [°C]
GC%
Δ G [kcals/mol]
Activity [µg/OD]
Degeneracy
Ta Opt [°C]
Sense 66 1 25 59.7 44.0 -43.3 31.4 1 --
Anti-sense 67 25 25 59.7 44.0 -43.3 32.3 1 --
Product 8 -- 25 67.1 44.0 -- -- -- 39.9
Hairpin
Dimer
False Priming
Cross Dimer
Sense Found Found Found Found
Anti-sense Found Found Found
Most stable Hairpin:
All
ΔG = -3.6 [kcals/mol] (3' Hairpin)
AGT TCTTCGCCAT 5'
CTA AGAAGAG 3'

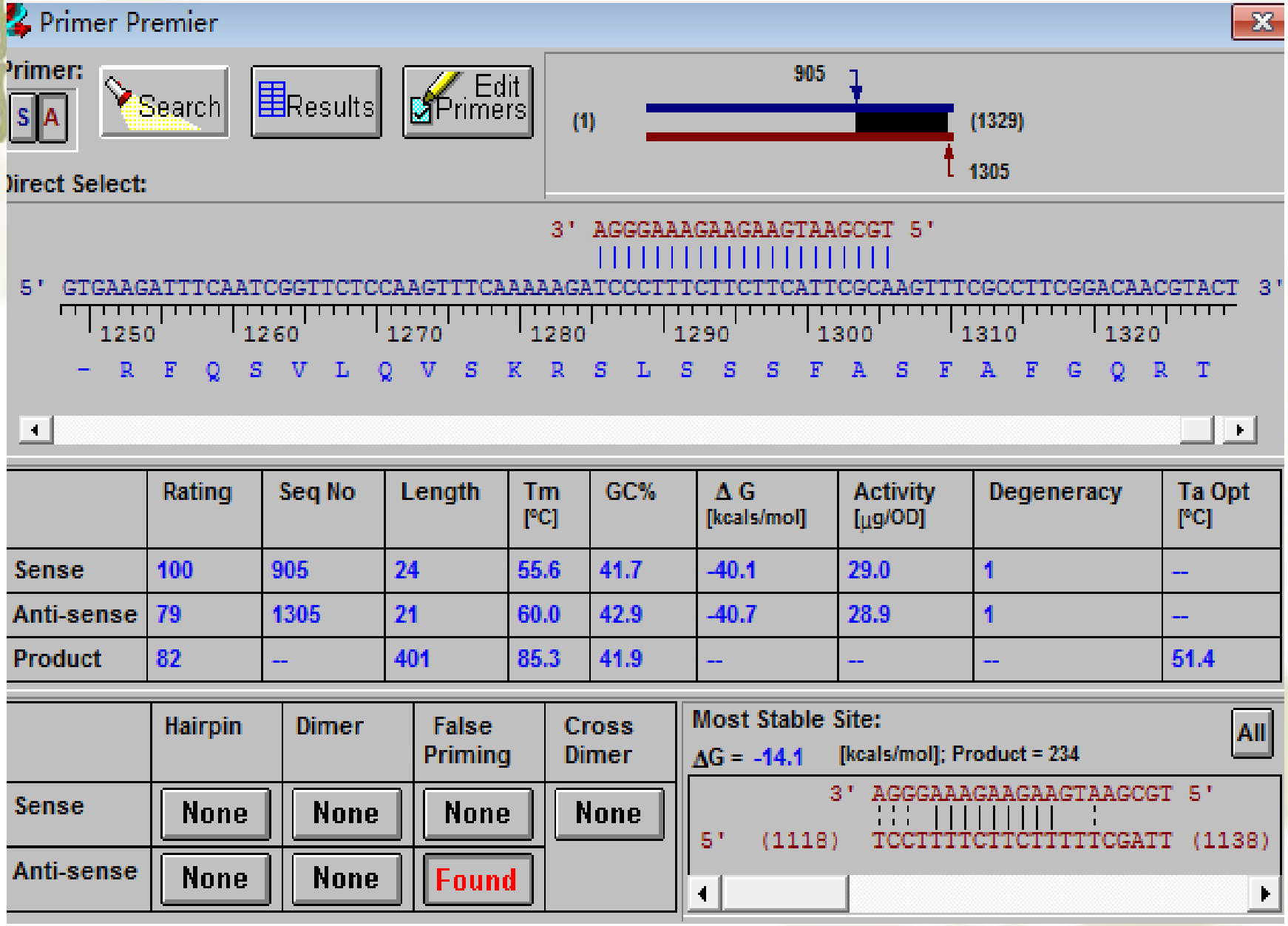

Search Criteria
Search For:
PCR Primers
Sequencing Primers
Hybridization Probes
Search Type:
Sense Primer
Compatible with Sense Primer
Anti-sense Primer
Compatible with Anti-sense Primer
Both
Pairs
Search Ranges:
Sense Primer:
1
to
1329
Anti-sense Primer:
1
to
1329
PCR Product Size:
100
bp
to
500
bp
Primer Length:
23
bp
?
2
bp
Search Mode:
Automatic
Manual
Search Parameters
OK
Cancel
Help

点击search

出现


Search Complete.
Primer Search Results:
Remaining/Rejected
Sense:
Anti-sense:
OK
Cancel
Total Possible
6535
6535
Tm
1241
1241
GC%
812
812
Degeneracy
0
0
3' End Stability
838
742
GC Clamp
732
780
Redundancy
2476
2577
Repeats/Runs
116
95
Dimer/Hairpin
207
186
False Priming
Optimal Primers
113
102
Stringency:
Very High
High
Moderate
Low
Very Low
Manual
Primer Pairs:
Pairs Found
100

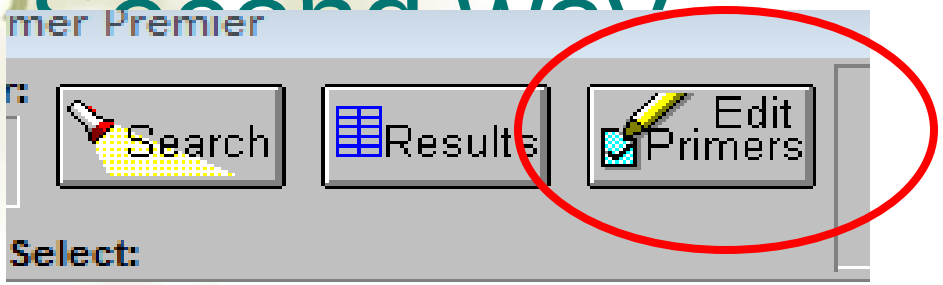

Search Results
Sense
Anti-sense
Pairs
100 pairs found.
# Rating Tm [發] Product Size Ta Opt [發] Mark
1 87 55.6 55.0 407 51.2
2 85 54.0 55.0 409 50.9
3 83 55.6 57.2 403 51.4
4 82 55.6 401 51.4
5 79 54.0 57.2 405 51.0
6 78 54.0 60.0 403 51.0
7 78 64.8 64.4 471 54.0
8 77 55.6 60.7 409 51.4
9 76 54.0 55.9 401 51.0
10 75 54.0 60.7 411 51.0
11 75 64.8 62.8 469 53.4
12 75 53.1 55.0 413 50.6

