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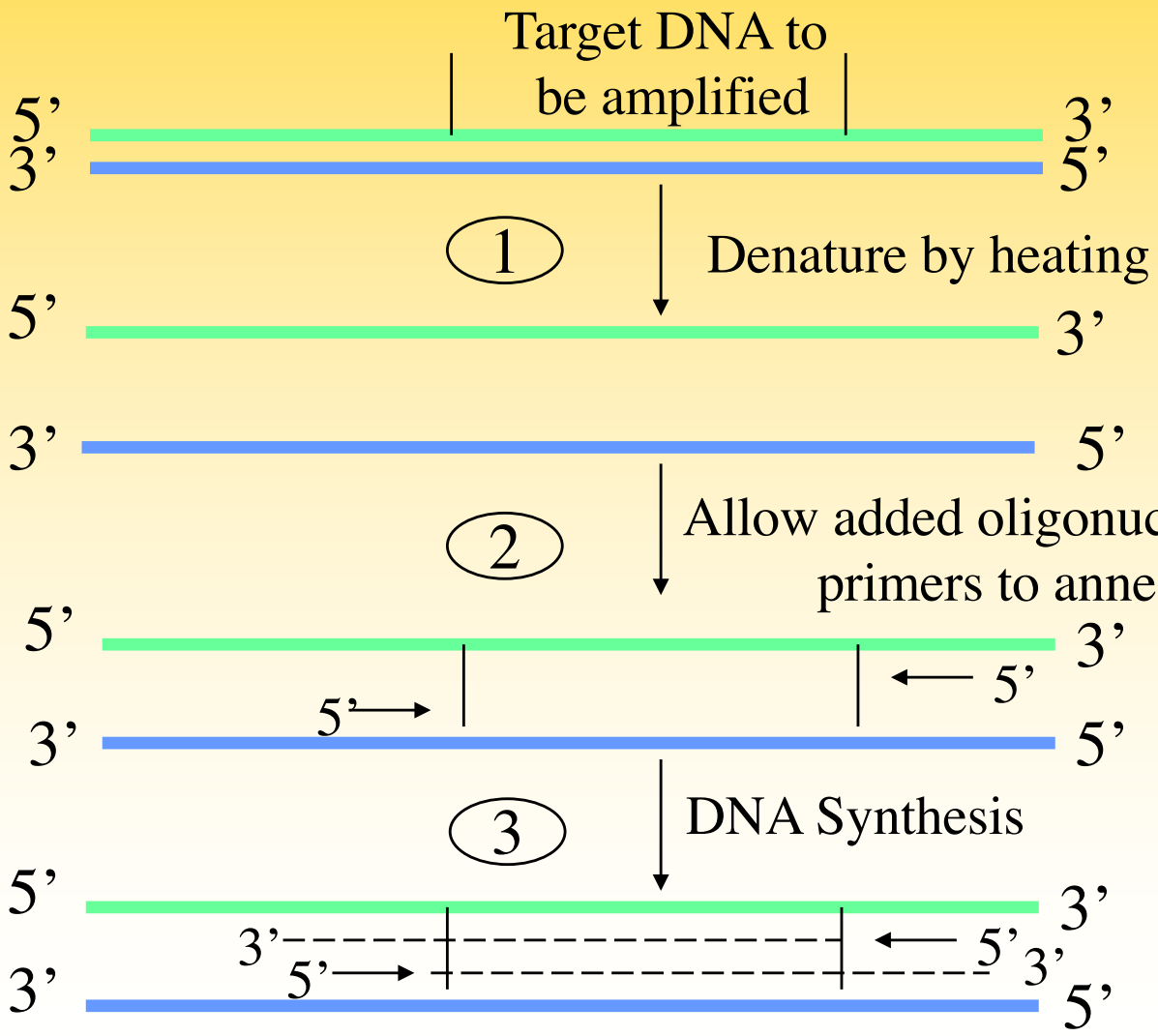
PCR

PCR CYCLE

NEEDED INFORMATION

- 📖 Target sequence for primers
- 📖 Thermostable DNA polymerase



- ➢ Chain reaction
- ➢ selective method to amplify specific DNA sequnces

ADVANTAGES

- different starting materials, even degraded ones
- High sensitivity
- Fast and simple
- Specific

LIMITS

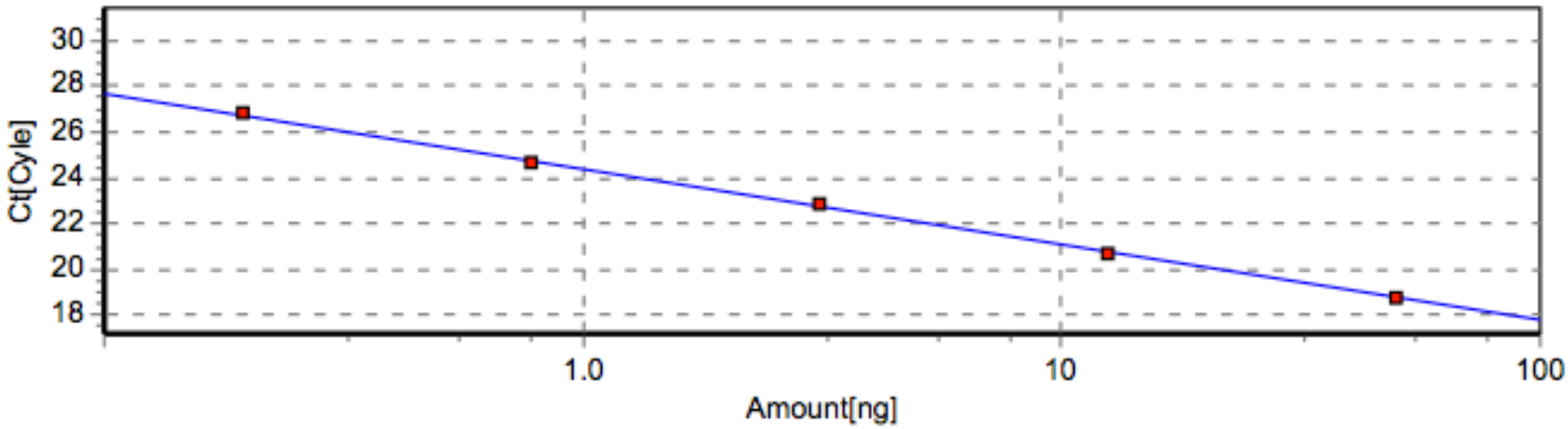
- It needs information on the target sequence
- Sensitivity- contamination (carry over)
- It amplifies relatively short sequences
- Errors in amplified sequences

APPLICATIONS

- Recognition of a specific sequence in a mixture of heterogeneous
- DNA Polymorphism analysis
- Analysis of mutations
- Analysis of unknown genes belonging to a family
- Analysis of DNA sequences adjacent to known sequences
- Generalized DNA amplification
- DNA amplification before sequencing
- Production of specific mutations

FACTORS INFLUENCING PCR

- ✓ **Annealing: Ta= Tm -2º**
- ✓ **Efficiency**



Slope	-3.307	R^2	0.999
Y-Intercept	24.43	Efficiency	1.01

- ✓ **Yield $X(1+E)^{n-2}$** where: X=amount (number) of starting molecules , E= efficiecy, n= cycles number.

Efficiency	Yield for n=30	Percentage
1	$268*10^6$	100
0.98	$202*10^6$	75
0.95	$132*10^6$	49

FACTORS INFLUENCING PCR

- ✓ **Magnesium:** *high concentrations stabilize the pairing of primers and can increase their sensitivity, but reduce the specificity of non-specific products*
- ✓ **$[Mg^{2+}]$= 0.7 mM + [dNTPs]** *If each dNTP is 0.2 mM* ⇒
- ✓ *$[Mg^{2+}]$= 0.7 mM + 4*0.2= 1.5 mM*
- ✓ **Error:**

Taq polymerase has no 3'-5 exonuclease activity, so it does not correct errors that result from incorrect incorporation of a nucleotide on DNA in summary 🎭 many errors (1 every 10,000 bases) 🎭 low PCR fidelity, especially for early errors, 🎭 first cycles. There are "high-fidelity" DNA polymerases, with correction activity in 3 '- 5' More common in PCR G➜T

- ✓ **DNA Polymerase**

Too much enzyme decreases the specificity, while a few enzyme returns small amounts of product.

Throughput:

Number of nucleotides added by the enzyme

Rate :

Number of nucleotides added by the enzyme in a unit of time.

In optimal conditions Taq makes a mistake every 10^6 bases, in normal conditions 1 every 1000- 10^5 bases.

- **Inhibitors**

Too much DNA or RNA, haemoglobin due to of Fe^{2+}, metal ions, aromatic compounds or dyes.

- **Co-solvents**

Lower the denaturation temperature and save the enzyme. Most used are DMSO, glycerol, formamide (CG rich sample).

