Troubleshooting. Nested PCR. Primer design

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Troubleshooting

1. **DNA extraction**
2. **DNA amplification (PCR)**
3. **Sequencing**

Troubleshooting —— **DNA extraction** **some notes**

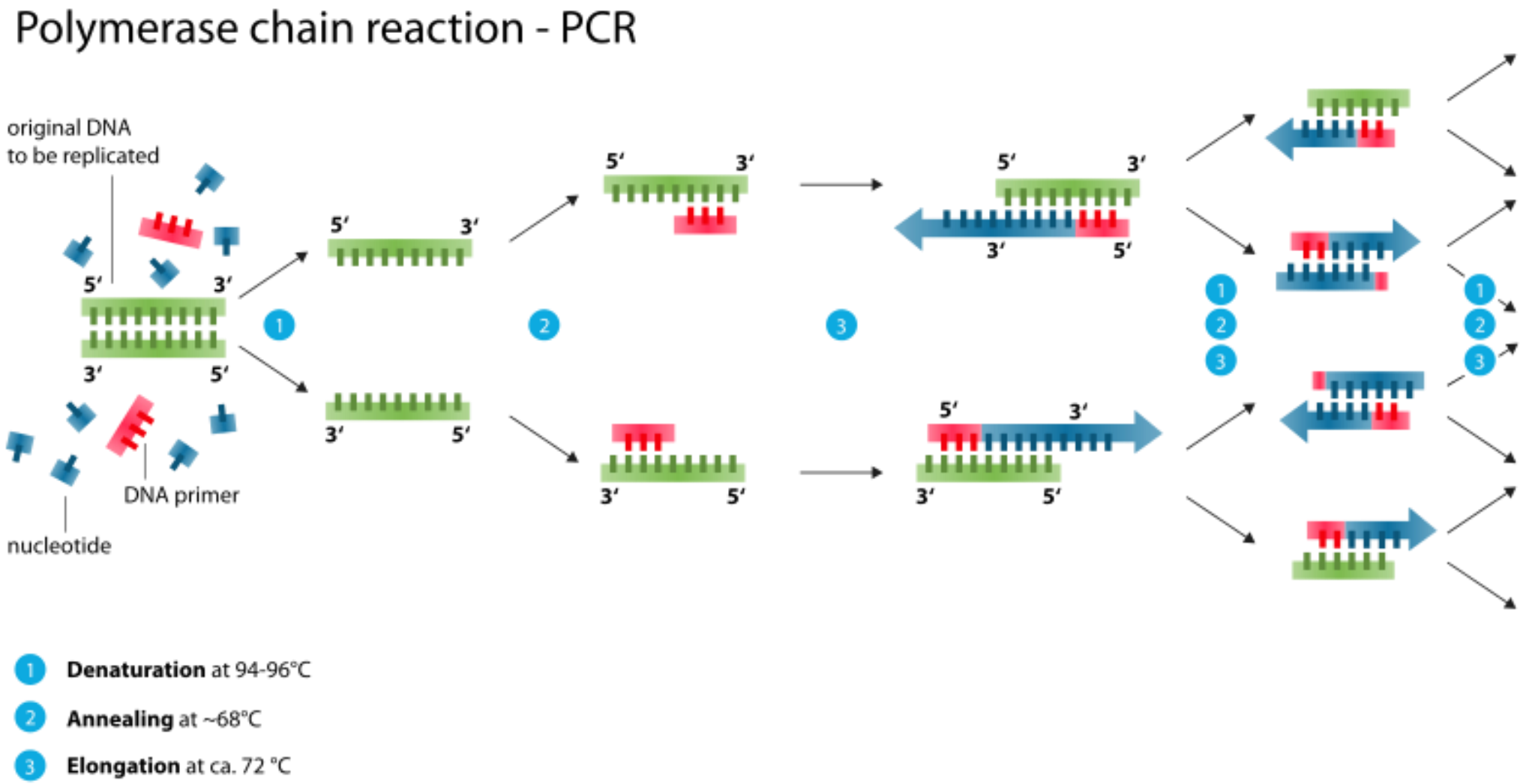
Collecting materials:

1. In a field trip, materials should be **quickly** dried using silica gel, salt, or ethanol
2. Avoid drying samples by heat
3. Avoid wash dried materials before extract DNA

Check DNAs:

1. To detect genomic DNA: make 0.8% of agarose gel (a diffuse smear should appear)
2. However, even if nothing is detected, it may still be ok