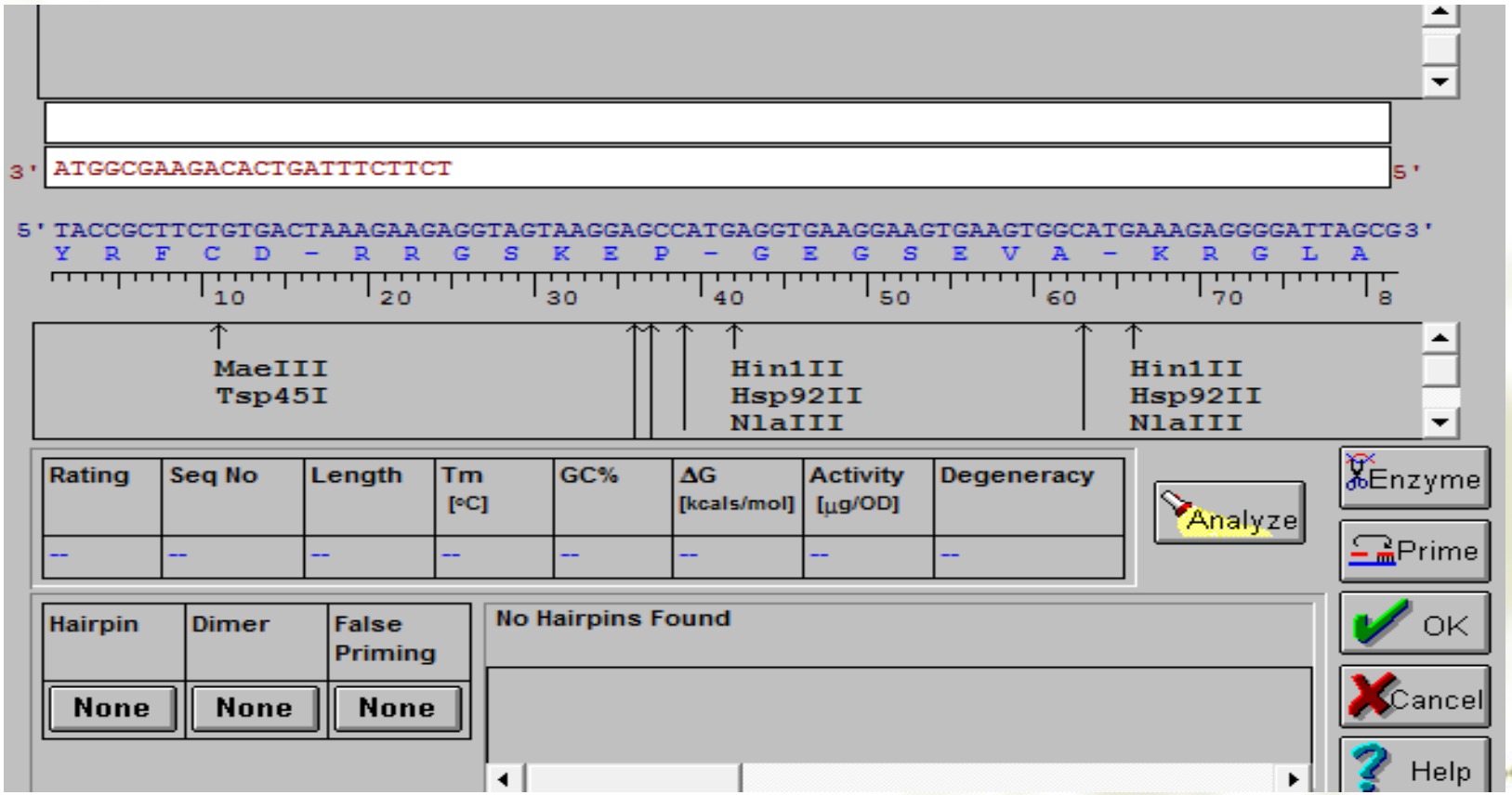


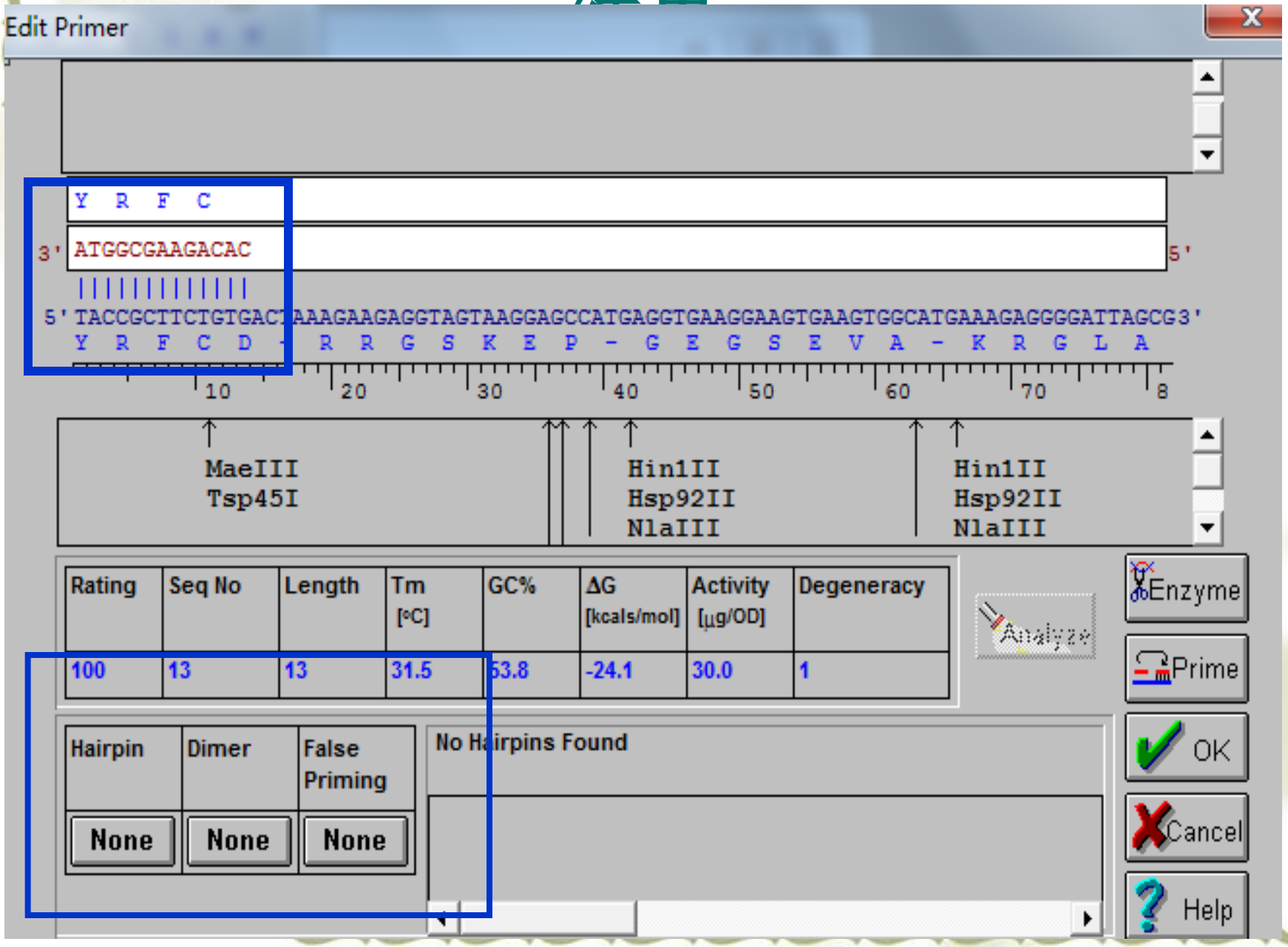

| | Rating | Seq No | Length | Tm [°C] | GC% | Δ G [kcals/mol] | Activity [µg/OD] | Degeneracy | Ta Opt [°C] |
|---|---|---|---|---|---|---|---|---|---|
| Sense | 100 | 905 | 24 | 55.6 | 41.7 | -40.1 | 29.0 | 1 | -- |
| Anti-sense | 79 | 1305 | 21 | 60.0 | 42.9 | -40.7 | 28.9 | 1 | -- |
| Product | 82 | -- | 401 | 85.3 | 41.9 | -- | -- | -- | 51.4 |

| | Hairpin | Dimer | False Priming | Cross Dimer |
|---|---|---|---|---|
| Sense | None | None | None | None |
| Anti-sense | None | None | Found | |

# Second way


mer Premier
r:
Search
Results
Edit Primers
Select:


3' ATGGCGAAGACACTGATTTCTTCT 5'
5' TACCGCTTCTGTGACTAAAGAAGAGGTAGTAAGGAGCCATGAGGTGAAGGAAGTGAAGTGGCATGAAAGAGGGGATTAGCG3'
Y R F C D - R R G S K E P - G E G S E V A - K R G L A
10 20 30 40 50 60 70 8
MaeIII
Tsp45I
Hin1II
Hsp92II
NlaIII
Hin1II
Hsp92II
NlaIII
Rating
Seq No
Length
Tm [°C]
GC%
ΔG [kcals/mol]
Activity [µg/OD]
Degeneracy
Analyze
Enzyme
Prime
OK
Cancel
Help
Hairpin
Dimer
False Priming
None
None
None
No Hairpins Found


Edit Primer
Y R F C
3' ATGGCGAAGACAC 5'
5' TACCGCTTCTGTGACTAAAGAAGAGGTAGTAAGGAGCCATGAGGTGAAGGAAGTGAAGTGGCATGAAAGAGGGGATTAGCG3'
Y R F C D - R R G S K E P - G E G S E V A - K R G L A
10 20 30 40 50 60 70 8
MaeIII
Tsp45I
Hin1II
Hsp92II
NlaIII
Hin1II
Hsp92II
NlaIII
Rating
Seq No
Length
Tm [°C]
GC%
ΔG [kcals/mol]
Activity [µg/OD]
Degeneracy
100
13
13
31.5
53.8
-24.1
30.0
1
Analyze
Enzyme
Prime
OK
Cancel
Help
Hairpin
Dimer
False Priming
None
None
None
No Hairpins Found

# Oligo介绍

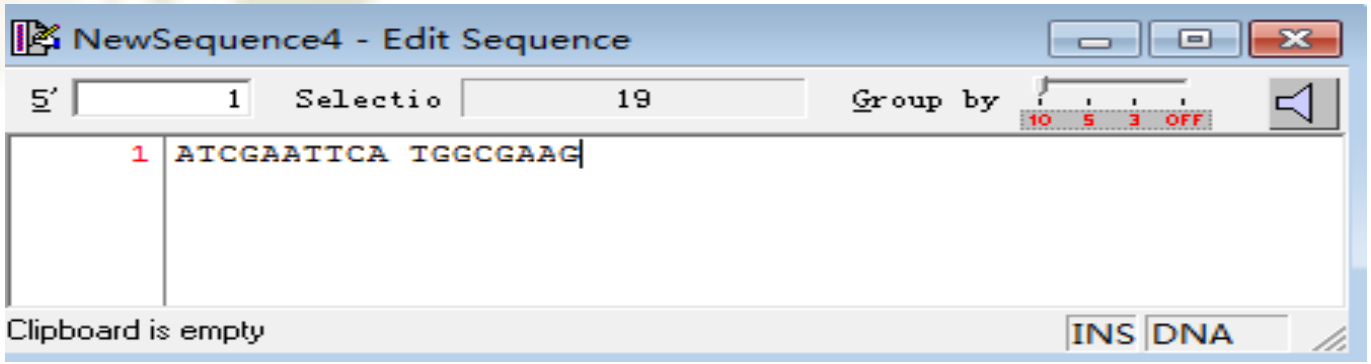
- 主要为对primer5设计出的引物进行更细致的评估。
- 得到两条引物，添加保护碱基，和酶切位点
- Sense：