10% glycerol + 5% formamide lower annealing Ta

PRIMER DESIGN

- *Balance between CG-AT*
- 15-25 bases in length for each one
- Tm included between 55 and 68° C
- Balance between oligos' Tm (+/- 2° C)
- non-complementary primers in 3'
- Proper use of Ta
- No secondary structures
- Use of the thermodynamic formula for calculating Tm

•TERMOCYCLER

Uniformità statica e dinamica

CARRY OVER

- *It is the contamination from previous reactions*
- *It can be caused by too concentrated C + or by collaborators*
- *To eliminate it: hypochlorite 5% at 95 ° C in an open block for 5 '*

Uracil N-glycosylase

•Nella mix si usa una miscela di dNTPs con la U al posto della T

• Carry over+ sample → UNG T=50° C it degrades carry over, but not the sample →95° C for UNG inactivation and Taq activation → it amplifies exclusively the template

•dsDNA and ssDNA with dUTP are substrates for UNG, while l'RNA and DNA with dT are not.

• it removes carry over

•Aumenta l'efficienza del clonaggio da prodotti di PCR

•It can increase the efficiency in the synthesis of specific site mutants (PCR based mutations)

•dUTP 20mM and 10 mM for each dA, dG e dC.

HIGH FIDELITY PCR

•Per ottenere amplificati senza errori

•To optaine amplicons without errors can cut amplicons and primers.[Mg2 +] ~ reduced to reduce the mismatch, low [dNTPs] (from 0.2 mM for each dNTPs to 0.05-0.04 mM), [Taq] lowest possible.Ta highest possible, t shortest possible extension, low cycles.

•Use of 3'exo proofreading enzymes, they have a < yield because they cut all the nucleotides unbound to the template ⇒it can cut amplicons and primers.

•Reduced [Mg^{2+}]~to lower mismatch, low [dNTPs] (from 0.2 mM

EXTRA LONG PCR

•To amplify 22 kb fragments. No use of normal Taq, it cannot return such long fragments. It is fundamental:

•DNA depurination at high denaturation temperatures lowers the yield. Favored by: acid pH of the tris → use of more basic buffers that are not so sensitive to T

•INTEGRITY OF DNA - adequate extraction - no phenol chloroform, but columns. Determination of DNA PM on denaturing gel

•DNA depurination at high denaturation temperature lowers the yield. It is promoted by: Tris acidic pH ⇒ More basic PCR buffers that are less sensible to ΔT.

•Misincorporation.

EXTRA LONG PCR

- Short denaturation times (to avoid depurination)
- Mg^{2+} quantification
- Secondary structures of DNA and Ta
- Co-solvents: glycerol + DMSO lower Tm $\Rightarrow$> t> but does not damage DNA.
- 2 Temp PCR: annealing / extension together 1 min / Kb
- Use of Taq mixtures.

Long distance PCR (LD-PCR)

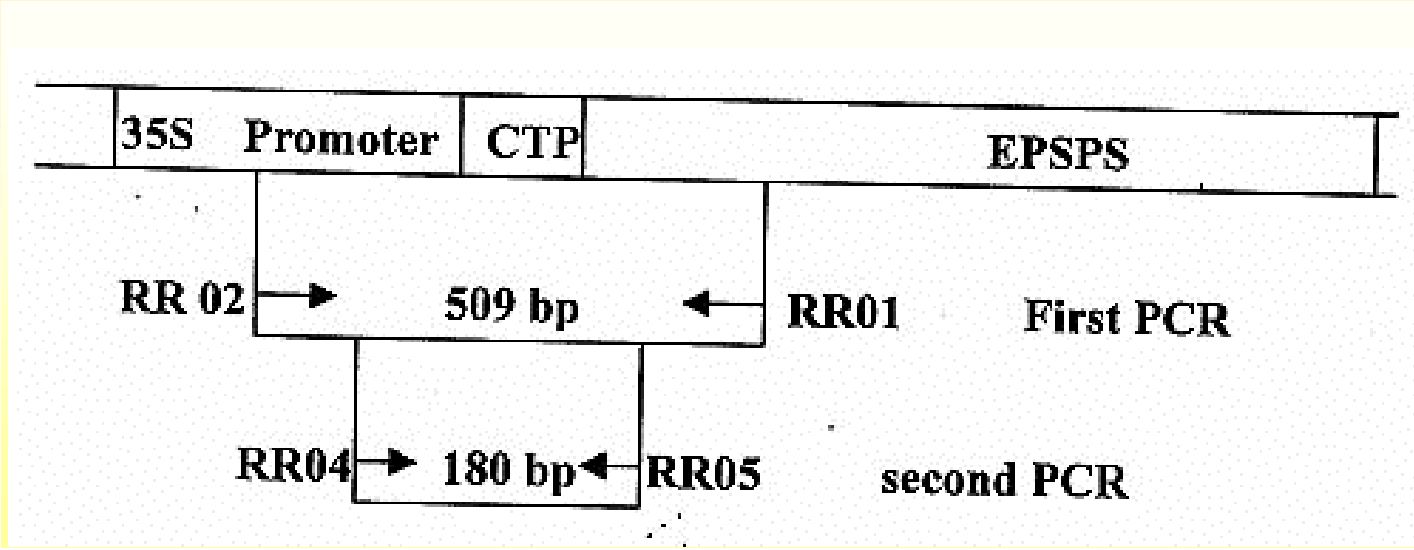
- Use of an enzyme with proofreading activity (eg Pfu polymerase) to correct possible mismatches and avoid mutations in the sequence.
- To amplify fragments longer than 40kb.
- [Mg^{2+}]~2.5 mM (range: 1.1÷3.5 mM), chloride or acetates.
- Primers 21-34 b long, Tm>58° C, [primers] included from 0.4 to 1 µM.

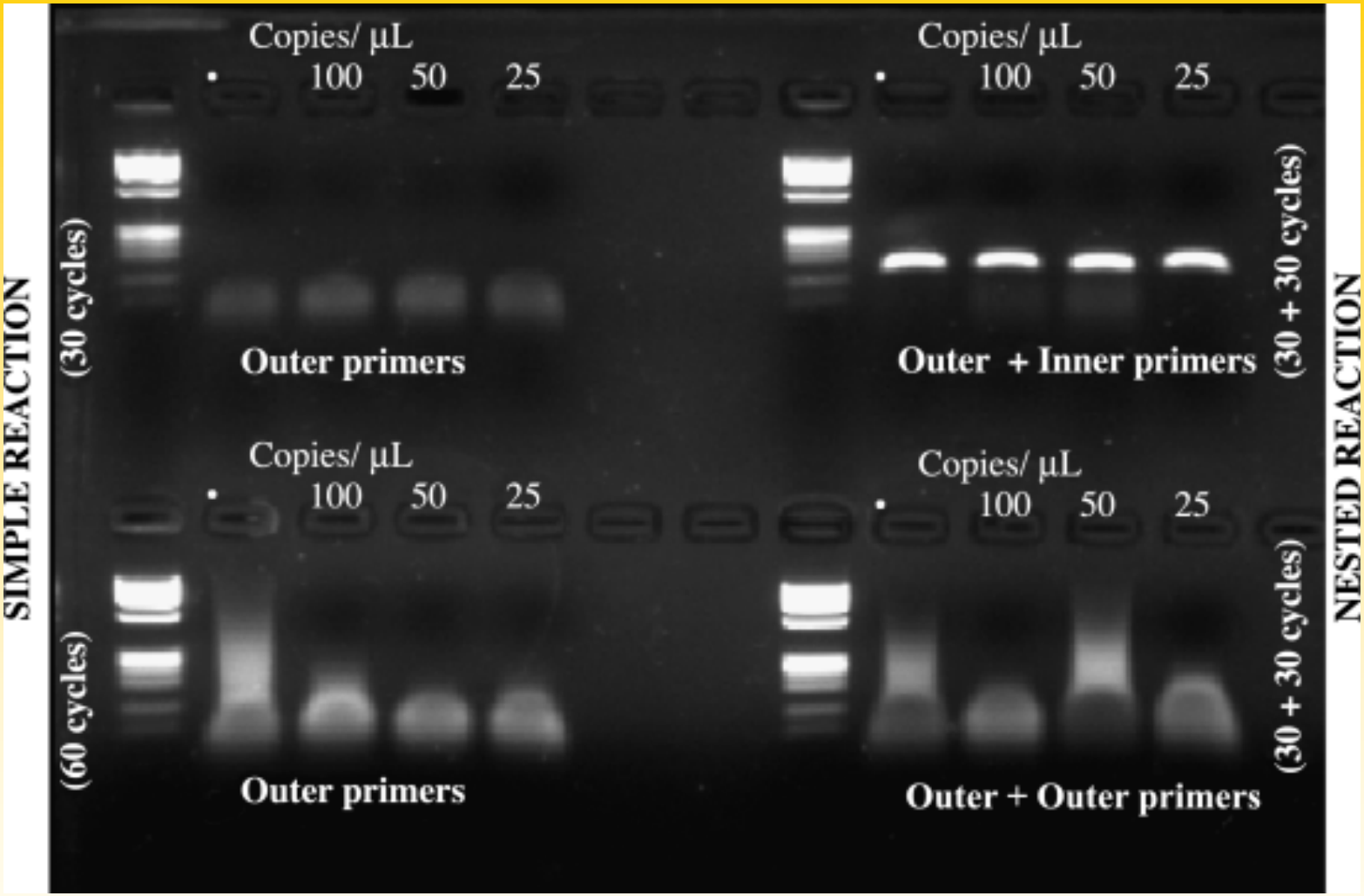
Long distance PCR (LD-PCR)

- Recombinant Taq polymerases are suggested because of the thermal stability.
- DNA template 125ng/10 µl reaction volume. Suggested reaction volume 50 µl
- Initial denaturation 92-95° C for more than 2 min.
- 10-30 cicli: 92-95° C/10-30”; Ta/30-60”; 68° C (if with DMSO or glycerol) or 72° C without co-slvents/3-15 min depending on amplicon length.

