

Troubleshooting. Nested PCR. Primer design



Troubleshooting

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

Troubleshooting — **DNA extraction** some notes

Collecting materials:

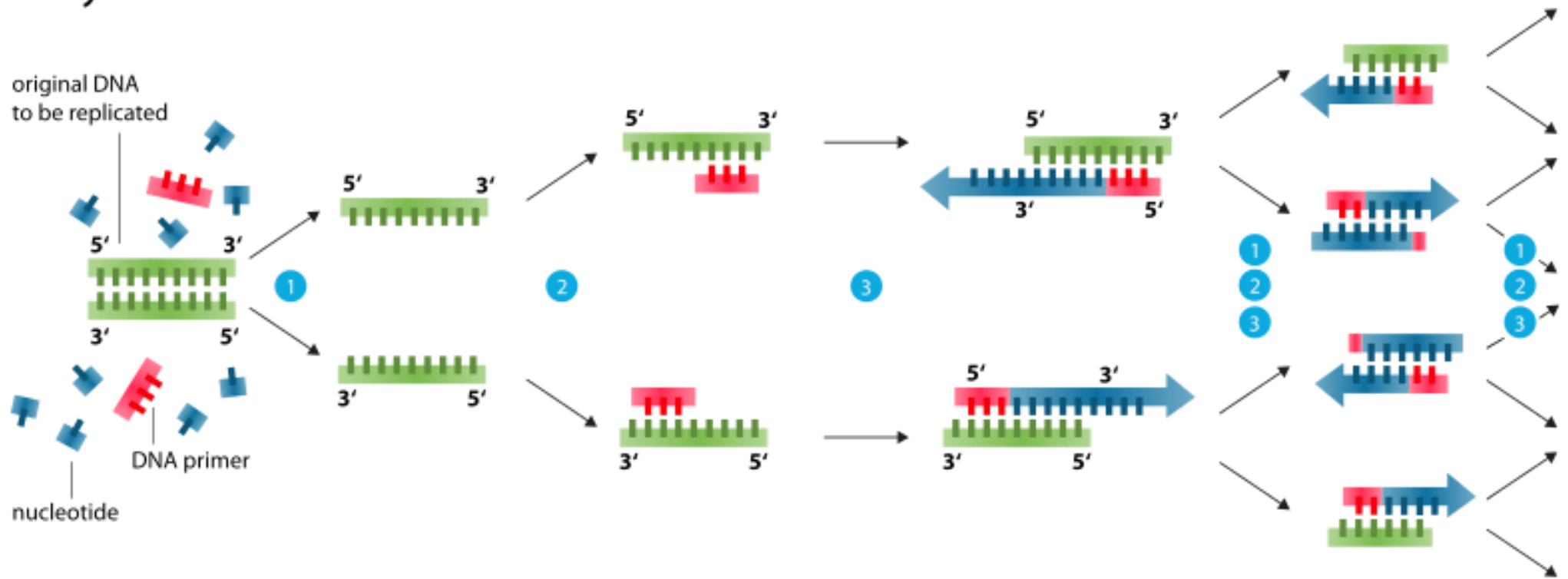
1. In a field trip, materials should be **quickly** dried using silica gel, salt, or ethanol
2. Avoid drying samples by heat
3. Avoid wash dried materials before extract DNA

