COLD-PCR: Very High Sensitivity Mutation Detection

New and Developing Technologies
for Genetic Diagnostics

Salisbury July 2010

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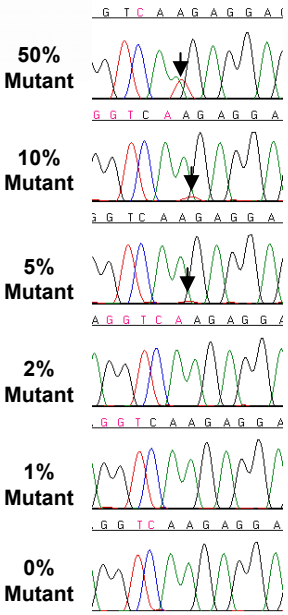
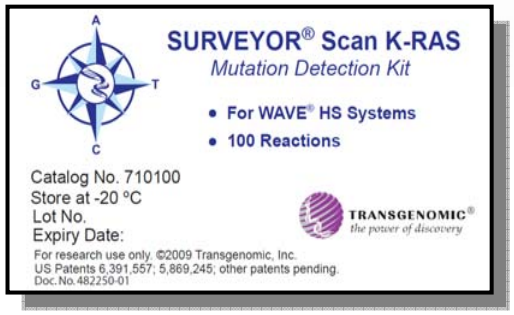
the power of discovery

Detection of Somatic Mutations



DNA/Pyro Sequencing



BEAMing

NextGen Sequencing

COLD-PCR

10% 1% 0.1%

Tumour Biopsies

Plasma/Serum

FNAs

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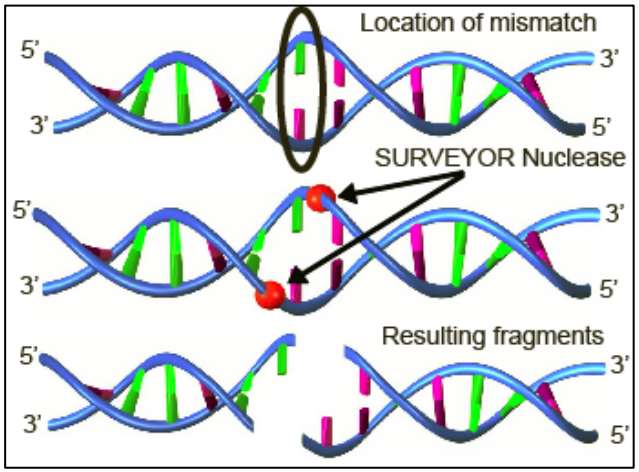
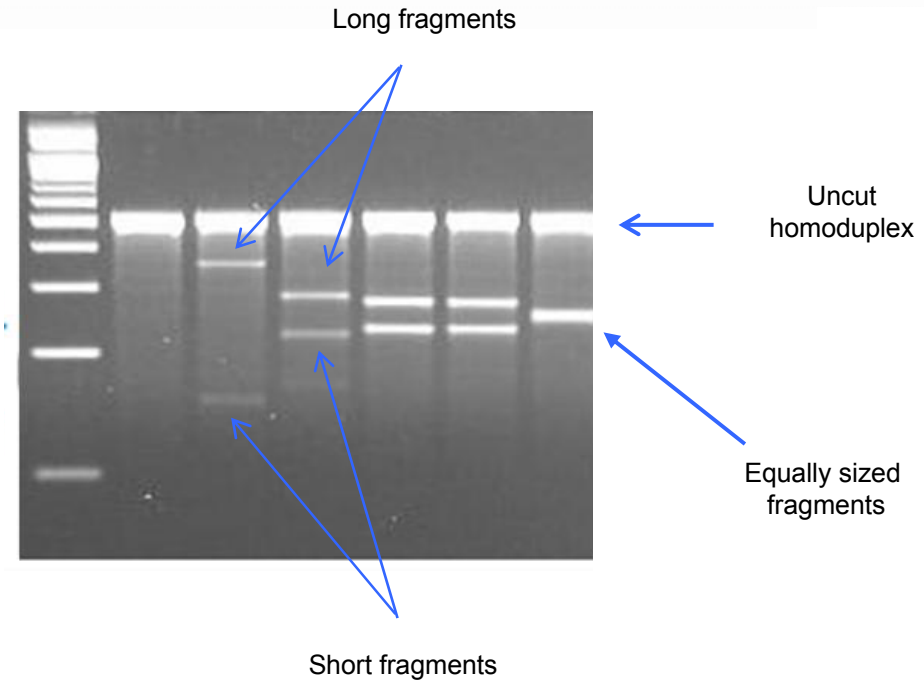
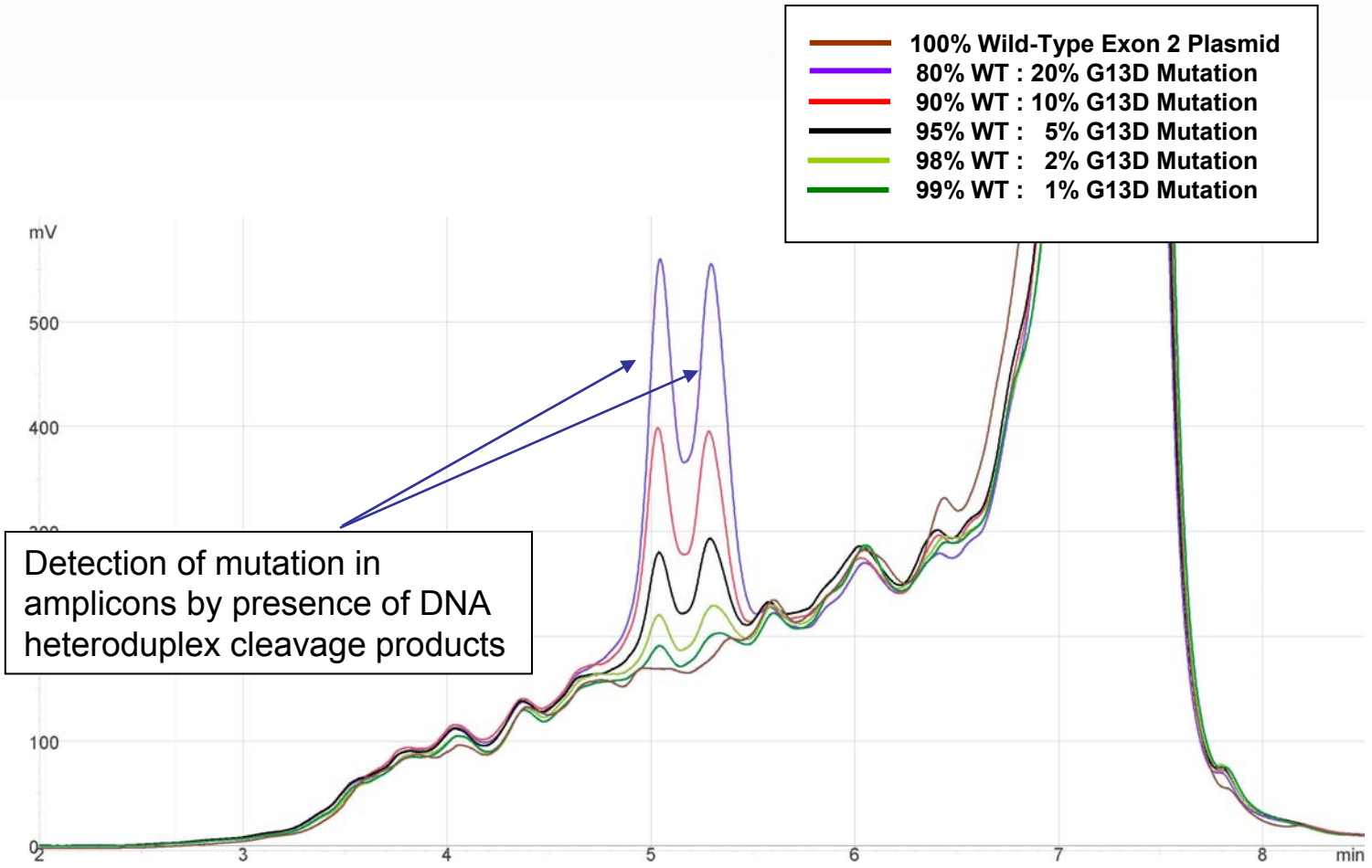
SURVEYOR Nuclease fragment cleavage