NESTED PCR

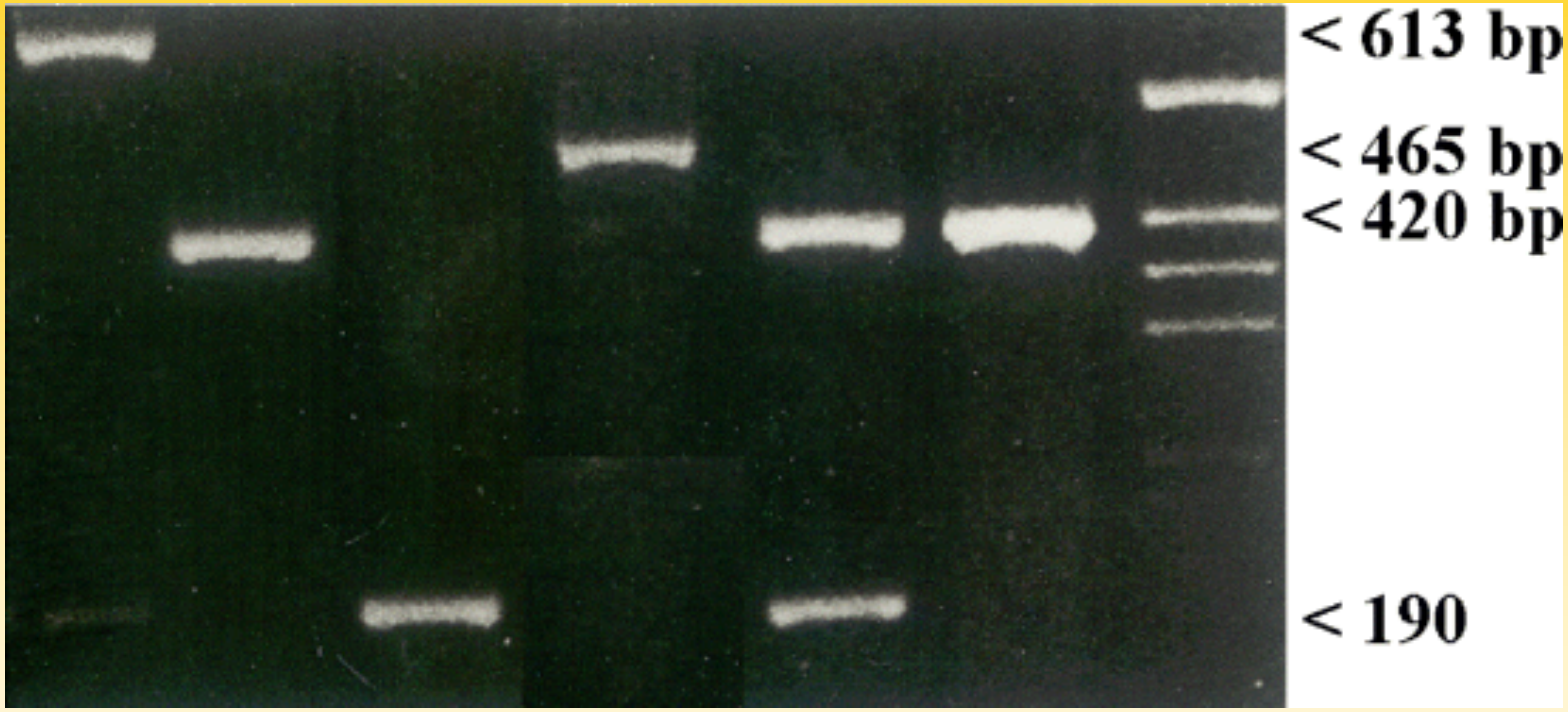
- To increase PCR sensitivity
- To minimize the amplification of aspecific products
- The product of the first amplification step includes the target of the second amplification
- In a single step-by inclusion of the second primer pairs ab initio
- In two steps: the product of the first amplification can be diluted and used as target for the second PCR.





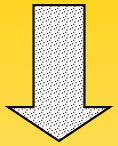
Left side PCR using exclusively external primers, 30 and 60 amplification cycles; right side ds nPCR (sensitivity of 10 a 100 copies/mL). nPCR external primers in both reactions

MULTIPLEX-NESTED PCR



Genotyping of clinical samples positive for CMVg. From left: gB2 (613 bp), gB 1 (420 bp), gB3 (190 bp), gB4 (465 bp).

TOUCH DOWN PCR



•Use of different

•Ta is higher in the early cycles, and decreases in the final ones

•Low extension time in the first cycles and in the final ones is increased especially if the input of starting material is low.

MULTIPLEX PCR

- Simultaneous amplification of multiple products by exploiting more than one pair of primers in a single PCR reaction.
- Useful for deletions, linkage, quantitative studies….
- Up to 15 pairs of primers per single PCR were used
- Fundamental the inclusion of a control to check the efficiency of the method in every assay

MULTIPLEX PCR- accorgimenti

- Selection of fragments with similar contents of G and C
- appropriate distribution of products based on length
- primers between 23 and 28 bases with% GC of ~ 40%
- Use 10% DMSO
- [Mg ++], dNTPs and enzyme should be increased compared to the single one
- Increase the extension in the PCR and optimize the conditions of the thermocycler
- **Advantages**: saving of t and reagents
- **Disadvantages**: laborious to search for optimal conditions.
- *Analisi dei risultati*

By length → electrophoresis

Real time PCR with ≠ fluorophores, max 4

Sequencing

PCR-ELISA

It is a PCR coupled with a detection system (PCR-ELISA).

Use of probes with complementary sequence to the amplified target.

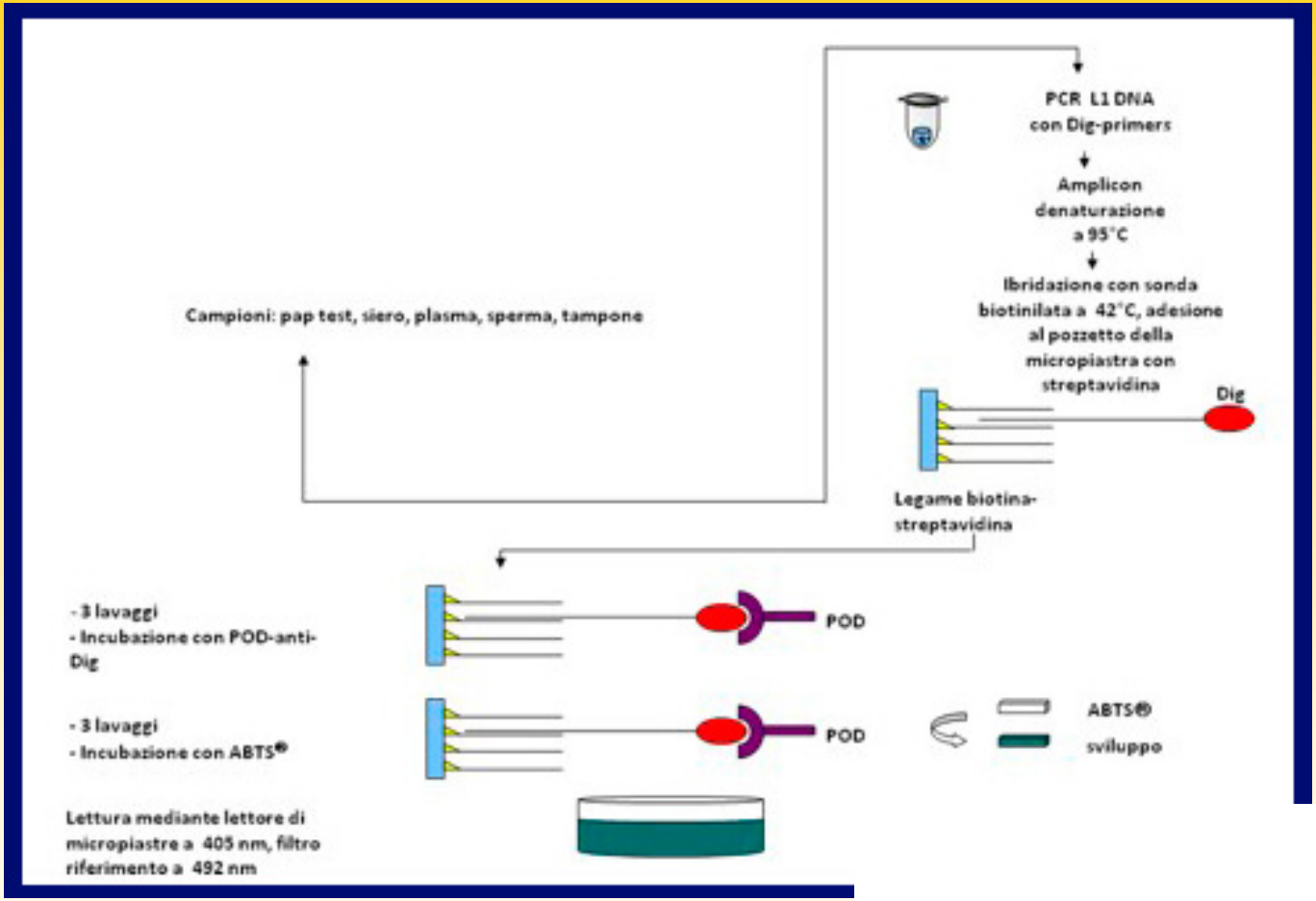
It can discriminate among different PCR products: specific products amplified with common consensus primers.