  ATCGAATTCATGGCGAAG
- Anti-sense

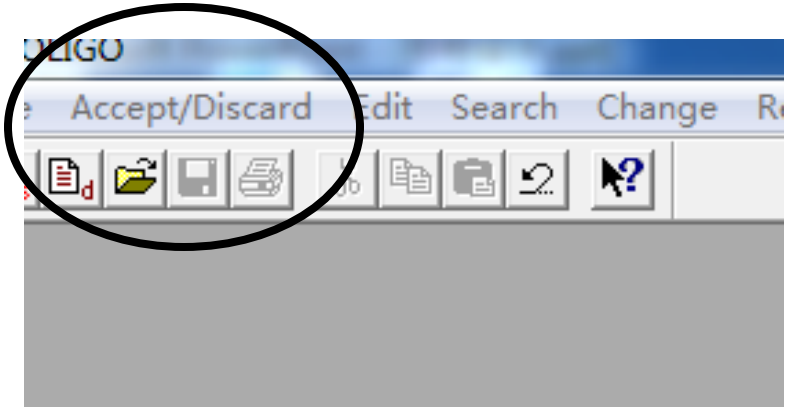
  GGAGGATCCAGTACGTTG

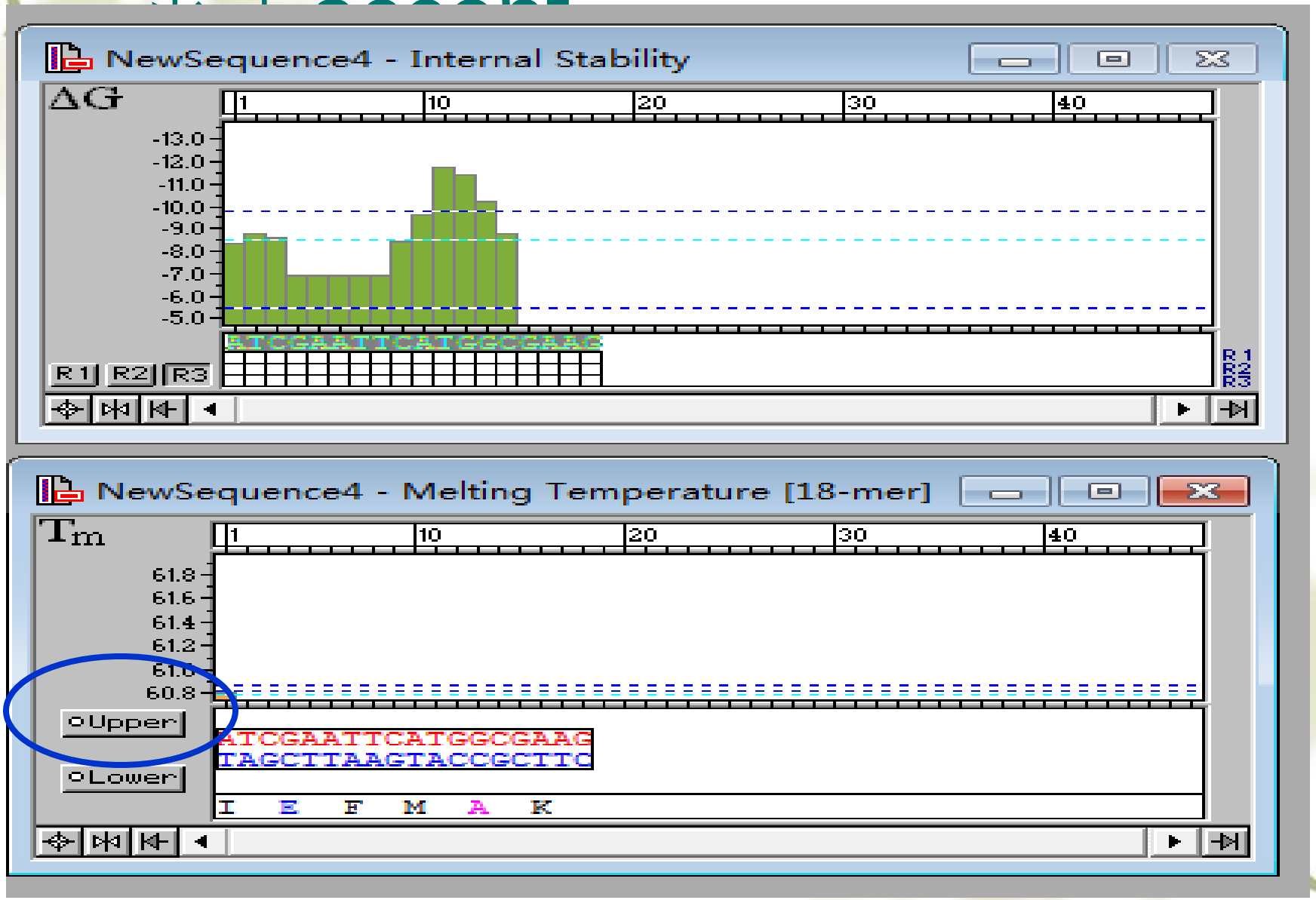
  将设计好的引物放入oligo6内分析

file → New sequence → manual



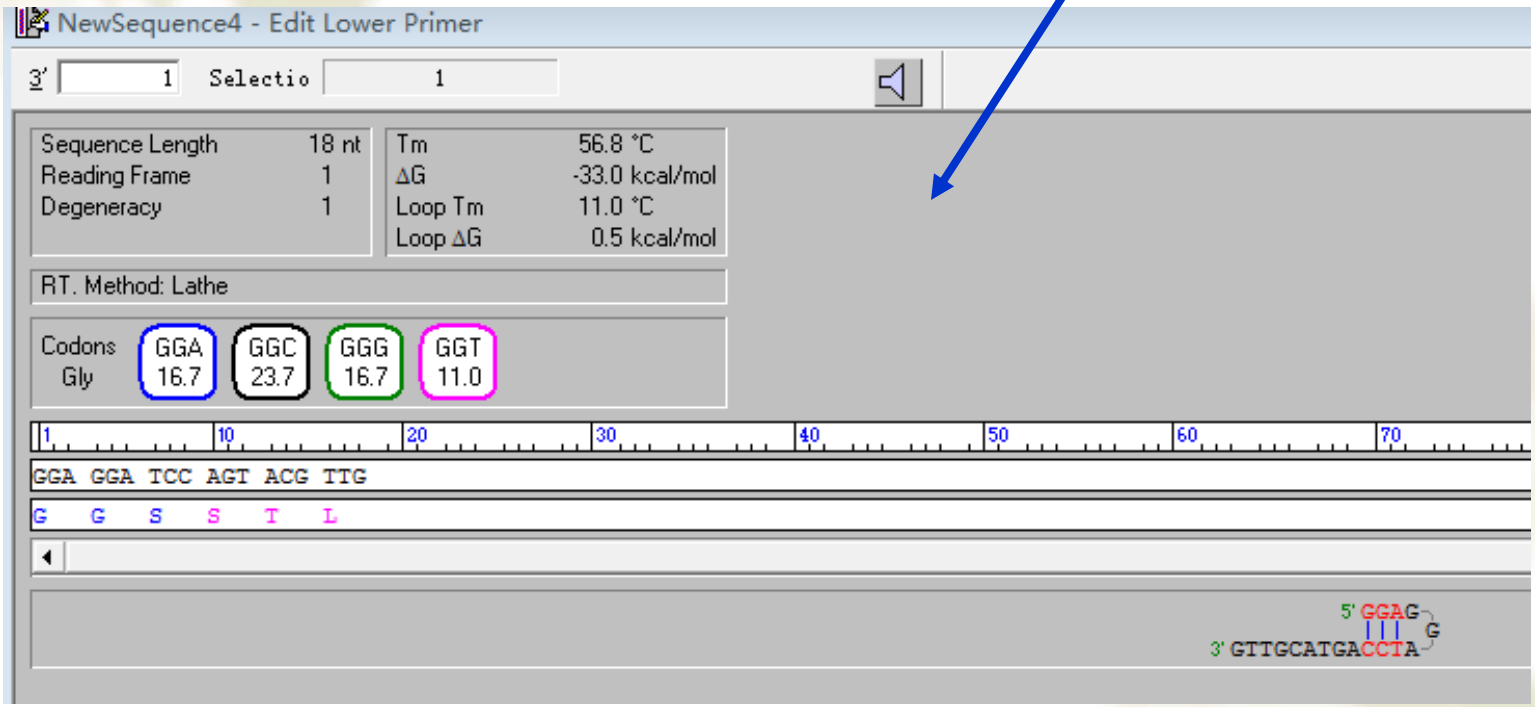

click



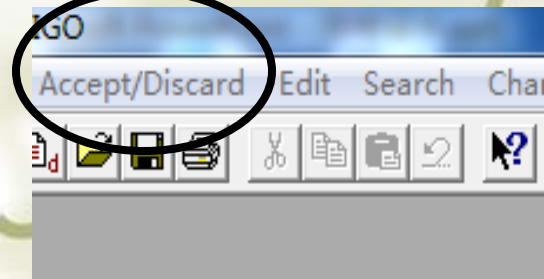

NewSequence4 - Internal Stability
ΔG
1
10
20
30
40
-13.0
-12.0
-11.0
-10.0
-9.0
-8.0
-7.0
-6.0
-5.0
R 1
R2
R3
NewSequence4 - Melting Temperature [18-mer]
Tm
61.8
61.6
61.4
61.2
60.8
Upper
Lower
ATCGAATTCATGGCGAAG
TAGCTTAAGTACCGCTTC
I E F M A K