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

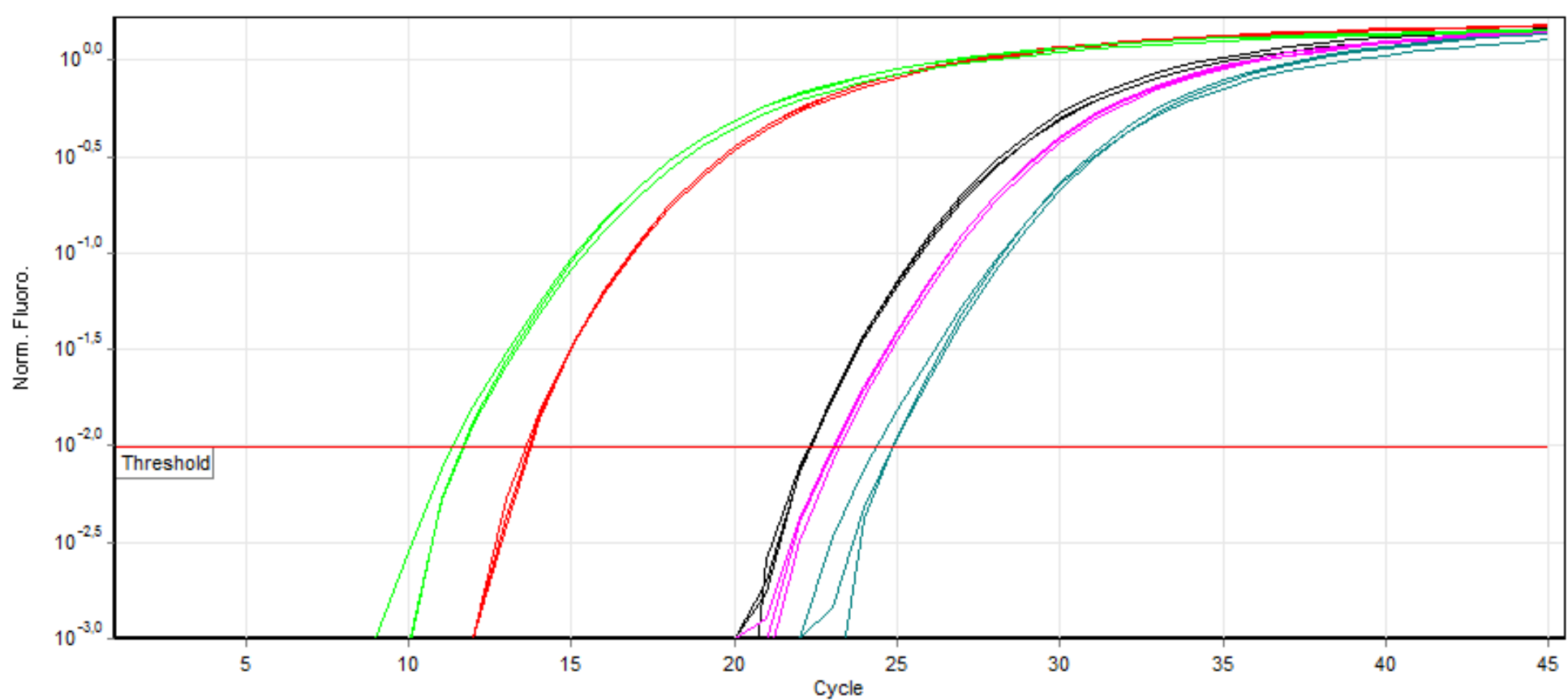
Polymerase chain reaction - PCR



- 1 **Denaturation** at 94-96°C
- 2 **Annealing** at ~68°C
- 3 **Elongation** at ca. 72 °C

Troubleshooting — PCR

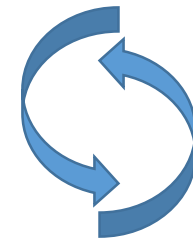
Real-time PCR:



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

Reason 2: new reagent/program?

Reason 3: PCR master mix error



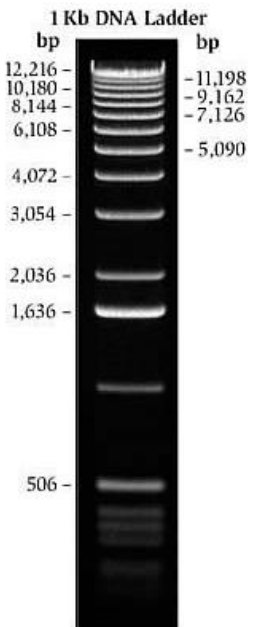
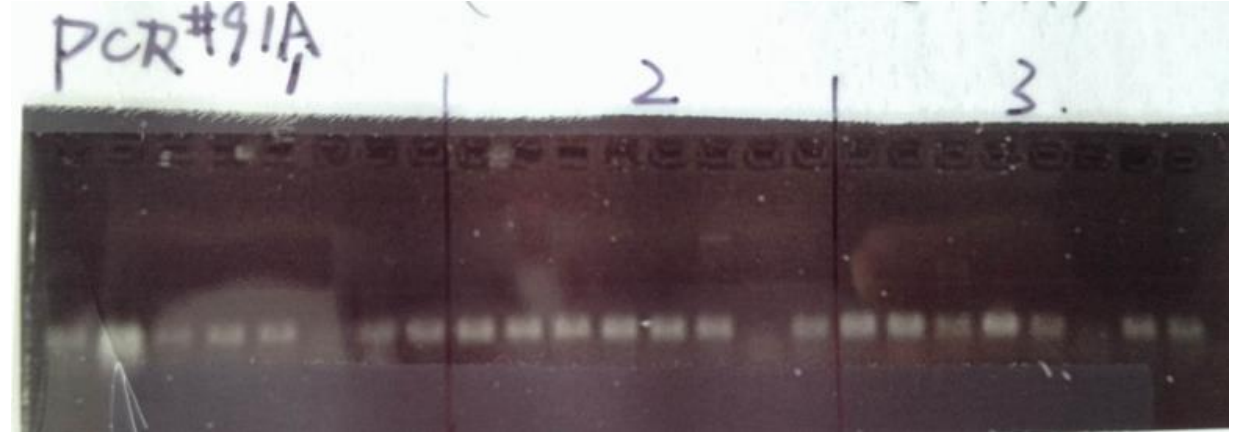
Use 1kb ladder