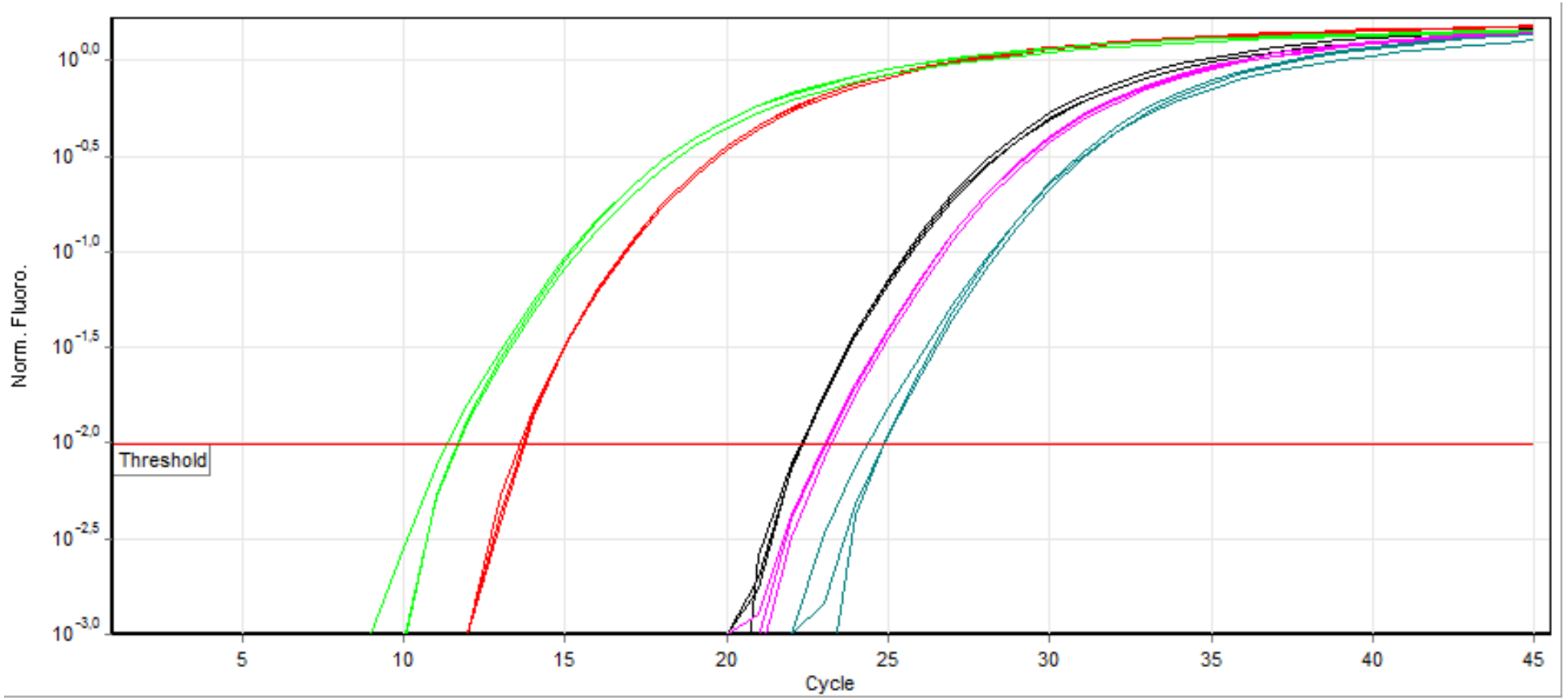
Troubleshooting — **PCR**



Enzoklop

Troubleshooting —— **PCR**

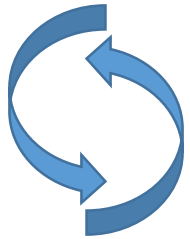
Real-time PCR:



Troubleshooting — **PCR**

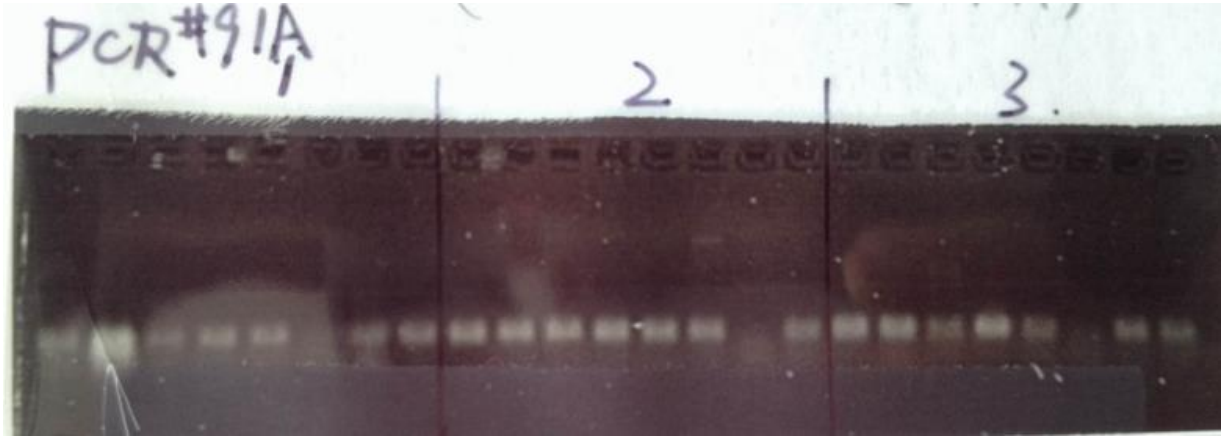
PCR program (Thermocycling conditions):

STEP	TEMP	TIME
Initial Denaturation	94 °C	3 minute
Denaturation	94 °C	30 seconds
Annealing	**45-68°C**	1 minute
Extension	70°C	**1 minute/kb**
Final Extension	70°C	10 minutes
Hold	4-10°C	



30-40 Cycles

Troubleshooting —— **PCR**



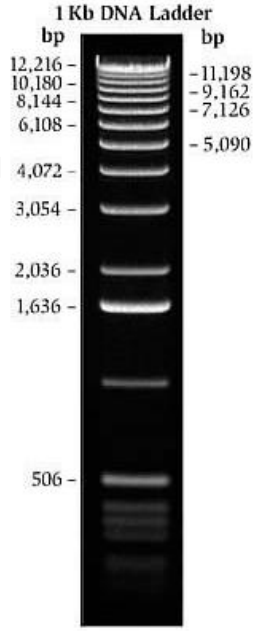
No samples worked

1. Check system:

Reason 1: failed gel staining → Use 1kb ladder

Reason 2: new reagent/program? → Check/change, try again

Reason 3: PCR master mix error → Check, try again



From Life Technologies

Troubleshooting —— **PCR**

No samples worked

2. Check sample:

- DNA failed ➡ Try other DNAs
- Primers ➡ Try other primers, something easy, i.e. trnL

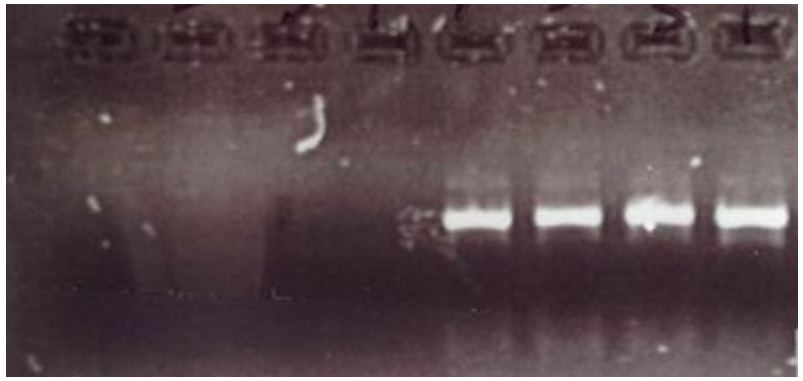
Troubleshooting —— **PCR**

No samples worked

3. Check PCR profile:

- DNA contains EDTA → Increase $MgCl_2$ concentration
- Lower the stringency → Lower annealing temperature
- Break secondary structure → Add Betaine/DMSO
- Improve primer → Design new primers