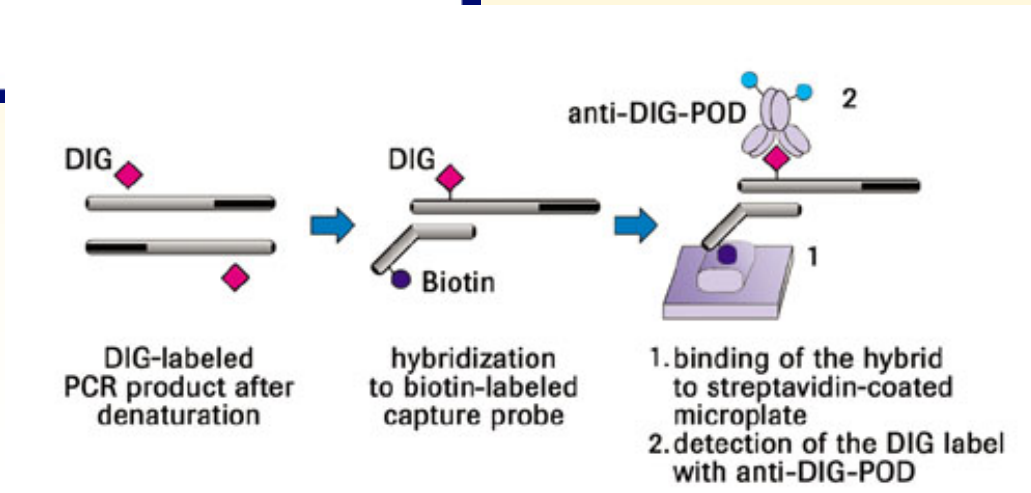
DIG-PCR / ELISA is based on 2 phases:

1. Amplification of the target sequence combined with digoxigenin (DIG),

2. Hybridization of the DIG-labeled amplified product to a biotin-labelled probe for colorimetric detection.

PCR-ELISA


PCR L1 DNA
con Dig-primers
Amplicon
denaturazione
a 95°C
Ibridazione con sonda
biotinilata a 42°C, adesione
al pozzetto della
micropiastra con
streptavidina
Dig
Campioni: pap test, siero, plasma, sperma, tampone
Legame biotina-
streptavidina
- 3 lavaggi
- Incubazione con POD-anti-
Dig
POD
- 3 lavaggi
- Incubazione con ABTS®
POD
ABTS®
sviluppo
Lettura mediante lettore di
micropiastre a 405 nm, filtro
riferimento a 492 nm


DIG
DIG
Biotin
anti-DIG-POD
2
1
DIG-labeled
PCR product after
denaturation
hybridization
to biotin-labeled
capture probe
1. binding of the hybrid
to streptavidin-coated
microplate
2. detection of the DIG label
with anti-DIG-POD

Tandemly repeated human DNA sequences

Satellite DNA (large arrays of tandemly repeated DNA-100 kb a 10 Mb) :

-Satellites 2 and 3 (5 bases reeats in most chromosomes).
-Satellite 1 (AT reach, 25-48 repeats, heterochromatin and centromers).
-Alpha (alphoid DNA, 171 b repeats, all chromosomes).
-Beta (Sau3A family, 68 b repeats, Centromeres of chromosomes 1,9,13,14,15,21,22,y).

DNA minisatellite (Minisatellites consist of repetitive, generally GC rich motifs that range from 10: >100 b in):

-Telomere (6 b repeats in telomers).
-Hypervariable minisatellites (9-24 b repeats in all chromosomes, often close to telomers.)

DNA microsatellite (ranging in length from one to 6 or more b, about 150 bp long):

- 1-4 brepeats in the entire genome, 15-30 repeats.

MICROSATELLITES

Polymorphic genes can be used to map certain diseases. The> part of the DNA in the chromosomes is redundant or anonymous.

It doesn't codify genes= no phenotypic manifestations

Since they have no function, those DNAs have high frequencies of polymorphic variation ⇒ inherited ⇒ they are used to evaluate the inheritance of the contiguous genes even if they have not been characterized

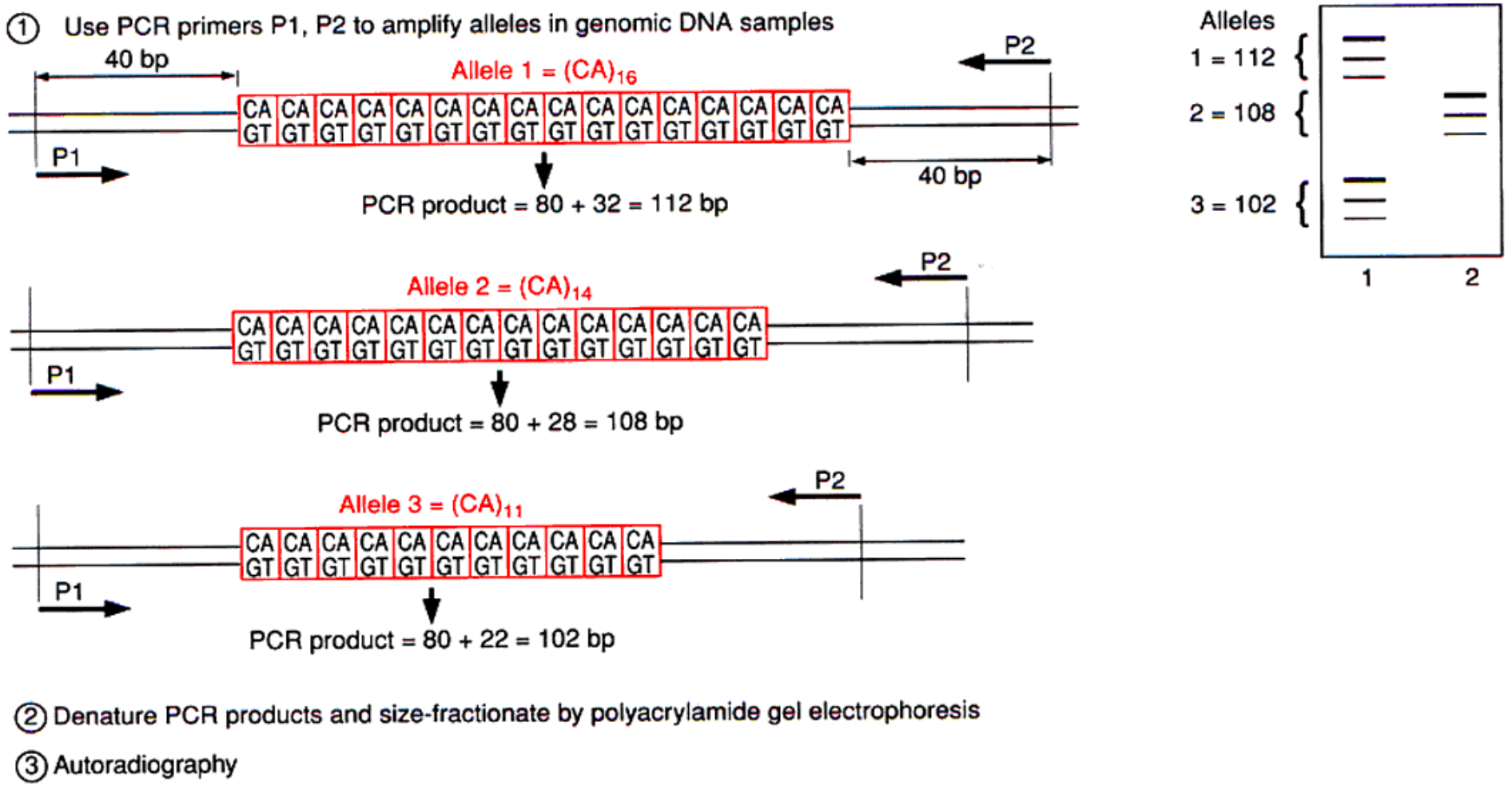
They are amplified using the sequences that flank the repetitions as primers. The specificity of the loci and the fact that two alleles are often different in length due to the different number of repetitions, makes the method interesting for the study of polymorphisms. Several neoplasms often present instability at the level of different microsatellite loci (3-4 bases).