Check/change, try again





Check, try again



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

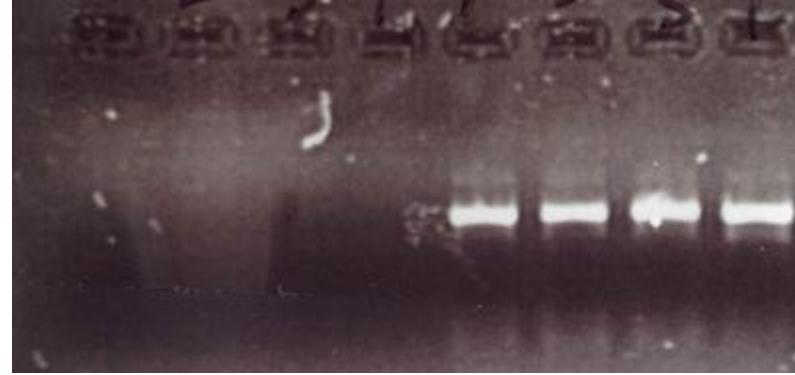


Design specific primers

2: not taxonomic sense



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

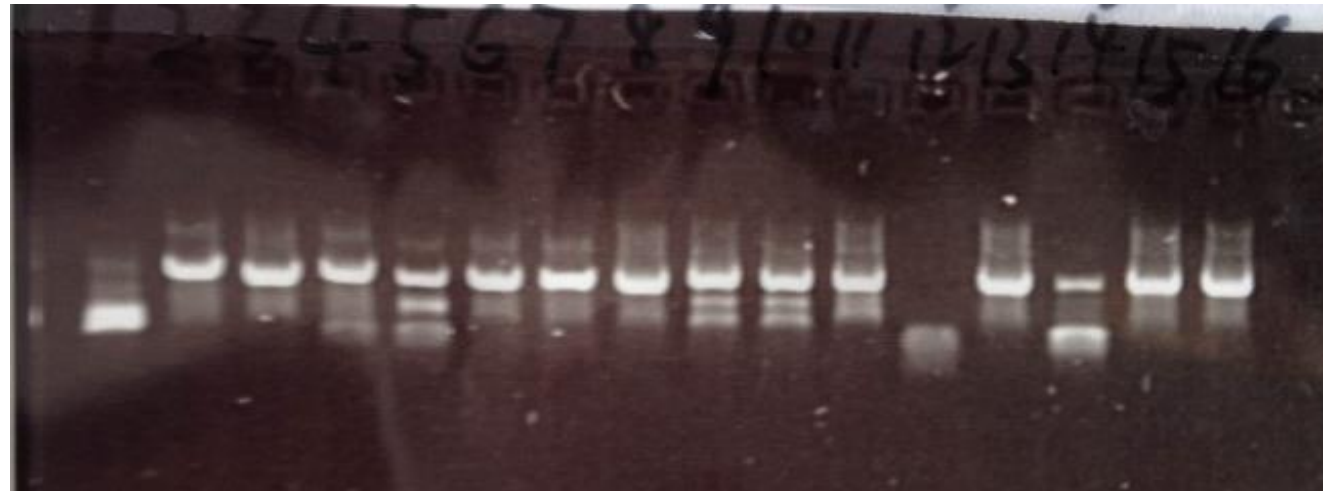


Troubleshooting — PCR

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

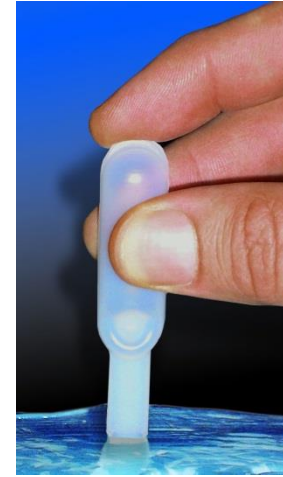


Troubleshooting — PCR

➤ Cut gel

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

Kit: NucleoSpin® Gel and PCR Clean-up