Troubleshooting —— **PCR**



Some samples worked

Check PCR profile:

1: taxonomic sense → Design specific primers

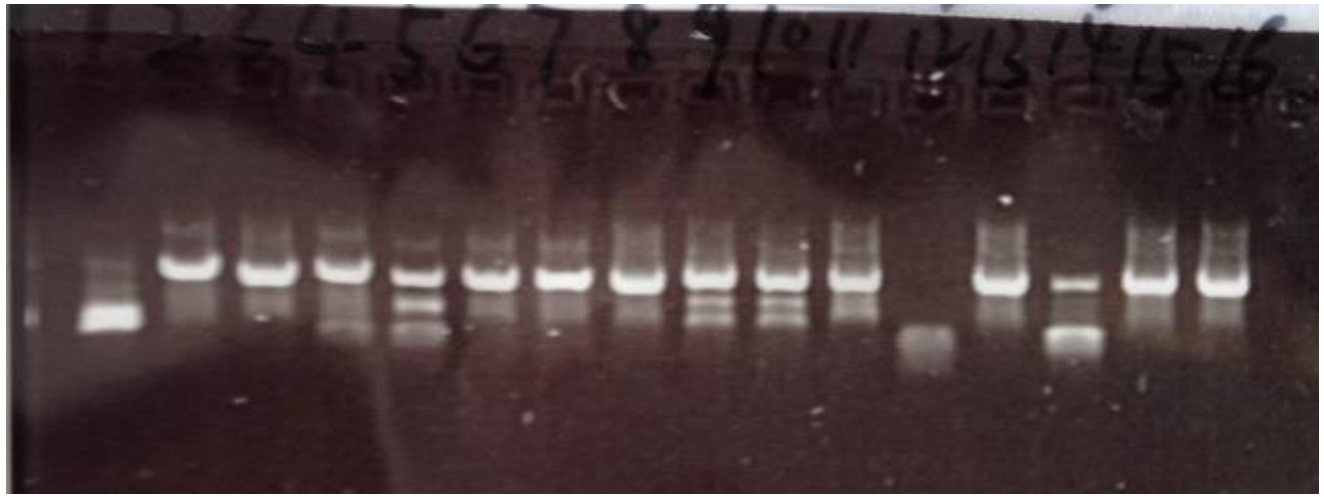
2: not taxonomic sense →

- Reduce annealing temp.
- Increase, or decrease DNA
- Add Betaine/DMSO
- Increase $MgCl_2$

Troubleshooting —— **PCR**

No specific products (double bands)

- Increase stringency, increasing annealing temp. (up to 62 °C)
- Reduce number of cycles (try 30 instead of 34)
- Longer unspecific products, decrease extension time
- Shorter unspecific products, increase extension time
- Add less primer and taq
- Contamination?



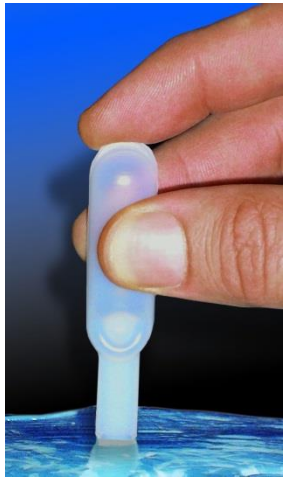
➢ Cut gel
➢ 2nd round PCR

Troubleshooting —— **PCR**

- Cut gel

http://www.youtube.com/watch?v=ZUZy0kydcUQ

Kit: NucleoSpin® Gel and PCR Clean-up



http://www.5prime.com

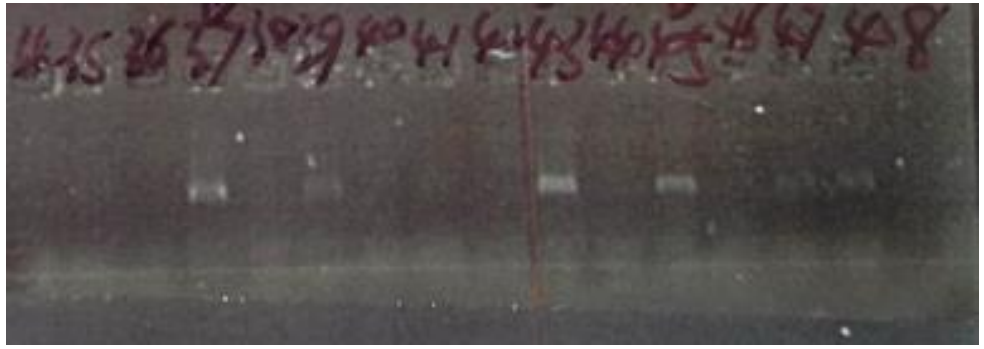
- 2nd round PCR

1. **Use pipette tip punch the target band on the gel**
2. **Proceed to PCR. Use the same primers, dip the pipette tip into the PCR tube as template**