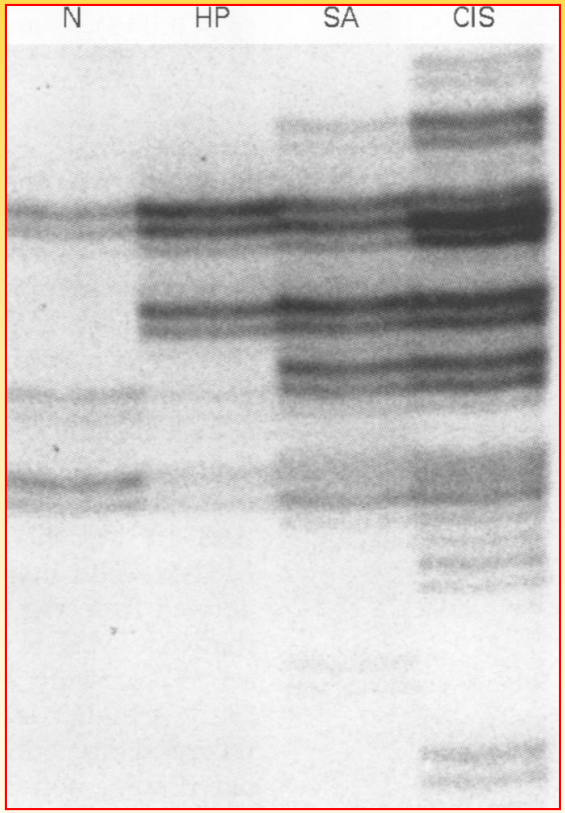
METHOD

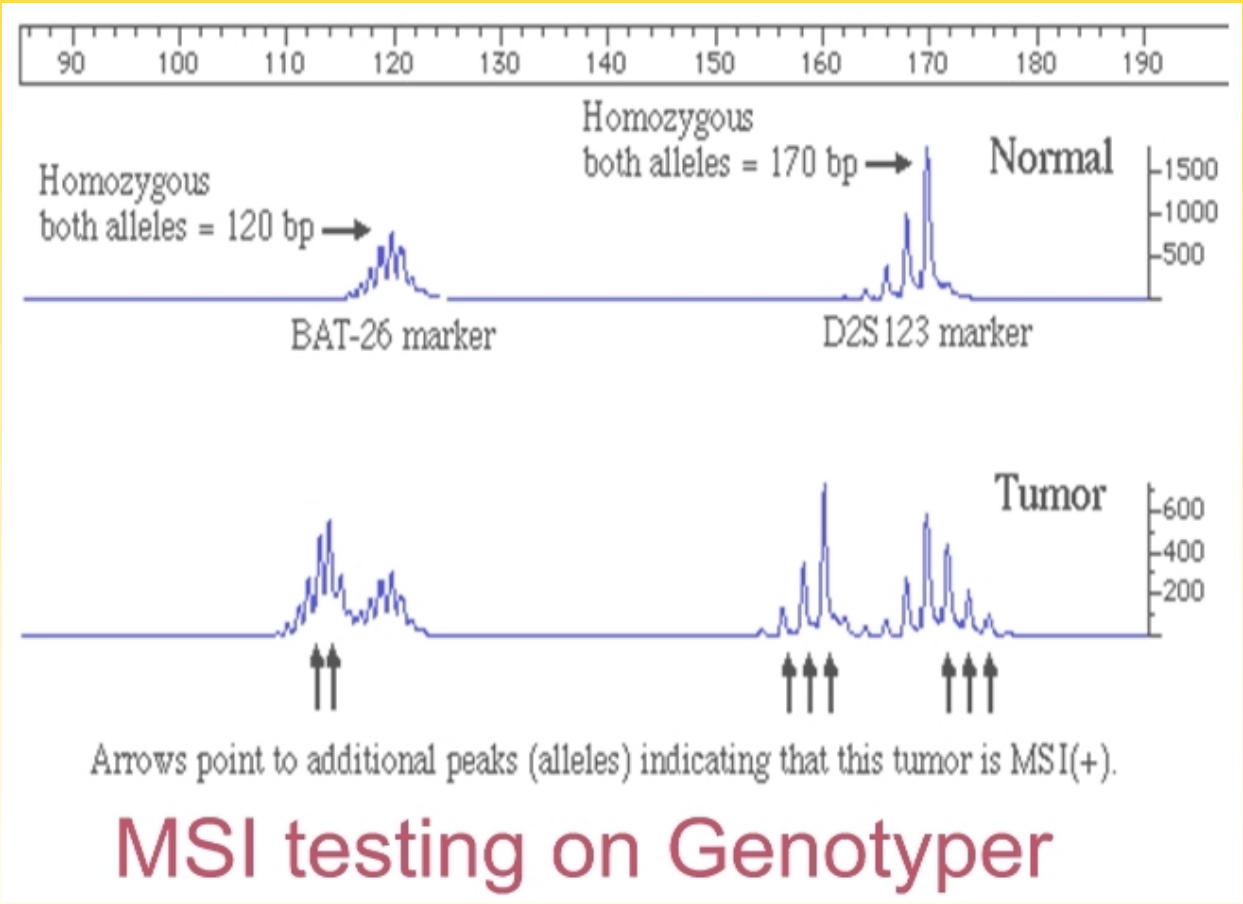
In the past SPECIFIC PCR for locus of interest

Electrophoresis using PAA

SHORT TANDEM REPEAT POLYMORPHISM (STRPs) MICROSATELLITE ANALYSIS







MSI testing on Genotyper

INVERSE PCR

To amplify by PCR unknown sequences flanking any identified segment of cDNA or genomic DNA.

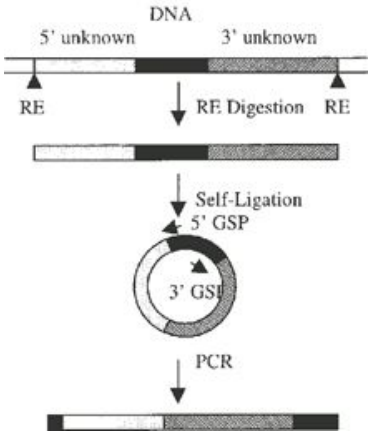
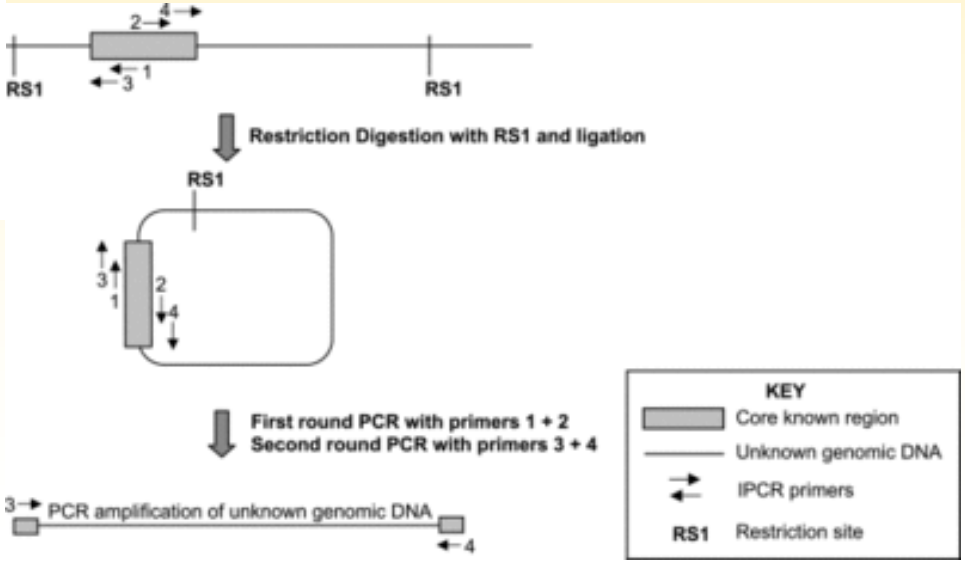


Fig. 1.