Edit

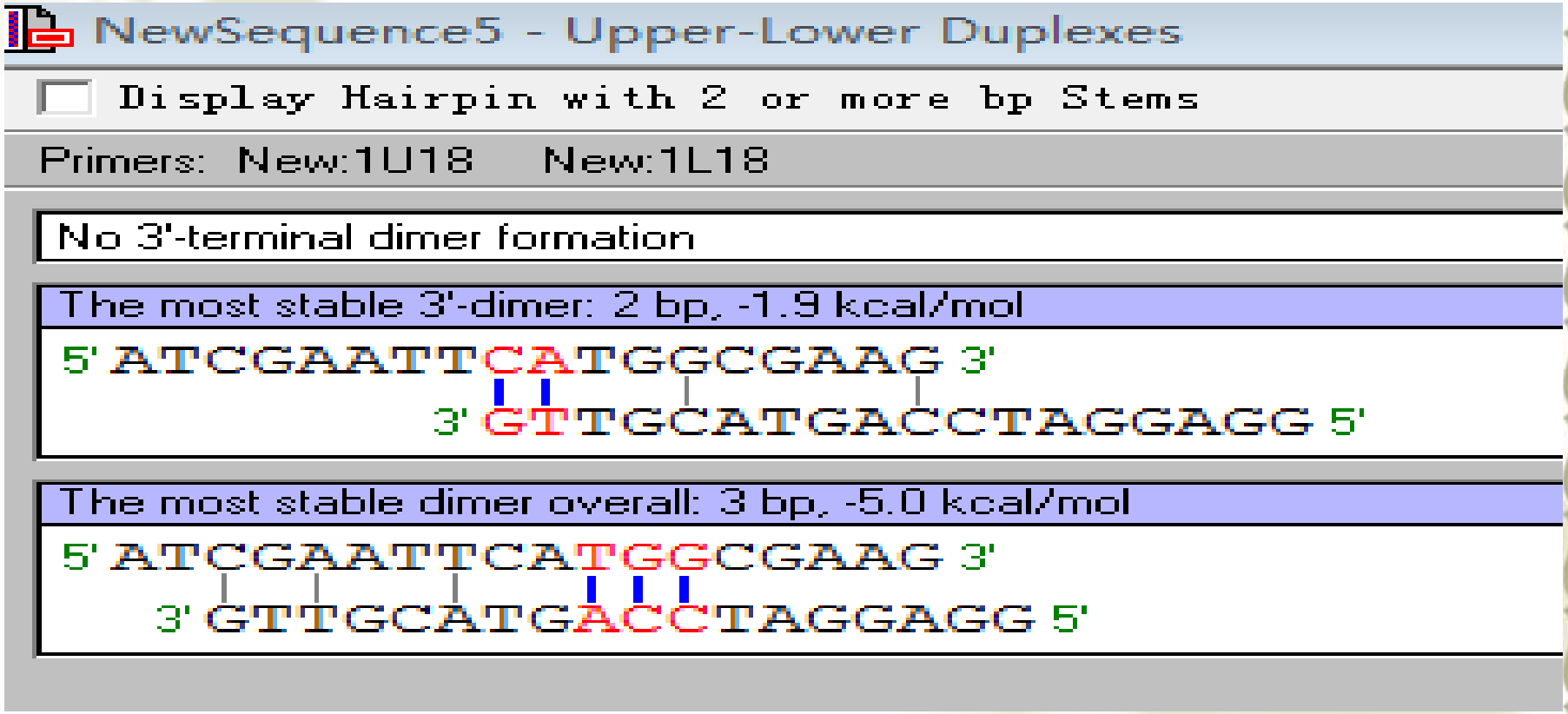
Lower primer



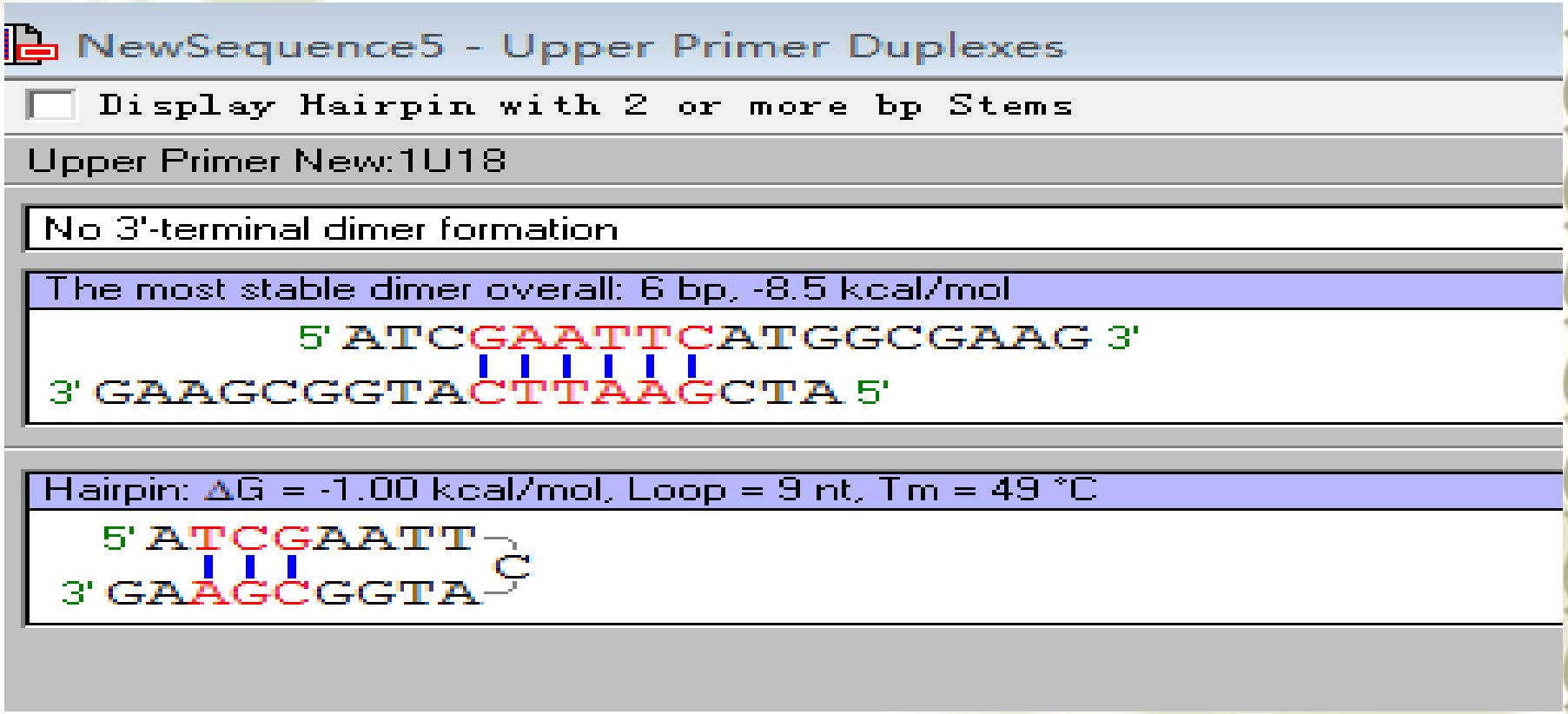

Accept and close



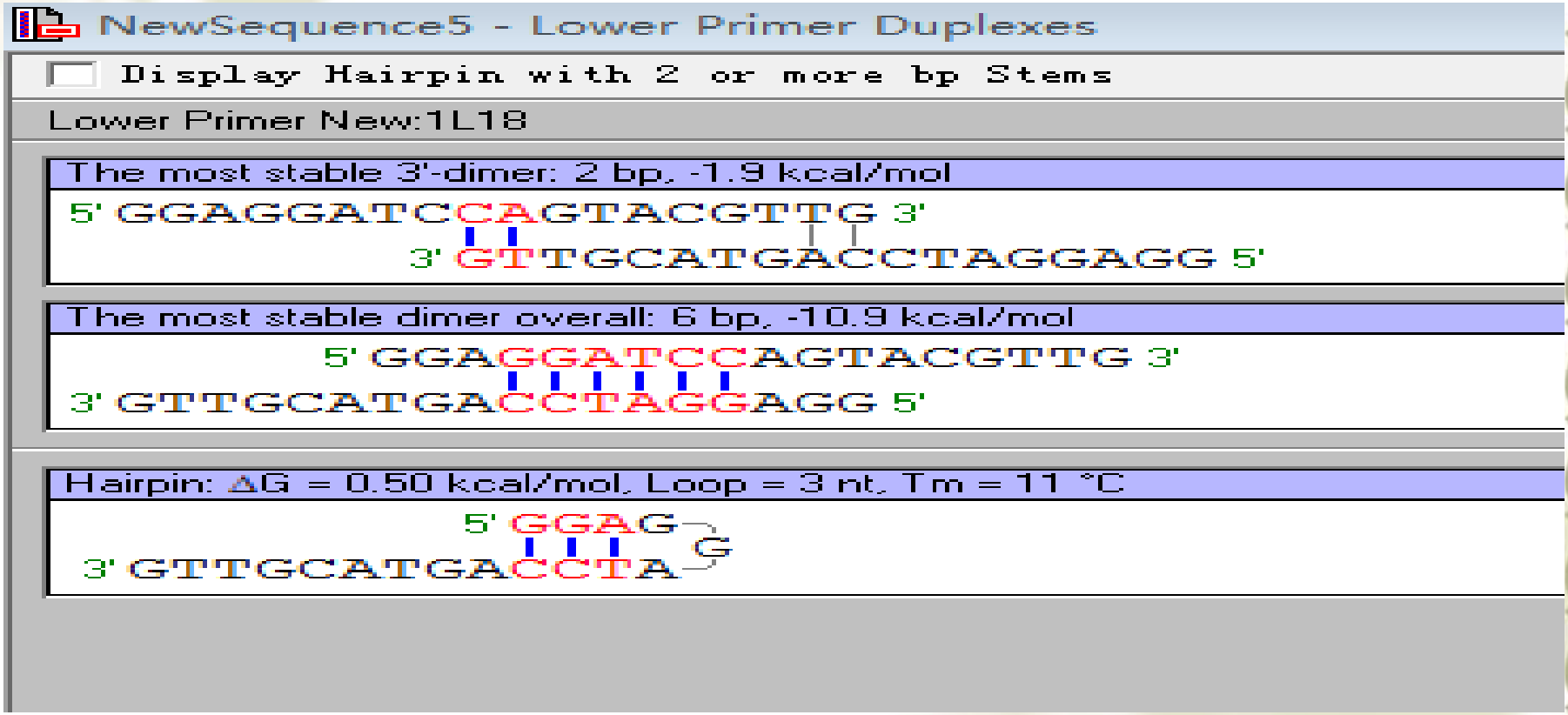
analyze


NewSequence5 - Upper-Lower Duplexes
Display Hairpin with 2 or more bp Stems
Primers: New:1U18 New:1L18
No 3'-terminal dimer formation
The most stable 3'-dimer: 2 bp, -1.9 kcal/mol
5' ATCGAATTCATGGCGAAG 3'
3' GTTGCATGACCTAGGAGG 5'
The most stable dimer overall: 3 bp, -5.0 kcal/mol
5' ATCGAATTCATGGCGAAG 3'
3' GTTGCATGACCTAGGAGG 5'