<http://www.5prime.com>

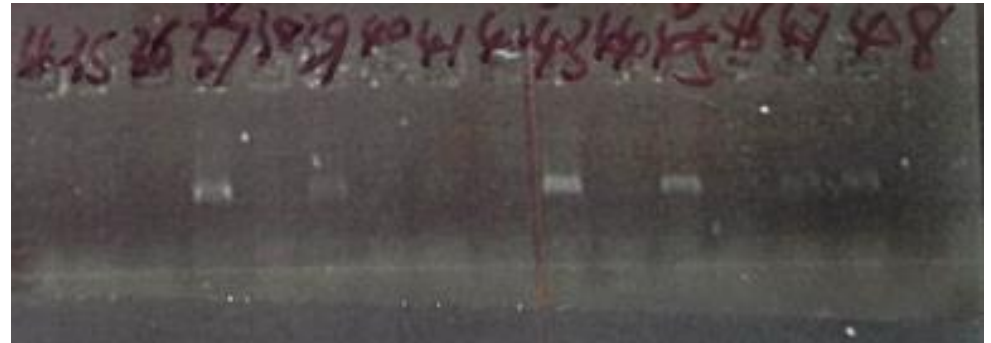
➤ 2nd round PCR

- 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

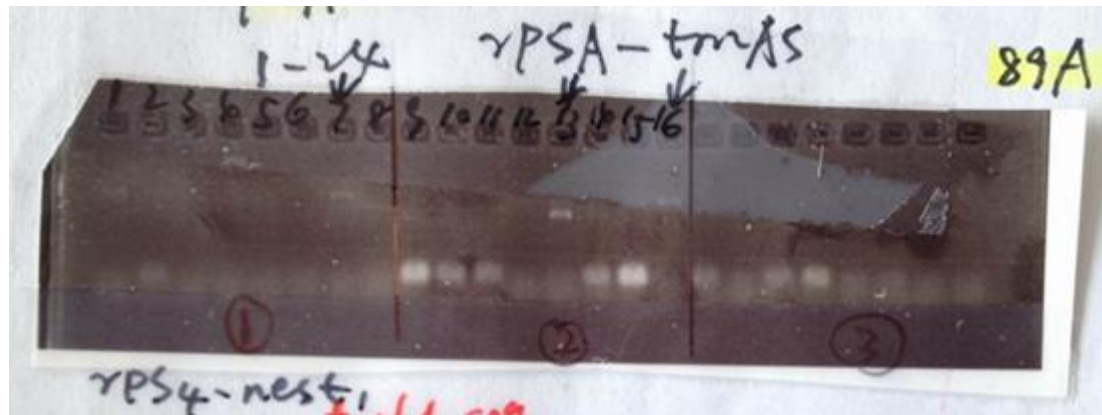
- Decrease stringency, use lower annealing temp. (down to 45 °C)
- Increase number of cycles (up to 40)
- Add Betaine/DMSO
- Add MgCl₂
- Add more primer and taq



Troubleshooting — PCR

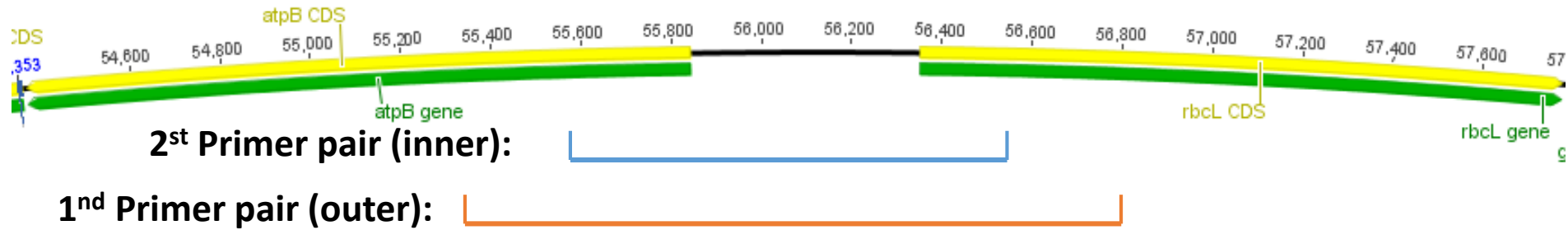
Tips

- 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

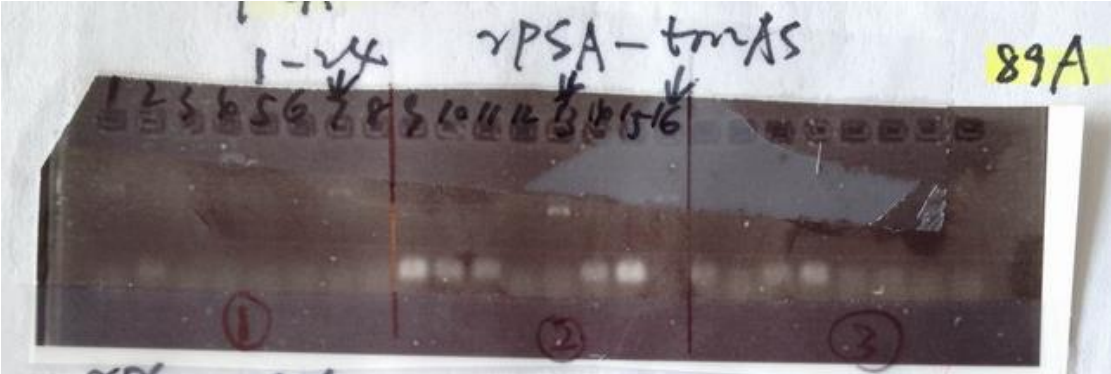


Proceeding:

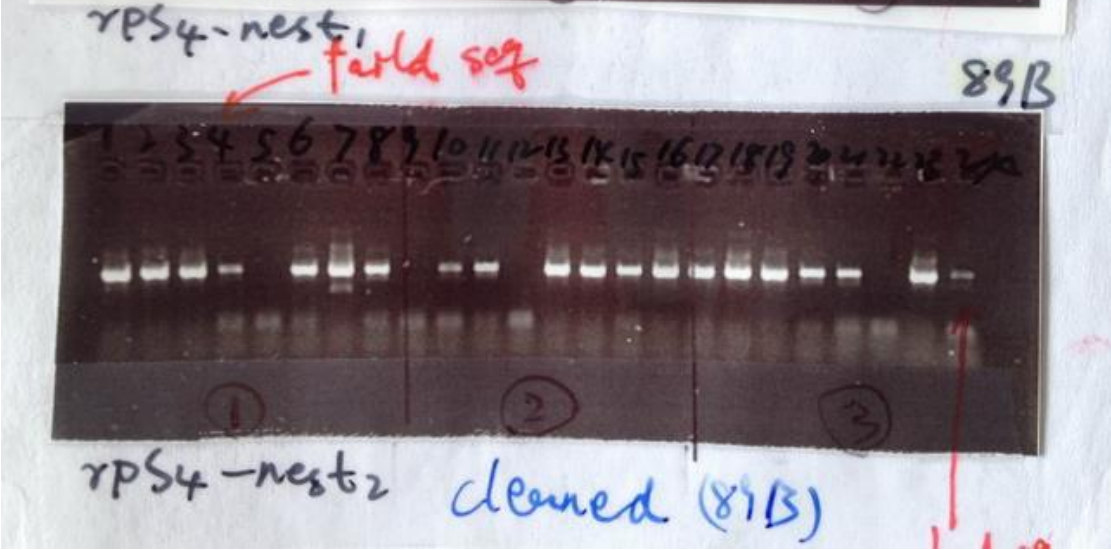
- PCR1: **outer** primer + genomic DNA as template
- PCR2: **inner** primer + PCR product from step1 as template
- Sequence : use **inner** primer

Nested PCR: results

Step 1st Primer pair:



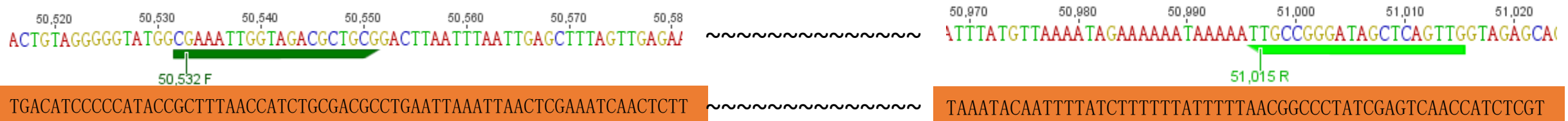
Step 2nd Primer pair:



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

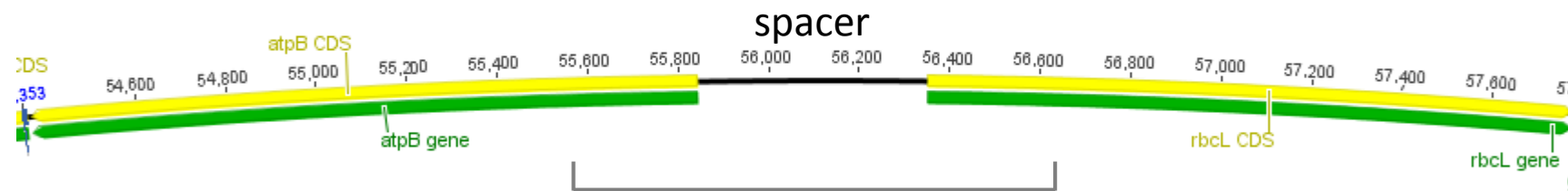
Strategies for choosing PCR target

- 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

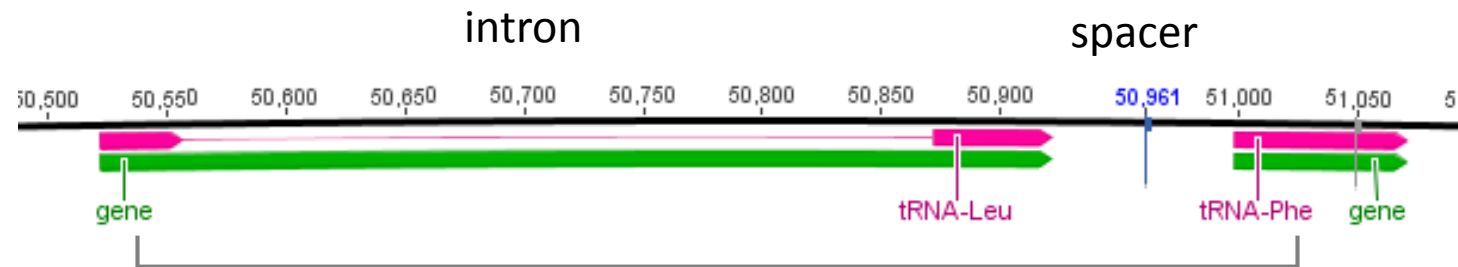
Primer design

Product position

atpB-rbcL



trnL-F



Primer design

Tips for primer designing

- Primer Length: 18-22 bp
- Melting Temperature (T_m): 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