Troubleshooting —— **PCR**

Weak bands

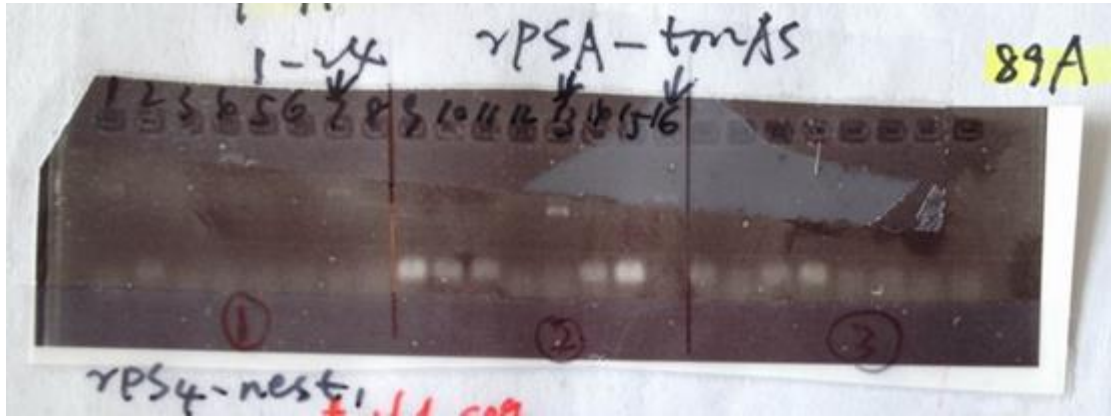
- Decrease stringency, use lower annealing temp. (down to 45 °C)
- Increase number of cycles (up to 40)
- Add Betaine/DMSO
- Add $MgCl_2$
- Add more primer and taq



Troubleshooting —— **PCR**

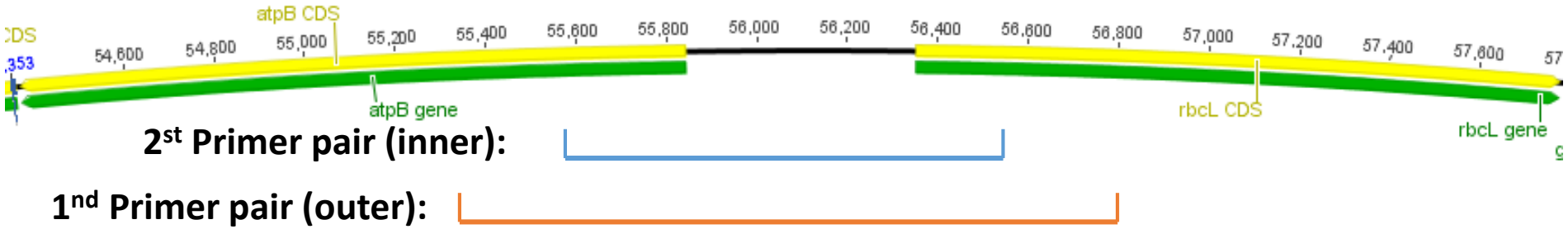
Tips

- To avoid false positive, include a **negative control**
- When running gel, add a **ladder**
- Include a **control DNA**, from previous experiment that worked well
- During troubleshooting, make a **gradient** of DNA amount, annealing temp. etc.



Nested PCR: 2nd round PCR

atpB-rbcL



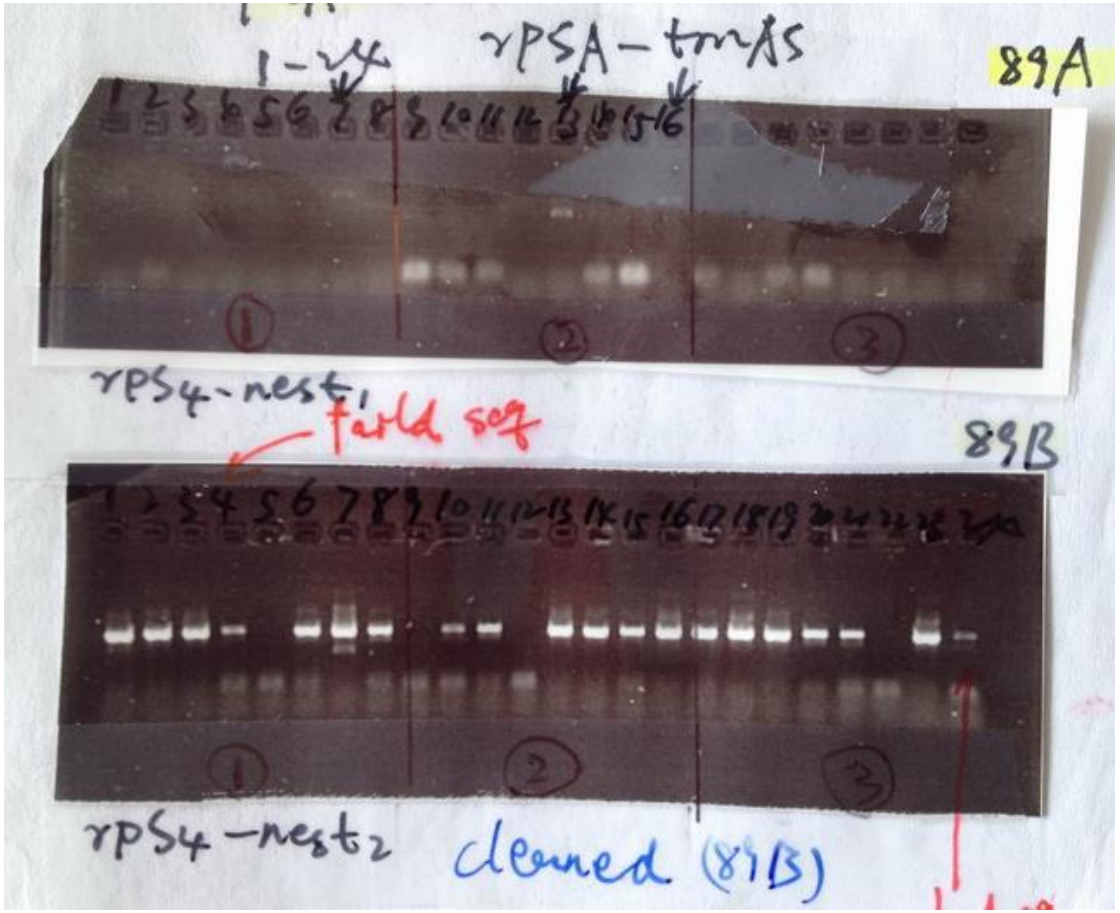
Proceding:

- PCR1: **outer** primer + genomic DNA as template
- PCR2: **inner** primer + PCR product from step1 as template
- Sequence : use **inner** primer

Nested PCR: results

Step 1st Primer pair:

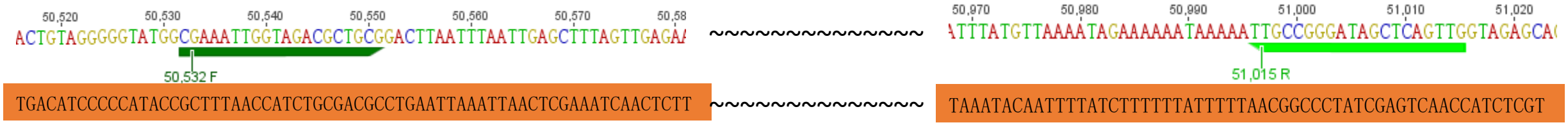
Step 2nd Primer pair:



Primer design

What is a primer

Primer: a strand of nucleic acid that serves as a starting point for DNA synthesis.