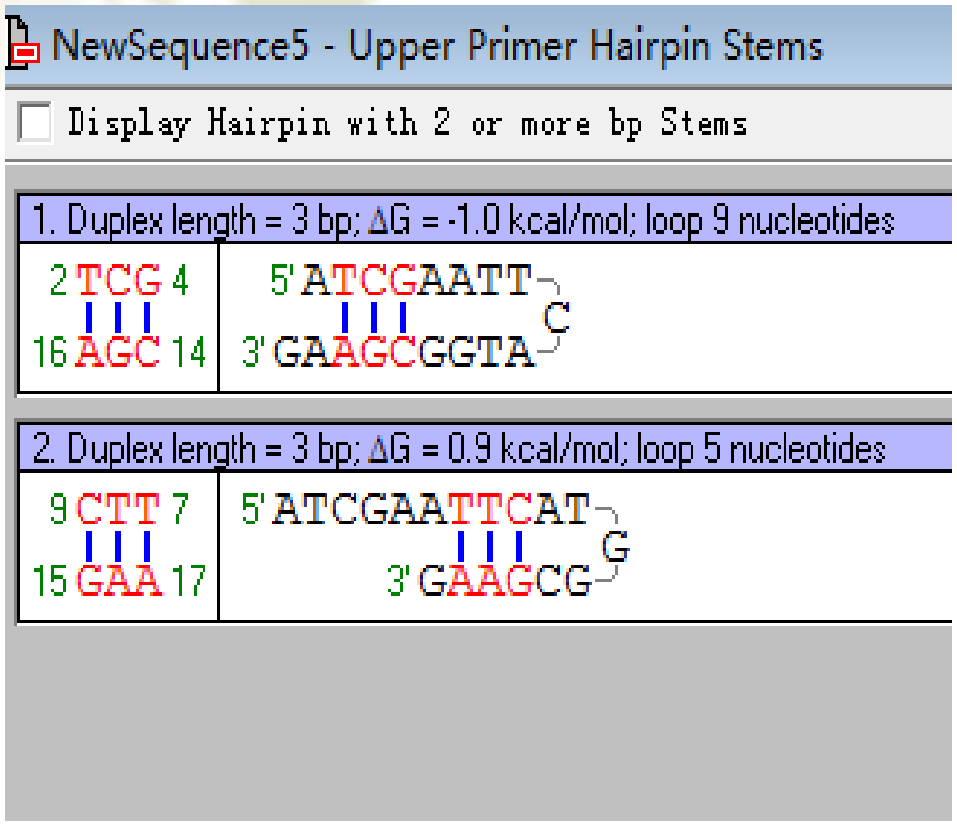
# Upper primer dimer

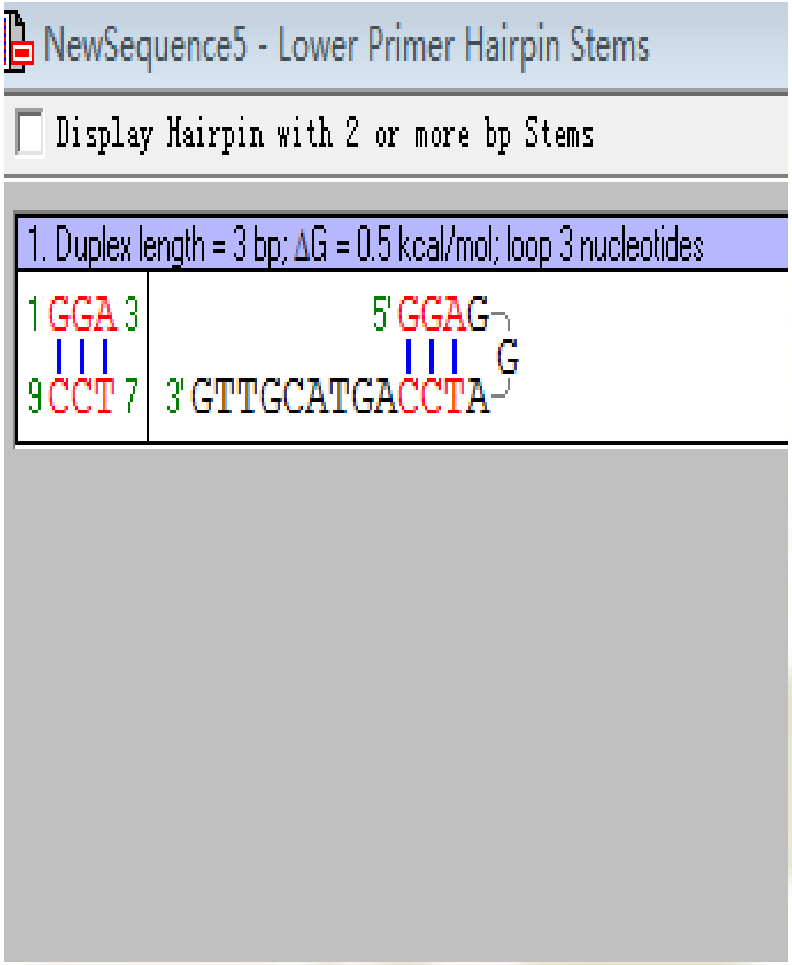


# Lower primer dimer

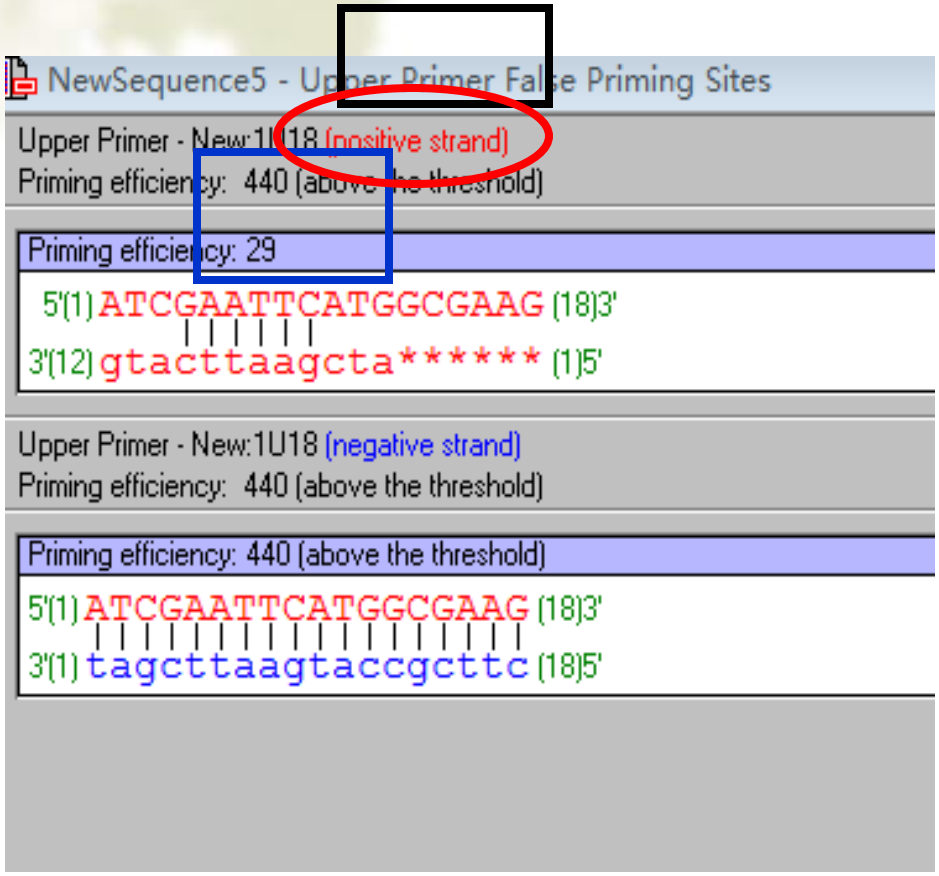


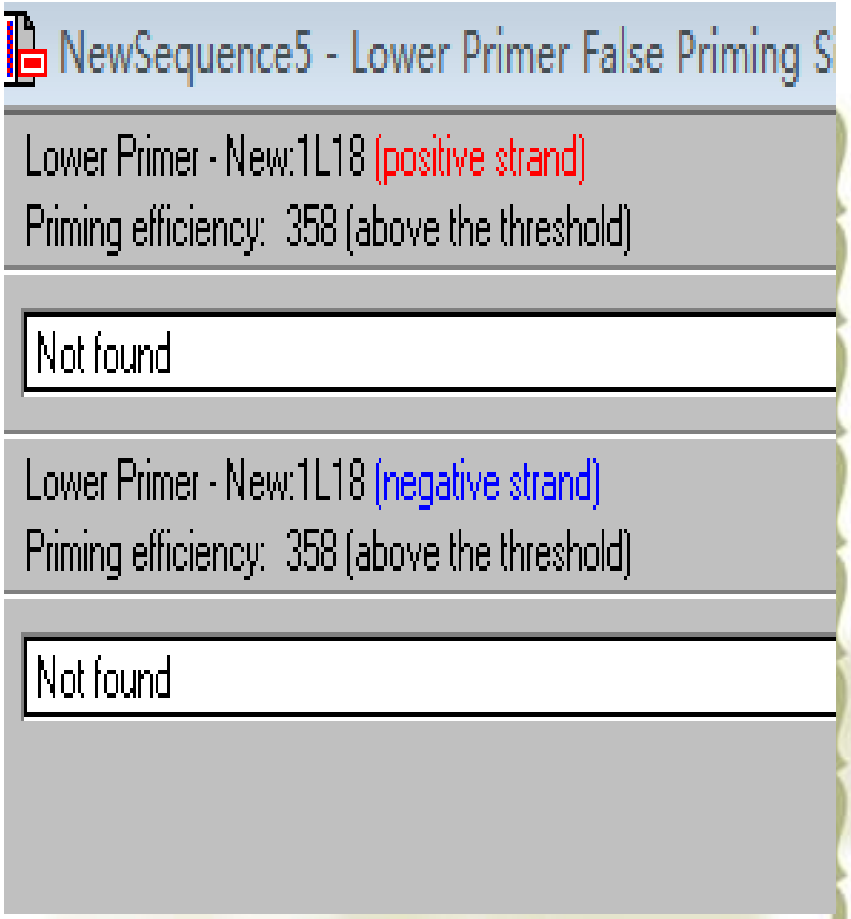
# hairpin


NewSequence5 - Upper Primer Hairpin Stems
Display Hairpin with 2 or more bp Stems
1. Duplex length = 3 bp; ΔG = -1.0 kcal/mol; loop 9 nucleotides
2 TCG 4