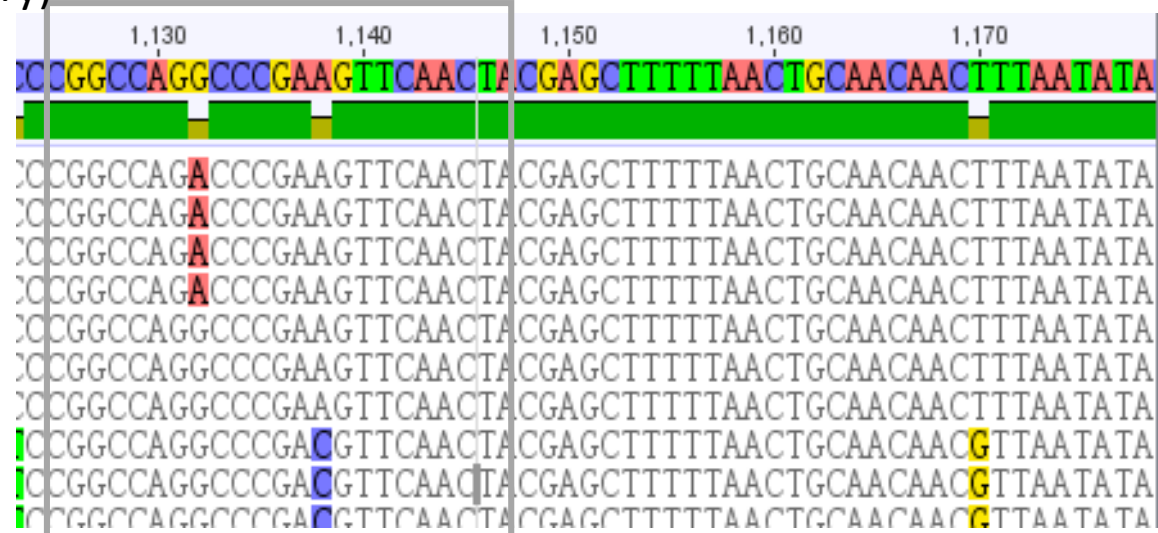
ACG -> V

ATG -> D

TCG -> B

ATC -> H

AGCT -> N



GGCCAGRCCCGAMGTTCAACTA

GGCCAGACCCGAAGTTCAACTA
GGCCAGACCCGACGTTCAACTA
GGCCAGGCCCGAAGTTCAACTA
GGCCAGGCCCGACGTTCAACTA

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

The screenshot shows the 'Edit Sequence' window for 'BRCA2 gene.seq'. The interface includes a menu bar (Accept/Discard, Edit, Search, Change, Rev.Translate), a toolbar with editing icons, and a 'pos:' field set to 2099. A statistics panel on the left shows sequence length (86101 nt), reading frame (1), and degeneracy (1). Thermodynamic data on the right includes t_m (73.9 °C), ΔG (-106.7 kcal/mol), Loop T_m (51.6 °C), and Loop ΔG (-2.5 kcal/mol). A table of codons for Valine is displayed, with the GTC codon highlighted. The main sequence view shows a DNA sequence with a red box around the insertion point (GTT TGA) and a corresponding amino acid sequence (V L P Q S H). A secondary sequence view shows a partial sequence with a primer pair (5' GTTCCAGGAGATGGGACTGAATTAGAATTCAAACAAATTTCCAGCGCTTCTGAGTTT and 3' CACTGACTCCAT) aligned. The clipboard contains 'TCTGAG' and the bottom right shows 'INS DNA' options.

File: BRCA2 gene.seq

Accept/Discard Edit Search Change Rev.Translate

5' 1 pos: 2099

Sequence Length: 86101 nt
Reading Frame: 1
Degeneracy: 1

t_m : 73.9 °C
 ΔG : -106.7 kcal/mol
Loop T_m : 51.6 °C
Loop ΔG : -2.5 kcal/mol

RT. Method: Lathe

Codons for Valine

GTA	GTC	GTG	GTT
6.6	15.3	29.6	10.6

2050 2060 2070 2080 2090 2100

GTT CCA GGA GAT GGG ACT GAA TTA GAA TTC AAA CAA ATT TTC CAG CGC TTC TGA GTT TTA CCT CAG TCA C

V P G D G T E L E F K Q I F Q R F - V L P Q S H

5' GTTCCAGGAGATGGGACTGAATTAGAATTCAAACAAATTTCCAGCGCTTCTGAGTTT
3' CACTGACTCCAT

Clipboard [6]: TCTGAG

INS DNA

Primer design

Primer3 as Geneious plugin

The image shows a screenshot of the Geneious software interface with the 'Primer Design' plugin open. The main window displays a sequence viewer for 'NC_016741 (Sequence)' with a scale from 25,000 to 510,519. The 'Primer Design' menu item is highlighted in the top toolbar. The 'Design New Primers' dialog box is open, showing the following settings:

- Select Task: Design New Design with Existing
- Primer design uses Primer3 2.3.4. Please cite [Primer3](#) if you publish results
- Forward Primer DNA Probe Reverse Primer
- Task: Generic
- Included Region: 366,762 To 366,833
- Target Region: 366,762 To 366,833
- Product Size Between: 300 And 1,800
- Optimal Product Size: 1
- Number of pairs to generate: 3
- ▶ Tm Calculation
- ▶ Characteristics
- ▶ Advanced
- OK Cancel

The sequence viewer shows the following DNA sequence around the target region:

```
366,740 366,750 366,762 366,770 366,780 366,790 366,800 366,810 366,820  
ATTTCAAATTGTTCTCTGGACTTTTATCAATATGAGGTGATCGTAACA CAGTATATAAGACTCGTGATTAGGCAATCCAATCTGCC TTGTC
```


Primer design

Primer3

Primer3web version 4.0.0 - Pick primers from a DNA sequence.

[disclaimer](#)

[code](#)

[cautions](#)

Select the [Task](#) for primer selection

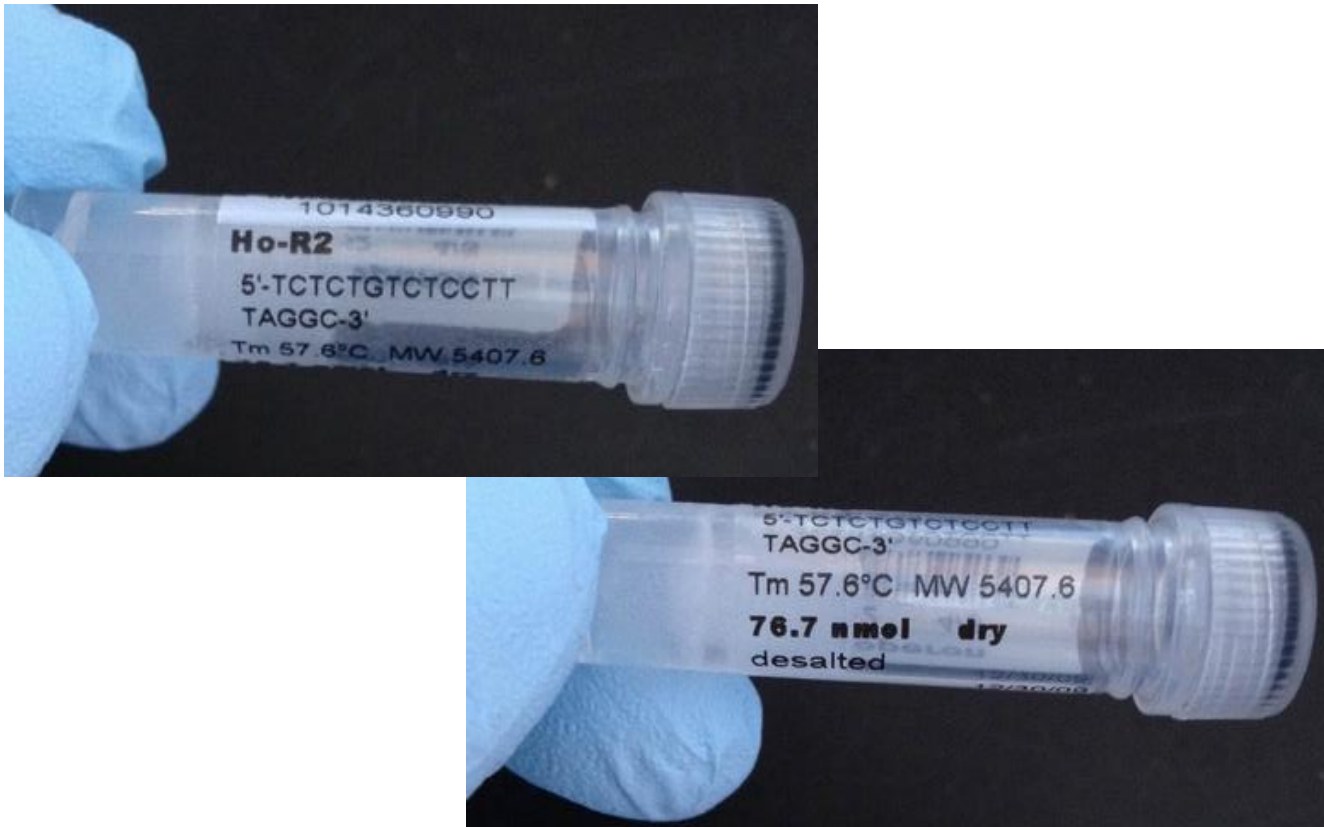
Paste source sequence below (5'→3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a [Mispriming Library \(repeat library\)](#)