Primers pair:
Forward: 5’-CGAAATTGGTAGACGCTGCG-3’
Reverse: 5’-CAACTGAGCTATCCCGGCAA-3’

Primer design

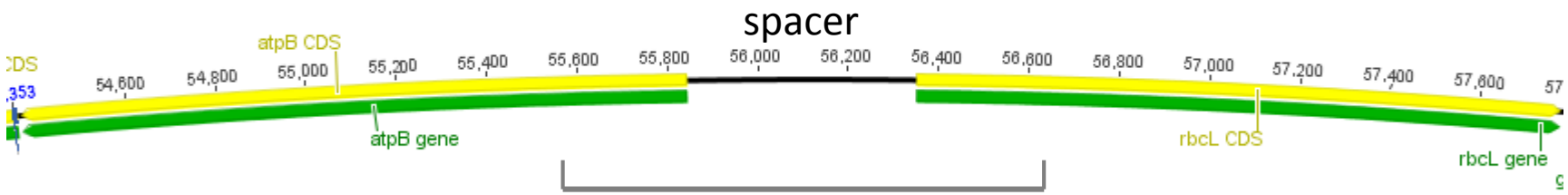
Strategies for choosing PCR target

- Product position: Keep primer in coding genes, avoid introns, intergenic spacer regions
- Amplicon length: normal PCR < 3000 bp; Long range PCR, up to 40 kb

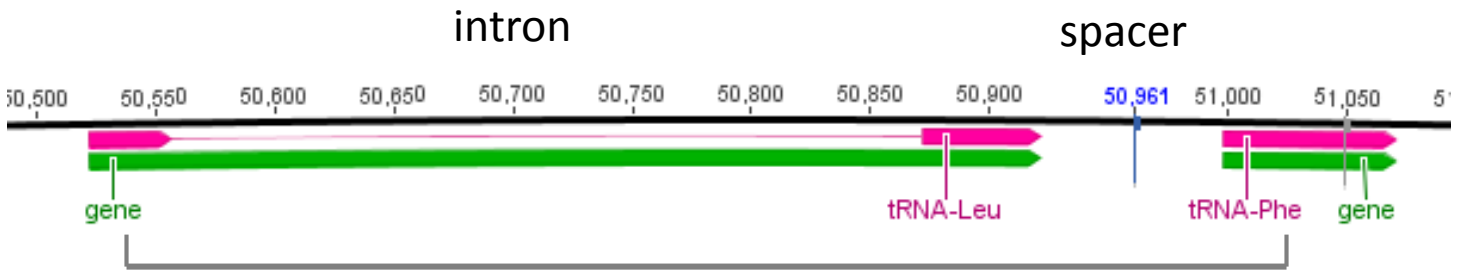
Primer design

Product position

atpB-rbcL



trnL-F



Primer design

Tips for primer designing

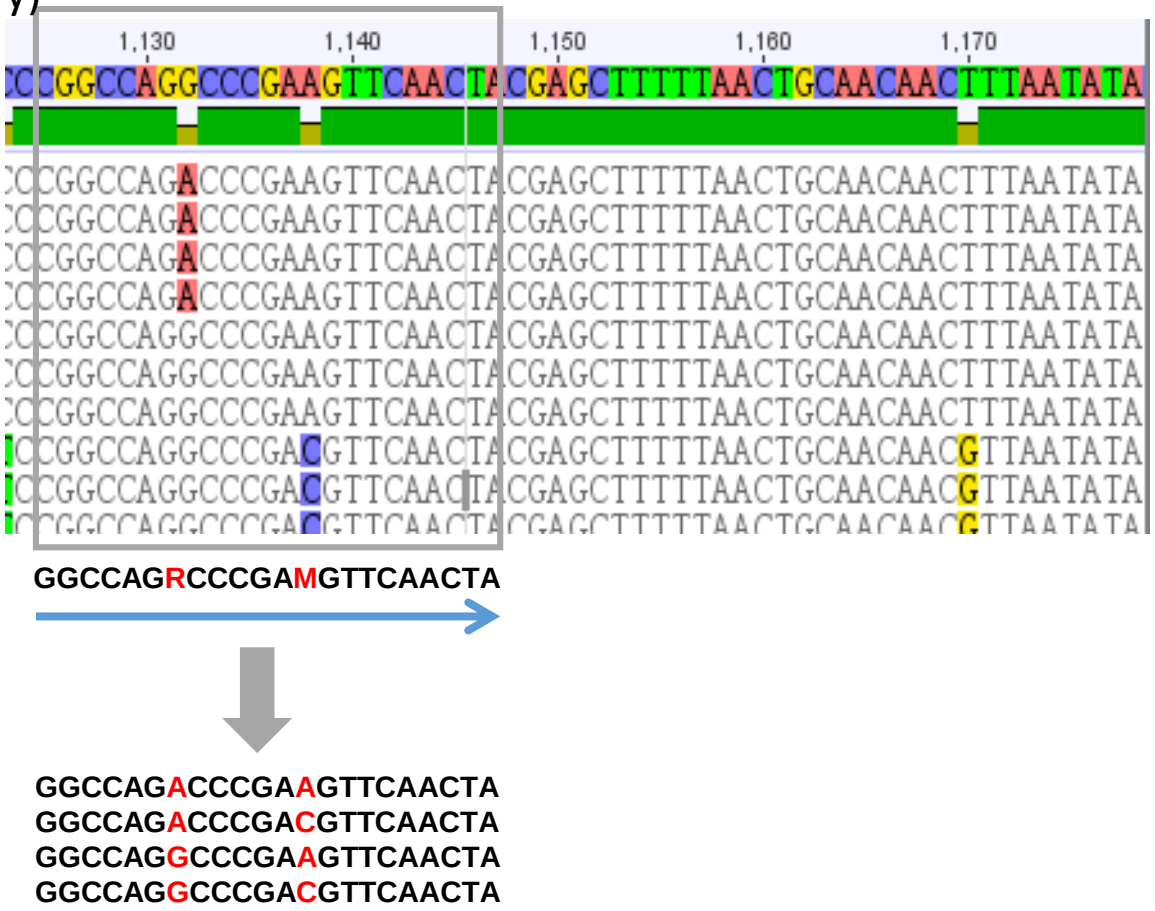
- Primer Length: 18-22 bp
- Melting Temperature (Tm): 55-65 °C, range < 5 °C between pair
- GC Content (percentage of G and C) : between 40 and 60%.
- If degeneracy is used , should be < 2 sites
- Avoid secondary structures: self dimer, cross dimer
- Avoid repeats (e.g. ACCCCC), or microsatellite (e.g. ATATATAT)
- Avoid cross homology: blast primers in the genome