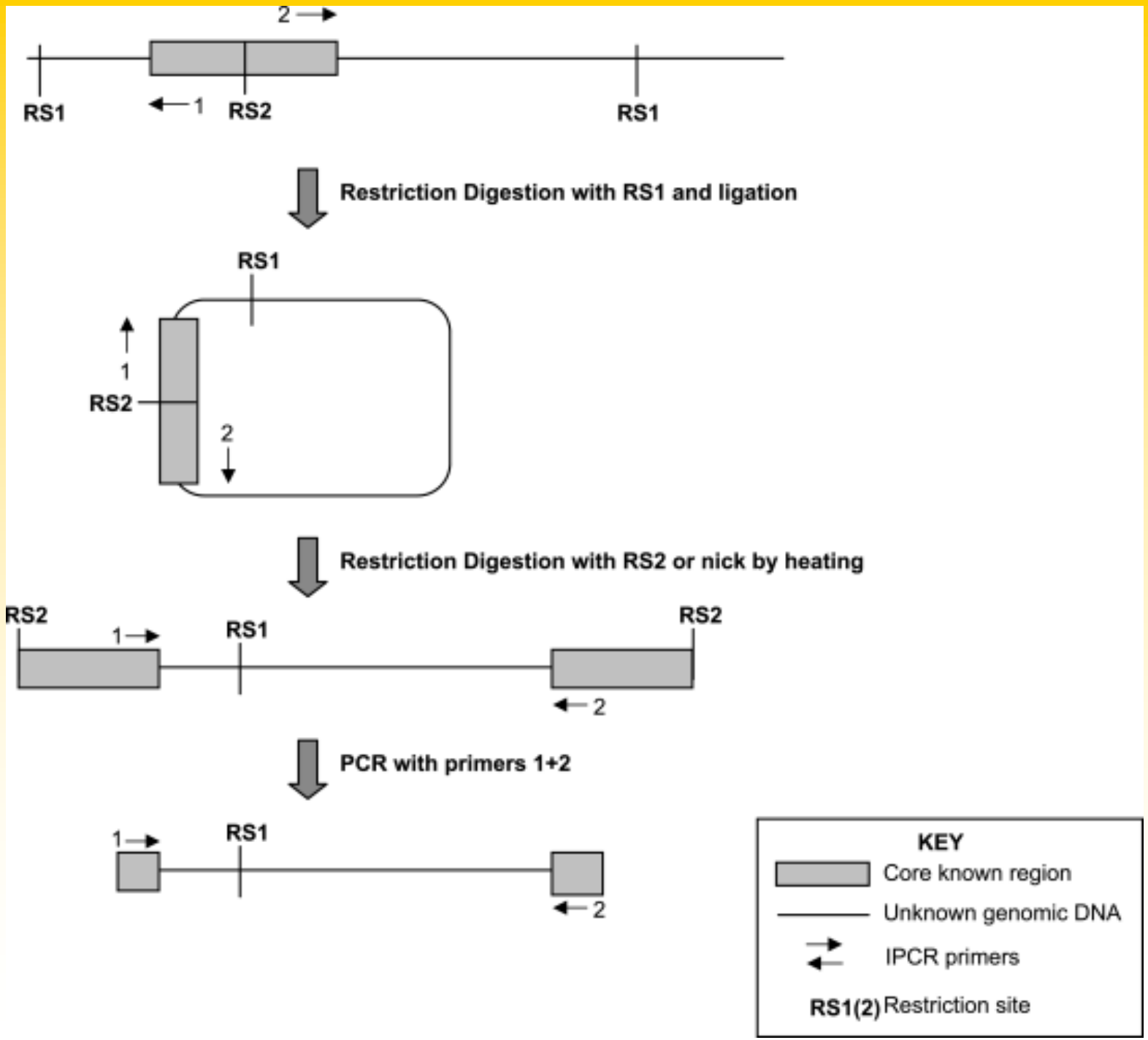
Diagram of IPCR for genomic DNA cloning. The procedure consists of four steps: genomic DNA isolation, circularization of double-stranded DNA, reopening of the circular DNA, and amplification of reverse DNA fragment. The black and open bars represent the known and unknown sequence regions of double-stranded cDNA, respectively. RE: restriction enzymesite; 65p: gene-specific primer.



Circular DNA is the template for PCR.
Nested PCR to increase sensitivity.

IPCR

Linearization



CONSIDERAZIONI E APPLICAZIONI

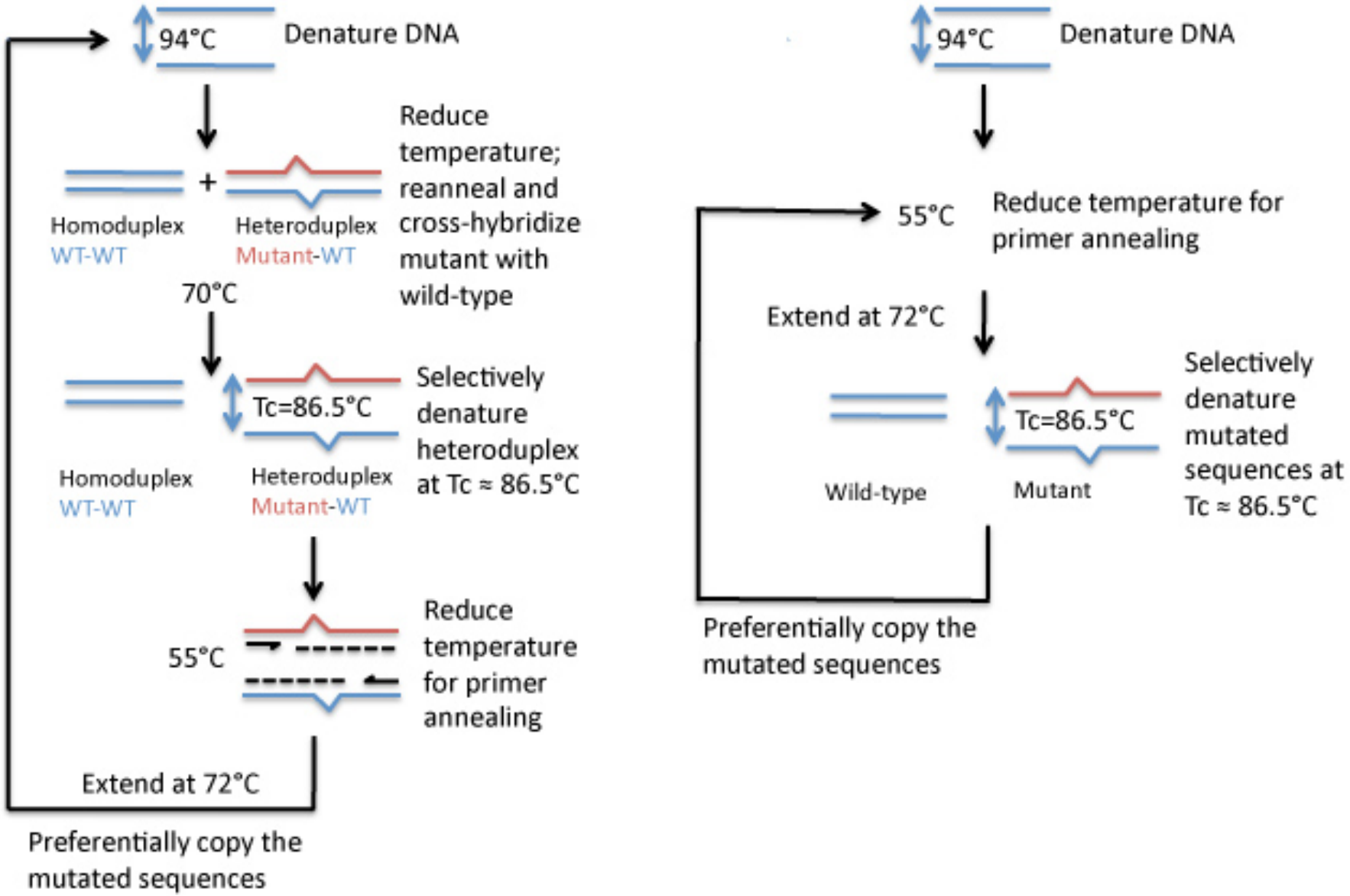
⇒ The crucial step is the choice of the restriction enzyme to cut the target DNA. If too short fragments are generated they have difficulty to close, while too long can be a problem for the subsequent amplification – combination with long distance PCR.