16 AGC 14
5' ATCGAATT
3' GAAGCGGTA
C
2. Duplex length = 3 bp; ΔG = 0.9 kcal/mol; loop 5 nucleotides
9 CTT 7
15 GAA 17
5' ATCGAATTCAT
3' GAAGCG
G

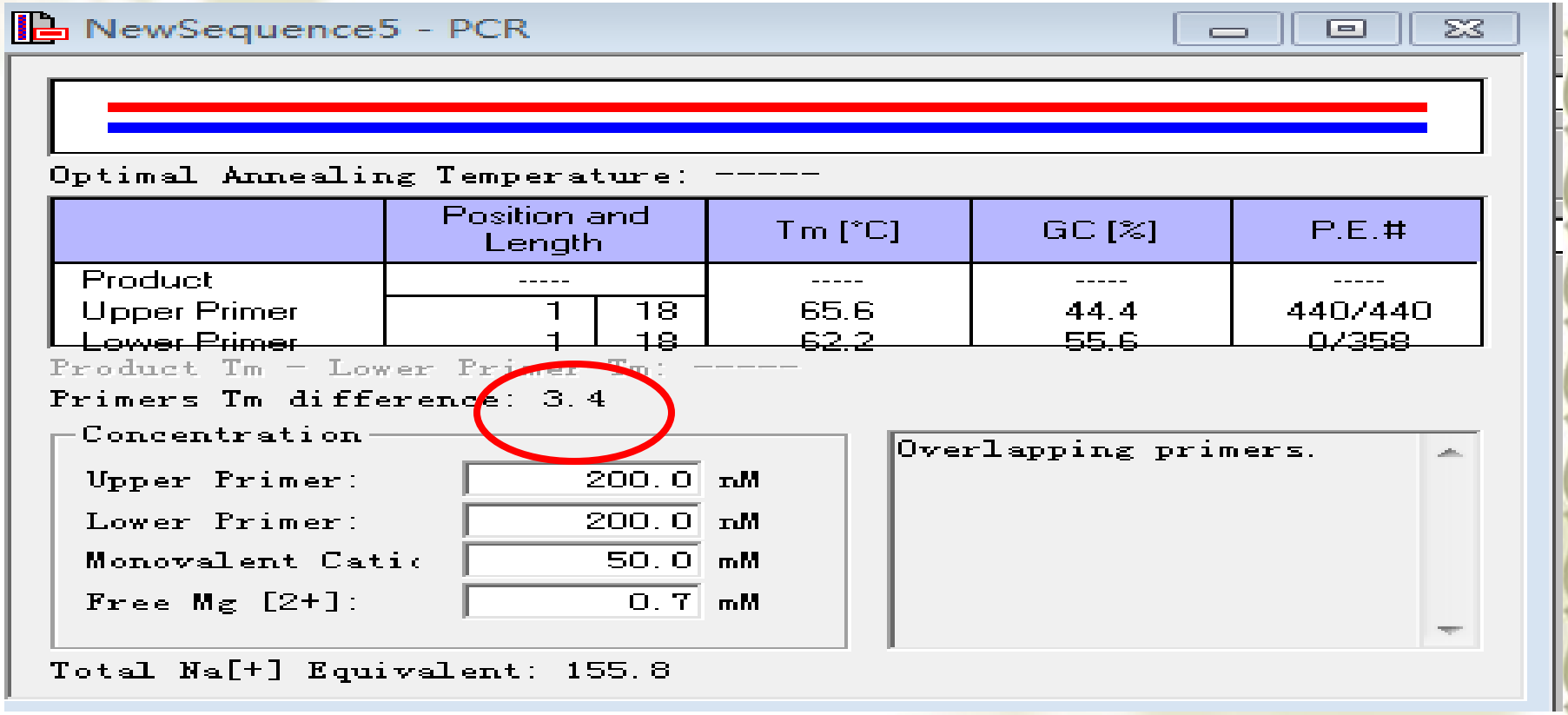

NewSequence5 - Lower Primer Hairpin Stems
Display Hairpin with 2 or more bp Stems
1. Duplex length = 3 bp; ΔG = 0.5 kcal/mol; loop 3 nucleotides
1 GGA 3
9 CCT 7
5' GGAG
3' GTTGCATGACCTA
G