Primer design

Degenerate primer. When should be used?

IUPAC (International Union of Pure and Applied Chemistry)

AT -> W
CG -> S
TG -> K
AC -> M
CT -> Y
AG -> R
ACG -> V
ATG -> D
TCG -> B
ATC -> H
AGCT -> N



Primer design

Software

Commercial:

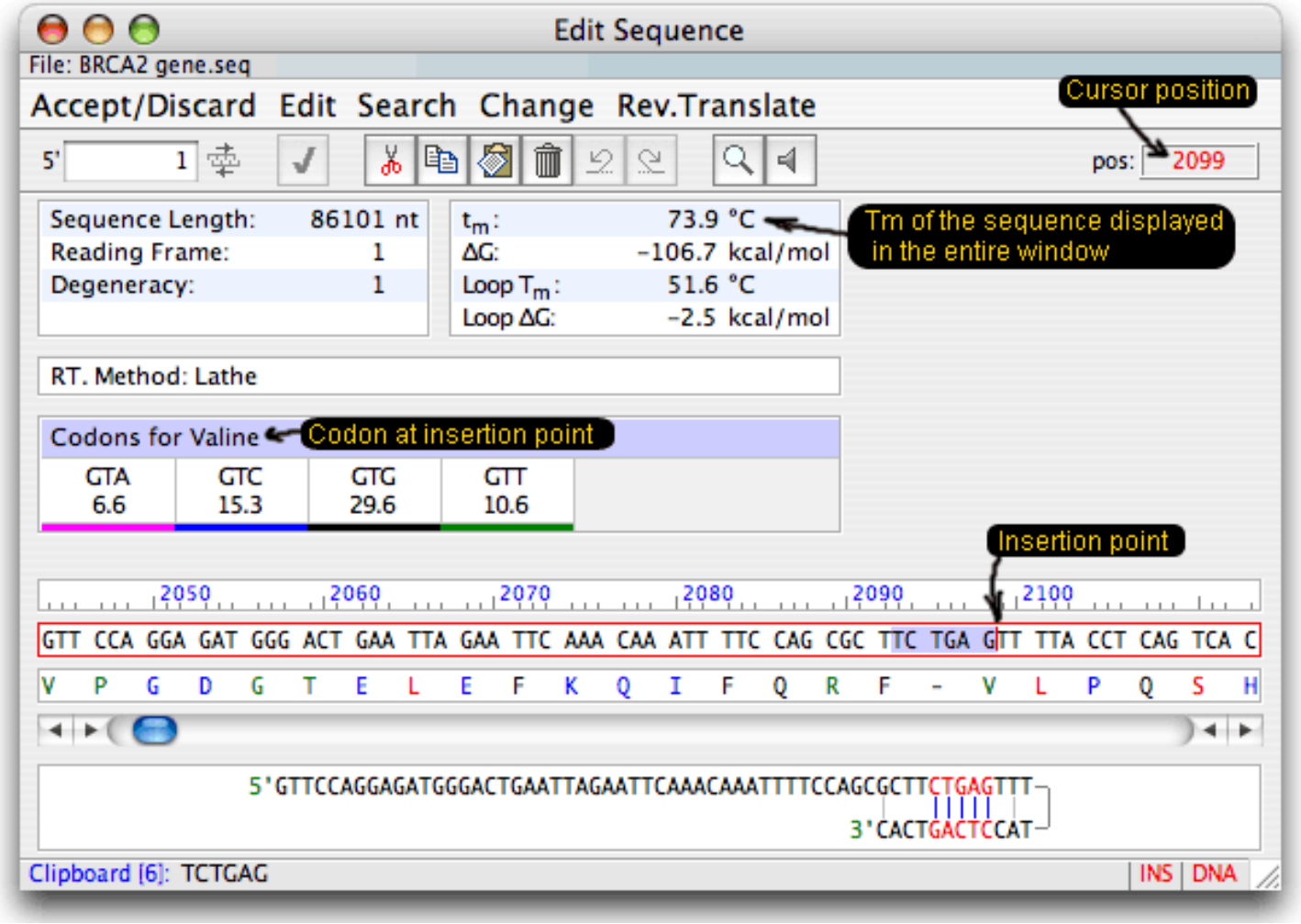
- DNASTAR v.12.1
- Oligo v.7

Free:

- OligoAnalyzer 3.1 (http://www.idtdna.com/calc/analyzer)
- Primer3 (http://primer3.ut.ee/)