1. **Characterization of long DNA fragments with unknown sequences e.g., chromosome mapping / walking and identification of regulatory elements (upstream and downstream)**
2. **2 Determination of insertion sites of mobile genetic elements, eg integration of viral genomes into the host**
3. **To support in the study of mutation mechanisms involved in translocations and deletions**
4. **For generating knock down vectors for genes deletion**
5. **Fingerprinting of microorganisms**
6. **Organisms' evolution**

COLD PCR
CO-amplification at Lower Denaturation Temp PCR

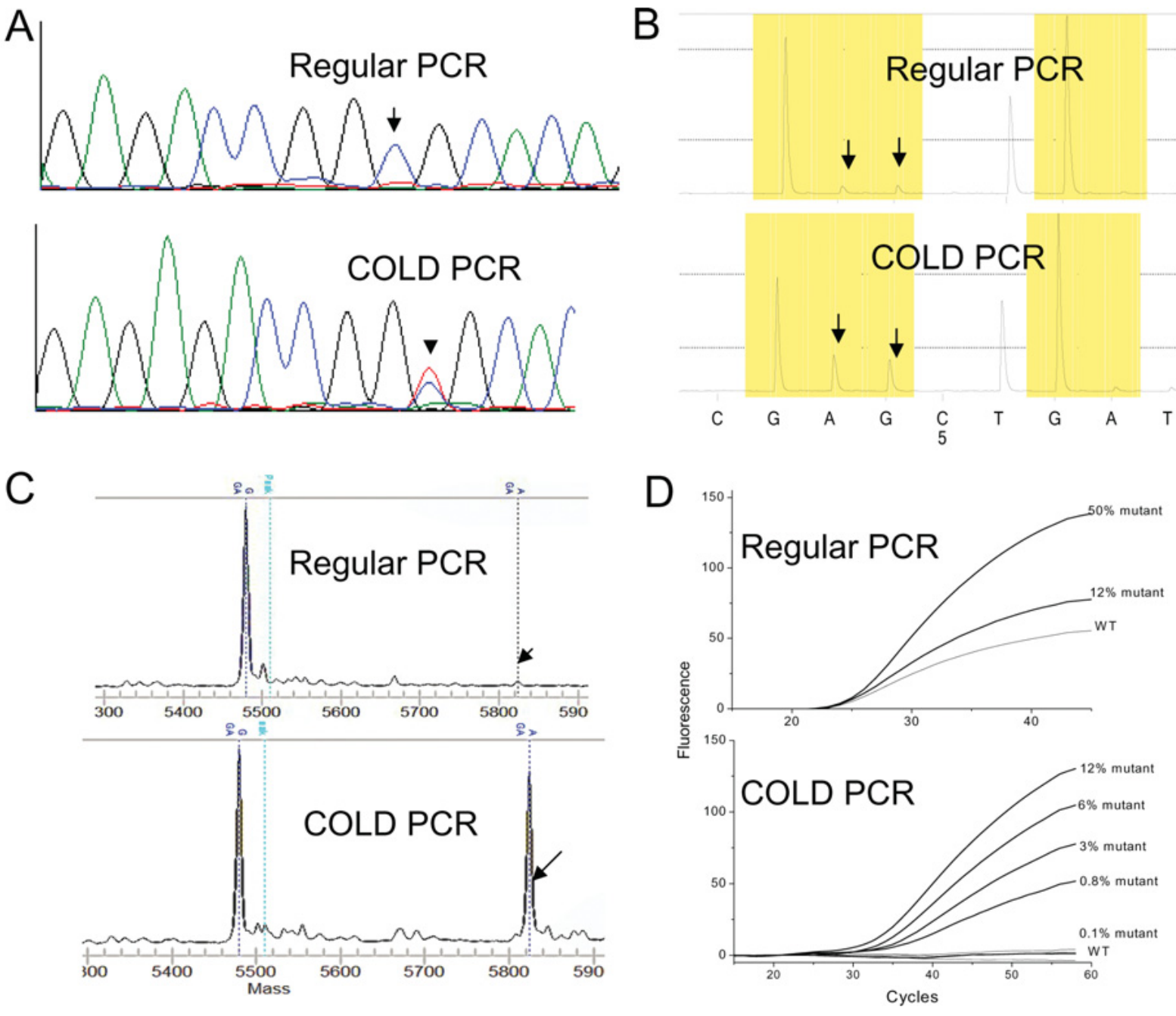
•Modified Polymerase Chain Reaction (PCR) protocol that enriches variant alleles from a mixture of wildtype and mutation-containing DNA

1. Denaturation stage. DNA is denatured at a high temperature, as usually 94 °
2. Intermediate annealing stage. Set an intermediate annealing temperature that allows hybridization of mutant and wildtype allele DNA to one another. Because the mutant allele DNA forms the minority of DNA in the mixture they will be more likely to form mismatch heteroduplex DNA with the wildtype DNA.
3. Melting stage. These heteroduplexes will more readily melt at lower temperatures. Hence they are selectively denatured at the Tc.
4. Primer annealing stage. The homo-duplex DNA will preferentially remain double stranded and not be available for primer annealing.
5. Extension stage. The DNA polymerase will extend complementary to the template DNA. Since the heteroduplex DNA is used as template, a larger proportion of minor variant DNA will be amplified and be available for subsequent rounds of PCR.

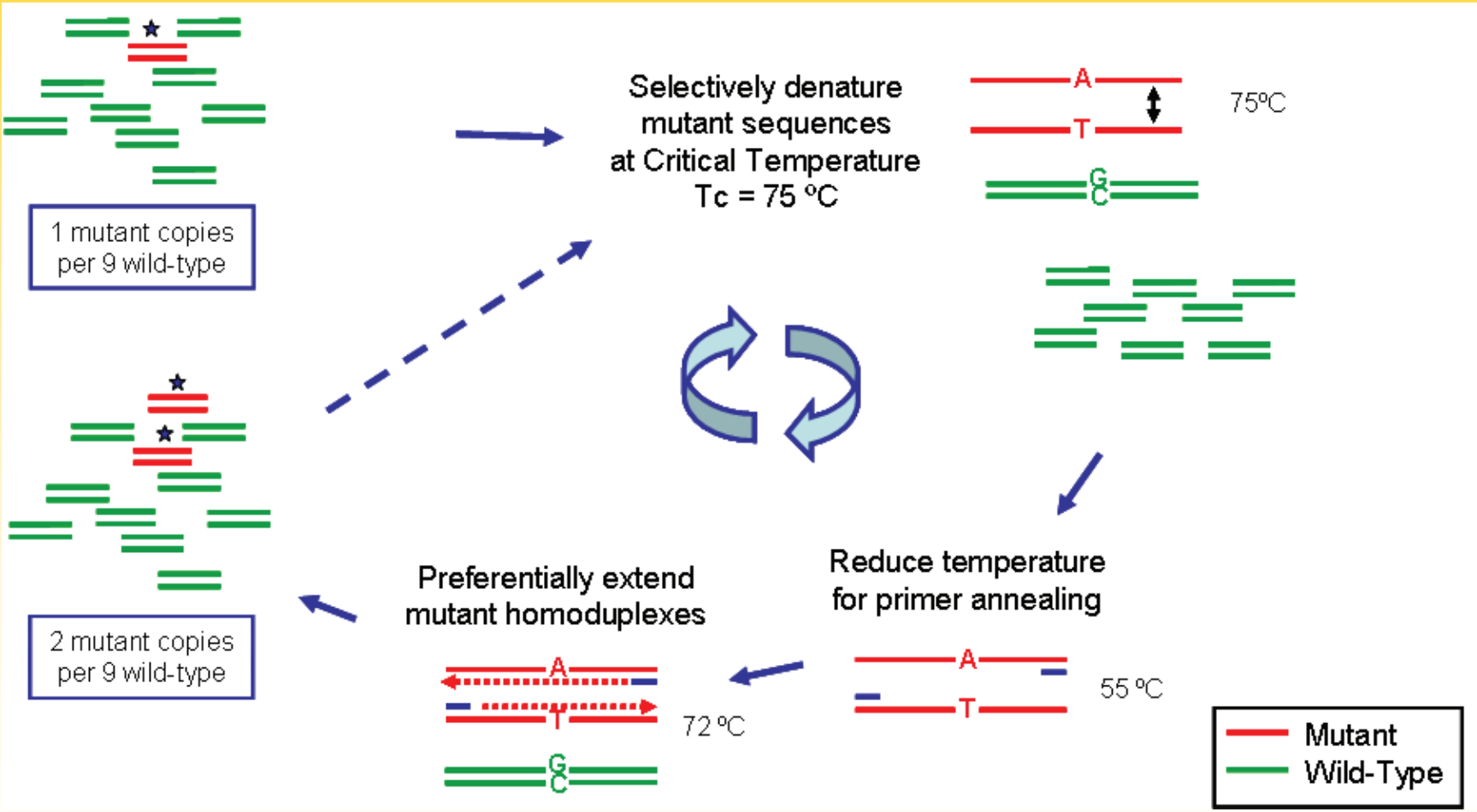


Full COLD-PCR
For enrichment of all mutations

Fast COLD-PCR
For enrichment of Tm-reducing mutations


A
Regular PCR
COLD PCR
B
Regular PCR
COLD PCR
C G A G C T G A T C
5
10
C
Regular PCR
COLD PCR
300 5400 5500 5600 5700 5800 590
Mass
D
Regular PCR
50% mutant
12% mutant
WT
COLD PCR
12% mutant
6% mutant
3% mutant
0.8% mutant
0.1% mutant
WT
Fluorescence
Cycles

PRINCIPLE OF FAST COLD-PCR



Ice COLD PCR

•ice-COLD-PCR employs a reference sequence (RS) the RS is engineered such that

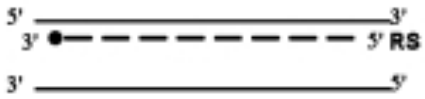
(i) it matches the WT-sequence of the anti-sense strand;
(ii) PCR primers cannot bind to it;
(iii) it is phosphorylated on the 3′-end so that it is non-extendable by the polymerase

•When incorporated into PCR reactions in excess relative to the template, the RS binds rapidly to the amplicons

•. At a critical denaturation temperature, the RS:WT duplexes remain double-stranded, thereby inhibiting selectively the amplification of WT alleles throughout the thermocycling.