# False priming site

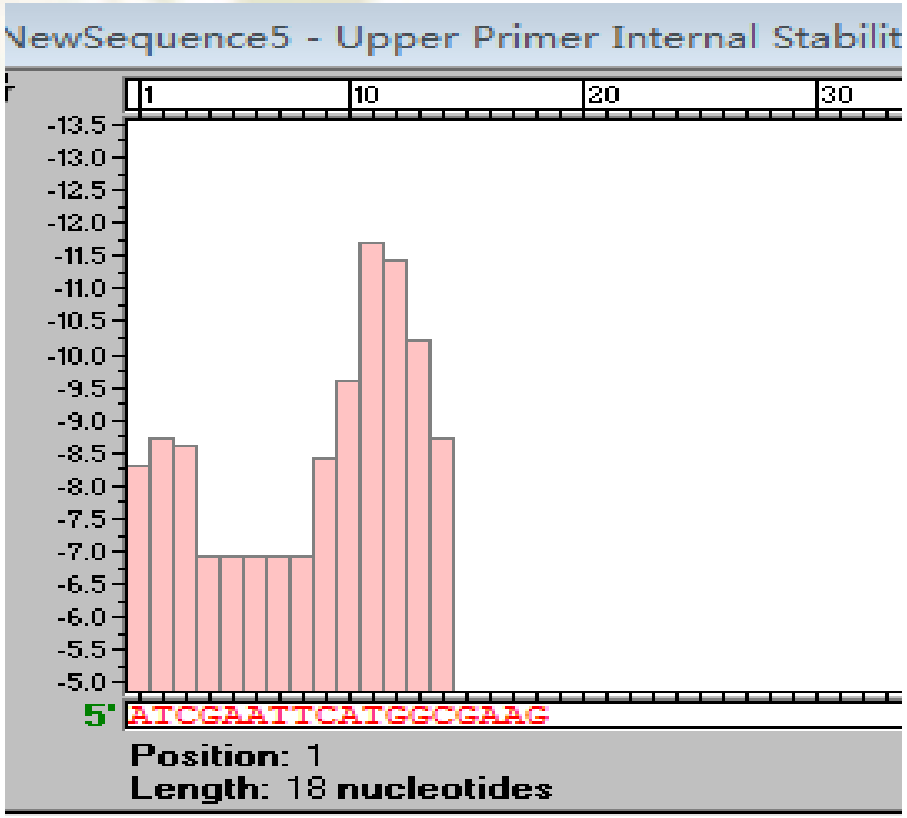


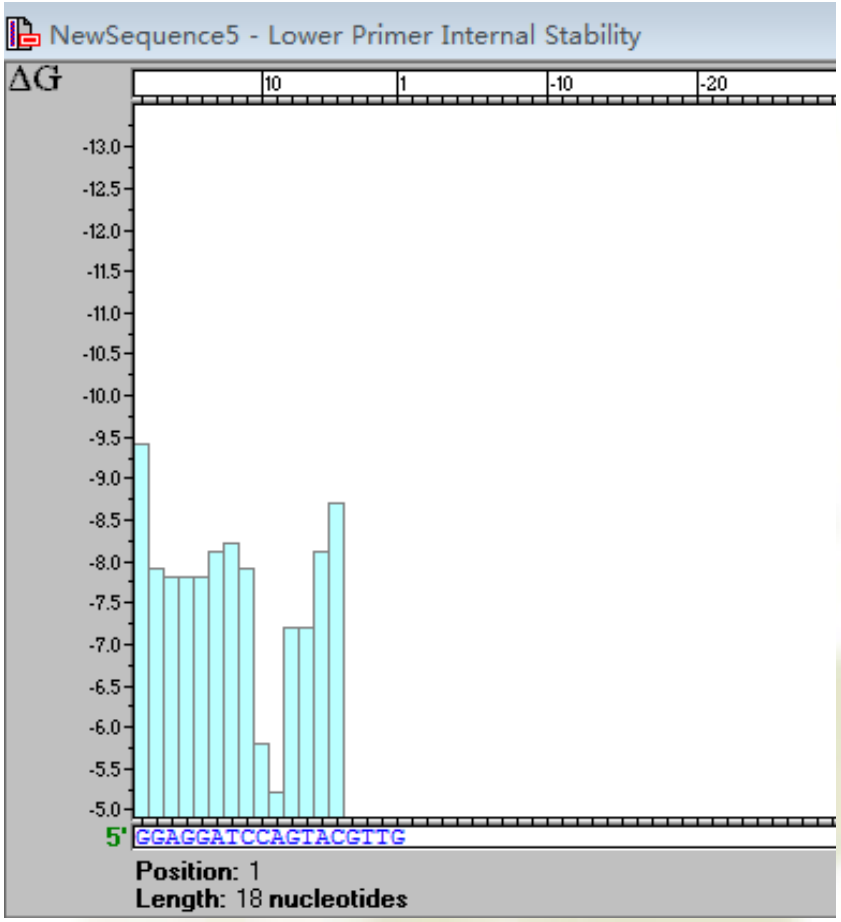



# PCR


NewSequence5 - PCR
Optimal Annealing Temperature: -----
Position and Length
Tm [°C]
GC [%]
P.E.#
Product
Upper Primer 1 18 65.6 44.4 440/440
Lower Primer 1 18 62.2 55.6 0/358
Product Tm - Lower Primer Tm: -----
Primers Tm difference: 3.4
Concentration
Upper Primer: 200.0 nM
Lower Primer: 200.0 nM
Monovalent Cati 50.0 mM
Free Mg [2+]: 0.7 mM
Overlapping primers.
Total Na[+] Equivalent: 155.8

# Primer internal stability



# Composition and Tm

NewSequence5 - Upper Primer Composition

Upper Primer New:1U18

| | | |
|---|---|---|
| Td | = 60.8° | [nearest neighbor method] |
| Tm | = 68.1° | [%GC method] |
| Tm | = 52° | [2(A+T)° + 4(G+C)° method] |
| Tm(RNA:RNA)[1M Na] | = 77.5° | [%GC method] |
| Tm(DNA:RNA)[1M Na] | = 79.0° | [%GC method] |
| $A_{260}/A_{280}$ | = 2.02 | [single strand] |
| Mr | = 5.6K | [one strand] |
| Mr | = 11.1K | [two strands] |
| g/OD | = 47.8 | [dsDNA] |

| Base | Number & % | |
|---|---|---|
| A | 6 | [33.3%] |
| C | 3 | [16.7%] |
| G | 5 | [27.8%] |
| T | 4 | [22.2%] |
| A + T | 10 | [55.6%] |
| G + C | 8 | [44.4%] |

DNA Melting Temperature in Various Salt and Formamide Concentrations [°C]

| [mM] | xSSC | 0% | 10% | 50% |
|---|---|---|---|---|
| 1 | 0.006 | 22.1 | 15.6 | -10.4 |
| 10 | 0.06 | 38.7 | 32.2 | 6.2 |
| 50 | 0.3 | 50.1 | 43.6 | 17.6 |
| 165 | 1 | 58.2 | 51.7 | 25.7 |

# 结语

- 这章ppt 只是对引物设计的入门式讲解，希望为大家起到一个抛砖引玉的效果
- PPt没有包括degenerate primer的设计，但原理是一致的，可以参考abc网站中的primer design
- 原理和工具就像是圣斗士的战衣，穿什么战衣不重要，不要太过拘泥于这些条条框框，只要你能扩出你要的片段，那么你就胜利了。

# 引用文献


- General concepts for PCR primer design.C W Dieffenbach, T M Lowe, G S Dveksler，Volume: 3, Issue: 3, Publisher: Cold Spring Harbor Lab, Pages: S30-S37
- Efficient primer design algorithms Thomas Kämpke1, Markus Kieninger2 and Michael Mecklenburg Bioinformatics (2001) 17 (3): 214-225.
- 百度百科，http://baike.baidu.com/view/2764.htm
- Primer design，belong to the Board of Regents of the University of Wisconsin System.

Thank you