Primer design

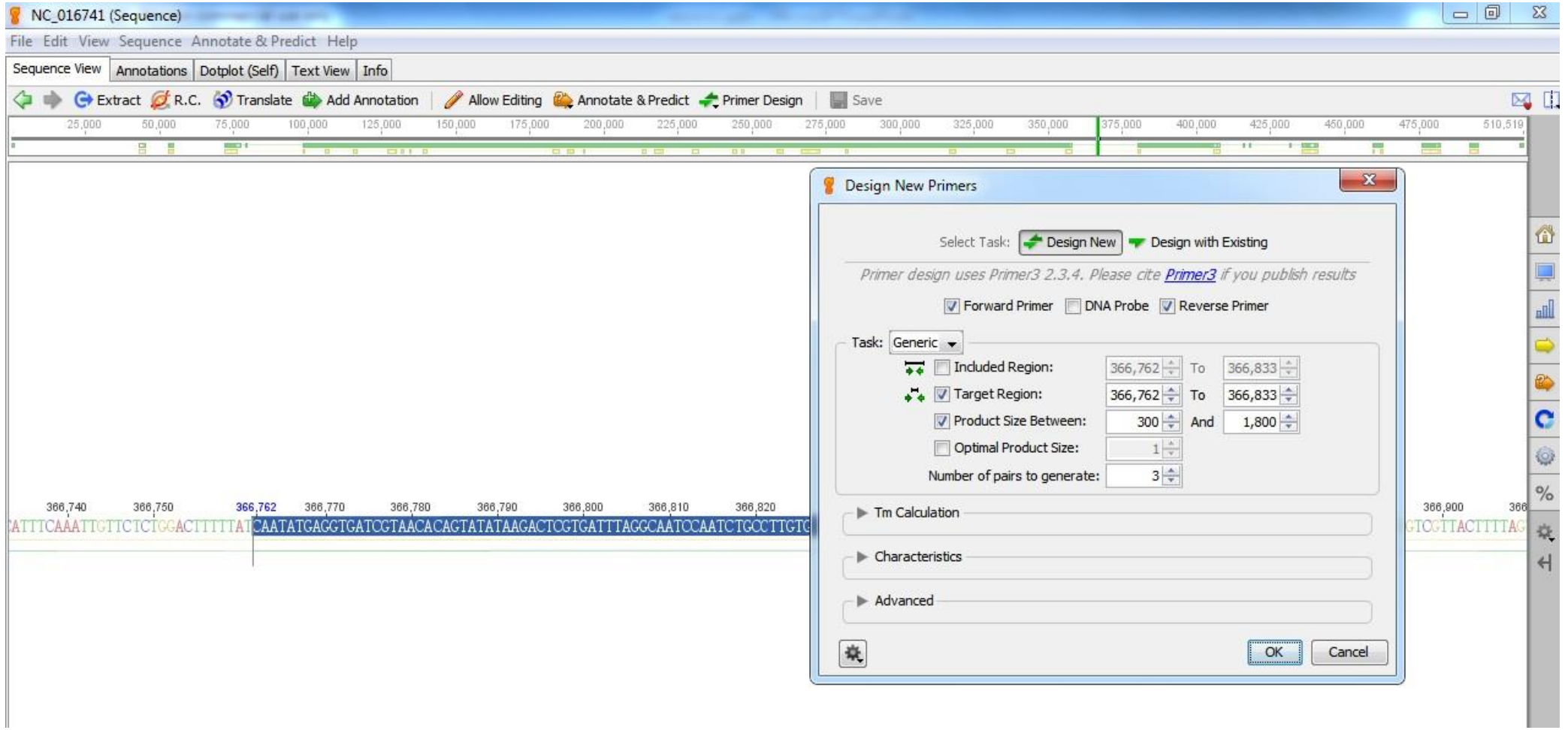
Oligo



From http://www.oligo.net

Primer design

Primer3 as Geneious plugin



Primer design

Primer3

Primer3web version 4.0.0 - Pick primers from a DNA sequence.

disclaimer | code | cautions

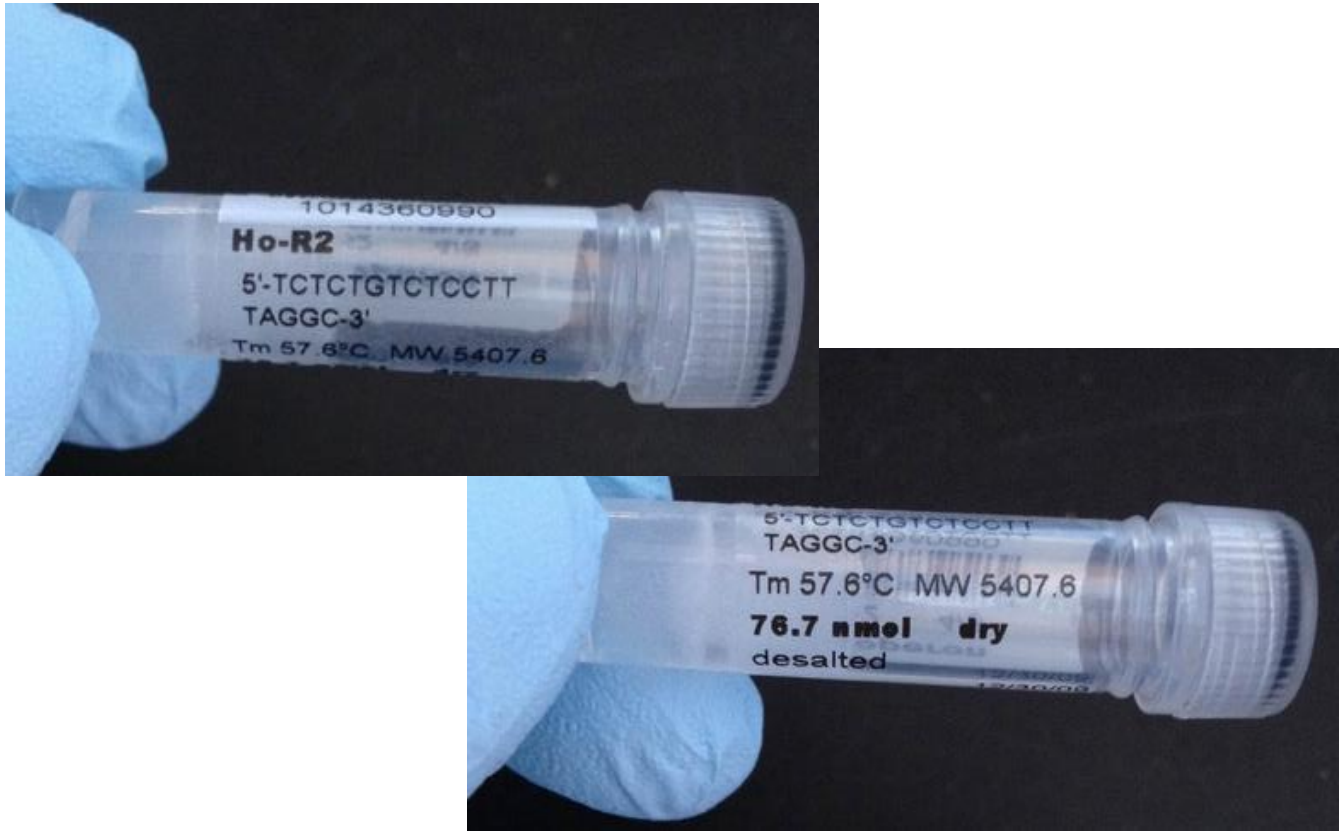
Select the Task for primer selection: generic