<input checked="" type="checkbox"/> Pick left primer, or use left primer below	<input type="checkbox"/> Pick hybridization probe (internal oligo), or use oligo below	<input checked="" type="checkbox"/> Pick right primer, or use right primer below (5' to 3' on opposite strand)
<input type="text"/>	<input type="text"/>	<input type="text"/>

Order primers

IDT: <http://www.idtdna.com/>

Fisher Scientific: <http://www.fishersci.com/>



Prepare:

- Annealing Temp. = $T_m - 5\text{ }^\circ\text{C}$
- Stock solution (10x): 100 pmol/ μl
- Working solution (1x): = 10 pmol/ μl

Note: 1 nmol = 1000 pmol

Troubleshooting — Sequencing

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

Adams et al. 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.

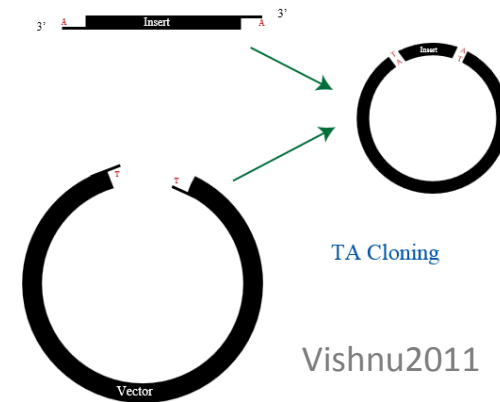
Zhao et al. J Biomol Tech 2000. 11:111

Kieleczawa et al. J Biomol Tech 2005. 16:220

- Repeats: Microsatellite or poly A/T or C/G stretches up to 40 times, cause PCR sliding

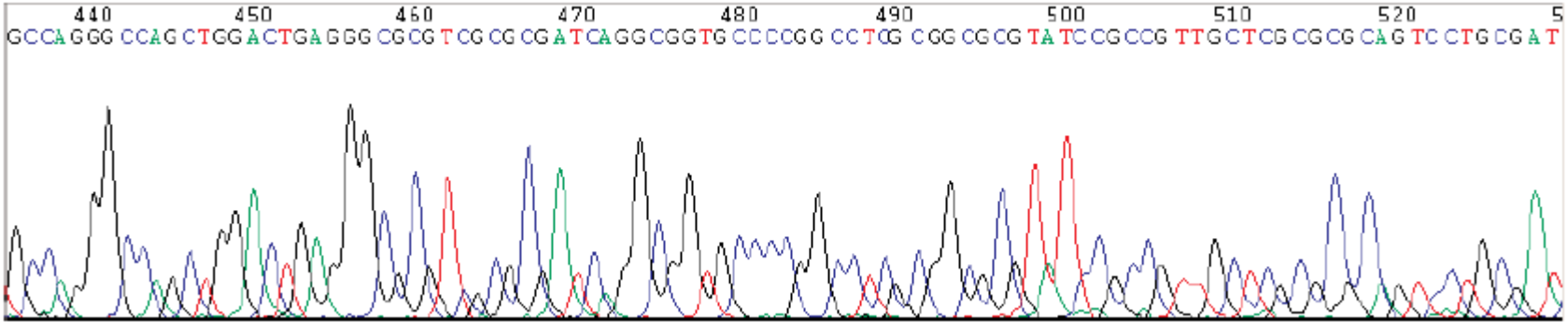
1. specialist taq, e.g. Hi-Fi Taq

2. cloning, extract vector, sequence vector



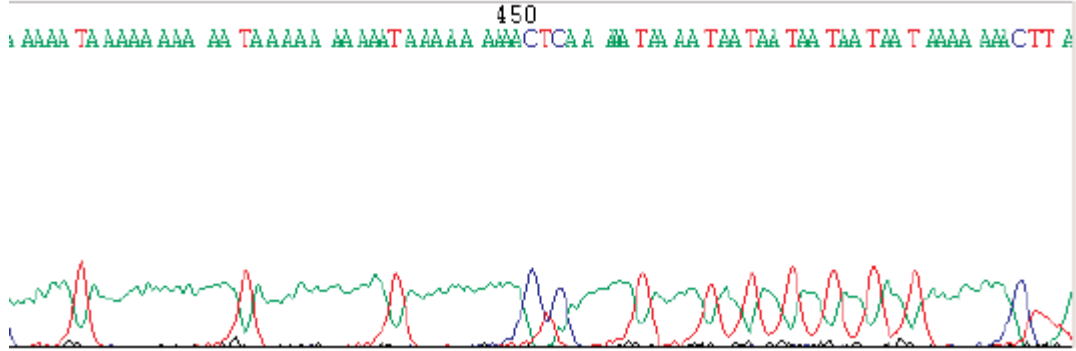
Troubleshooting — Sequencing

GC rich



www.appliedbiosystems.com

Repeats



www.appliedbiosystems.com

Poly C