•the RS:mutant duplexes are preferentially denatured and amplified. By using a WT-specific RS, all variants can be effectively amplified, regardless of mutational type and position.

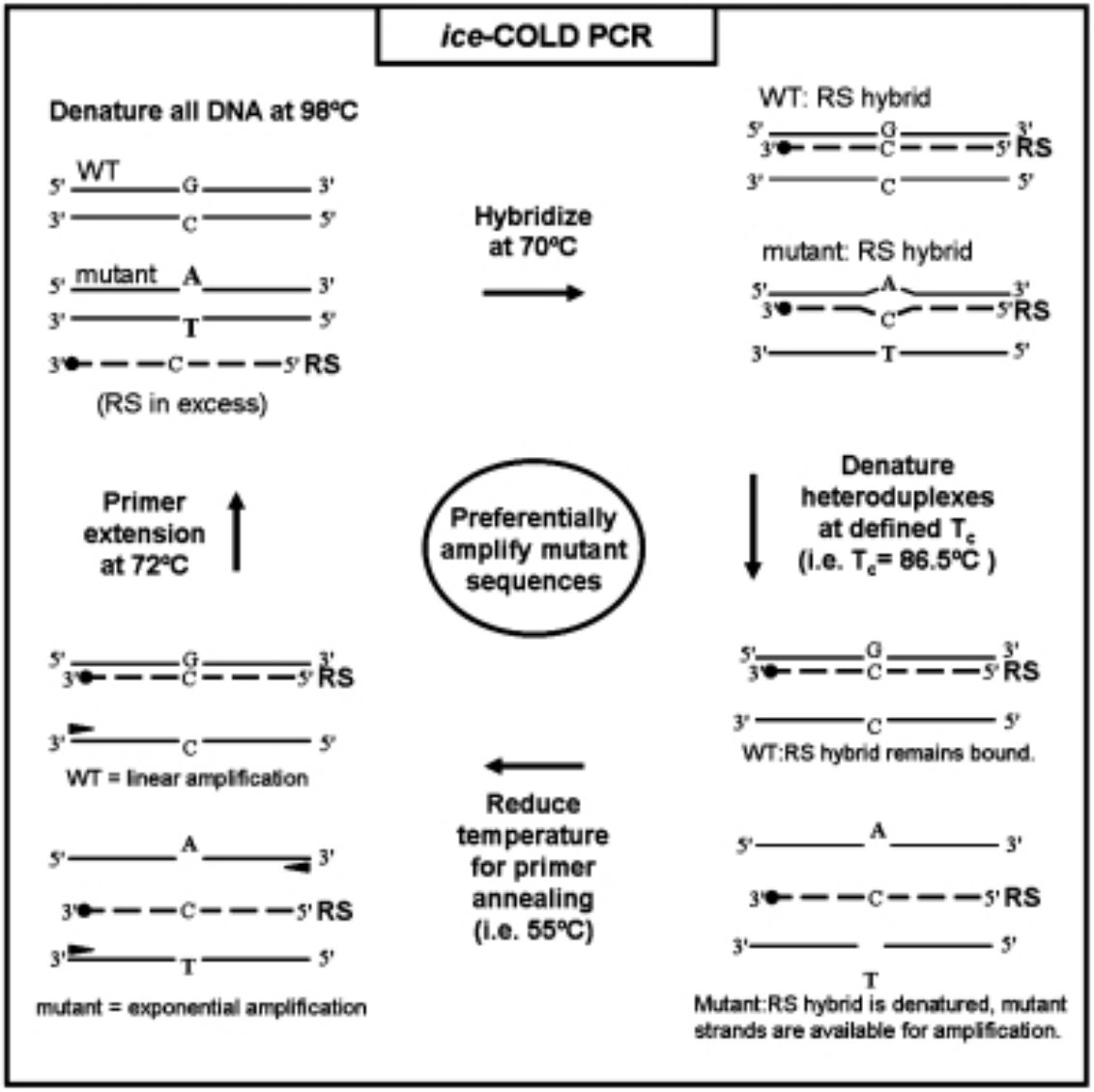
Reference Sequence (RS)

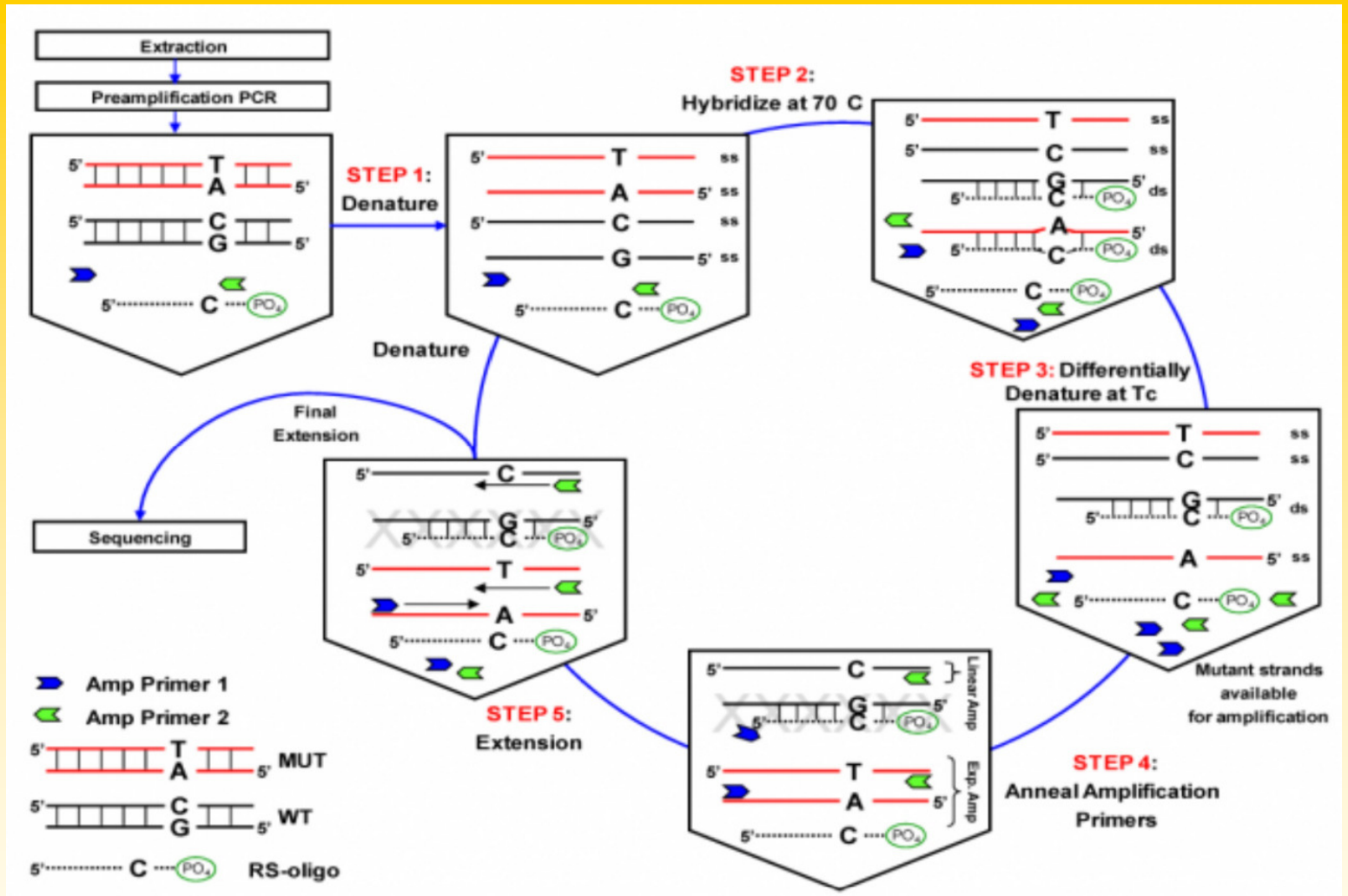


e.g. RS = 60 nt and RS = 90 nt used in this work

RS sequence properties:

- Complementary to WT sense-strand sequence
- 3'-PO_4 prevents polymerase extension
- ≤5 bp overlap with amplicon primers to prevent primer binding
- Selectively enhances denaturation of mutated sequences at the critical denaturation temperature (T_c)




Extraction
Preamplification PCR
STEP 1:
Denature
STEP 2:
Hybridize at 70 C
Denature
STEP 3: Differentially
Denature at Tc
Final
Extension
Sequencing
STEP 5:
Extension
STEP 4:
Anneal Amplification
Primers
Mutant strands
available
for amplification
Linear Amp
Exp. Amp
Amp Primer 1
Amp Primer 2
MUT
WT
RS-oligo