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored).
FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a Mispriming Library (repeat library)
NONE

☑ Pick left primer, or use left primer below	☐ Pick hybridization probe (internal oligo), or use oligo below	☑ Pick right primer, or use right primer below (5' to 3' on opposite strand)

Order primers

IDT: http://www.idtdna.com/
Fisher Scientific: http://www.fishersci.com/



Prepare:

- Annealing Temp. = Tm-5 °C
- Stock solution (10x): 100 pmol/ul
- Working solution (1x): = 10 pmol/ul

  Note: 1 nmol = 1000 pmol

Troubleshooting —— **Sequencing**

- GC rich: a DNA template, or a region (100-200 bp) > 60% GC content

  Adams etal. Microb Comp Genomics 1997;2:198.

  Adams et al. BioTechniques 1996;21:678.

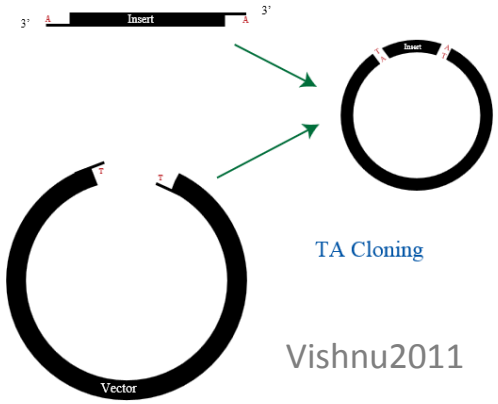
- Secondary structures: Hairpin from inverted repeats, tRNA or rRNA

  Sharp. Genes and Dev. 1999:13:139–141.

  Zhao etal. J Biomol Tech 2000. 11:111

  Kieleczawa etal. J Biomol Tech 2005. 16:220

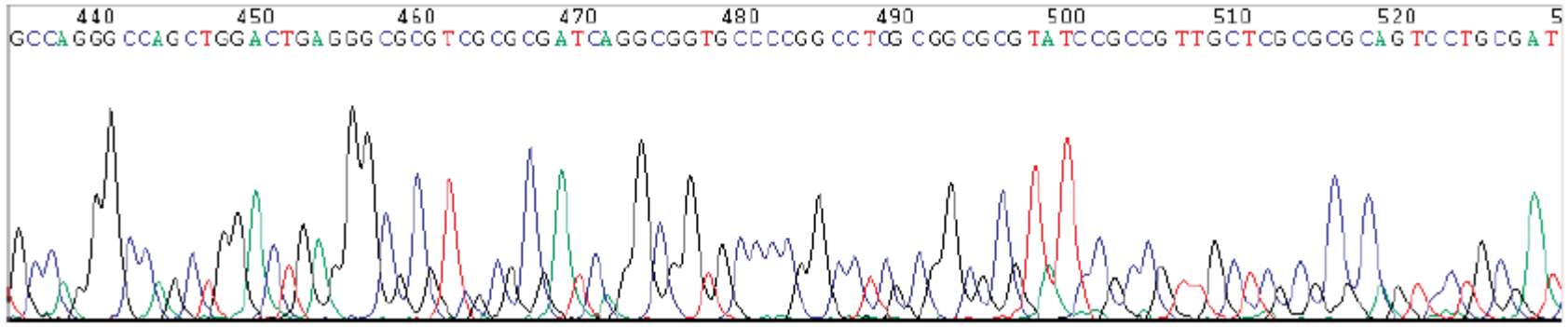
- Repeats: Microsatellite or poly A/T or C/G stretches up to 40 times, cause PCR sliding
  1. specialist taq, e.g. Hi-Fi Taq
  2. cloning, extract vector, sequence vector



Vishnu2011

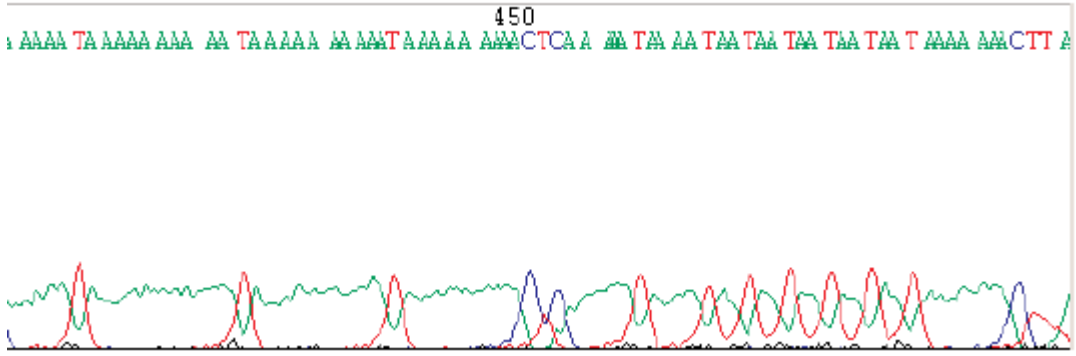
Troubleshooting — **Sequencing**

GC rich



www.appliedbiosystems.com

Repeats



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Poly C