Identifies both *presence* and *location* of sequence alteration

SURVEYOR Nuclease/WAVE DHPLC – based mutation detection of K-RAS Codon 13



Very high sensitivity mutation detection

COLD-PCR: COamplification at Lower Denaturation temperature PCR

nature medicine

Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing

Jin Li[1], Lilin Wang[1], Harvey Mamon[1], Matthew H Kulke[2], Ross Berbeco[1] & G Mike Makrigiorgos[1]

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the mutation type or position on the sequence. We replaced regular PCR with COLD-PCR before sequencing or genotyping assays to improve mutation detection sensitivity by up to 100-fold and identified new mutations in the genes encoding p53, KRAS and epidermal growth factor in heterogeneous cancer samples that had been missed by the currently used methods. For clinically relevant microdeletions, COLD-PCR

COLD-PCR technology

- Enriches mutant DNA in a mutant/wild-type mixture by preferential amplification in PCR
- Based on exploitation of the critical temperature, Tc, at which mutation-containing DNA is preferentially melted over wild-type
  - Tc determined empirically by real-time or gradient PCR
- Preference in synthesis is repeated over many PCR cycles
- The greater the Δ(Tm-Tc) the greater the enrichment of mutant DNA

Rationale for sensitivity of less than 1%

Applications:

1. Definitive genotyping of tumour samples with low % malignant cells
2. Blood/body fluid cancer mutation testing
3. Mutation detection in circulating tumour cells
4. Identification of resistance clones in primary tumors
5. Very low heteroplasmy detection in mtDNA
6. Virology – resistance mutation monitoring

COLD-PCR types

I. **Full** COLD-PCR

- Amplifies by preferentially melting **heteroduplexes**
- Amplifies **all** mutations

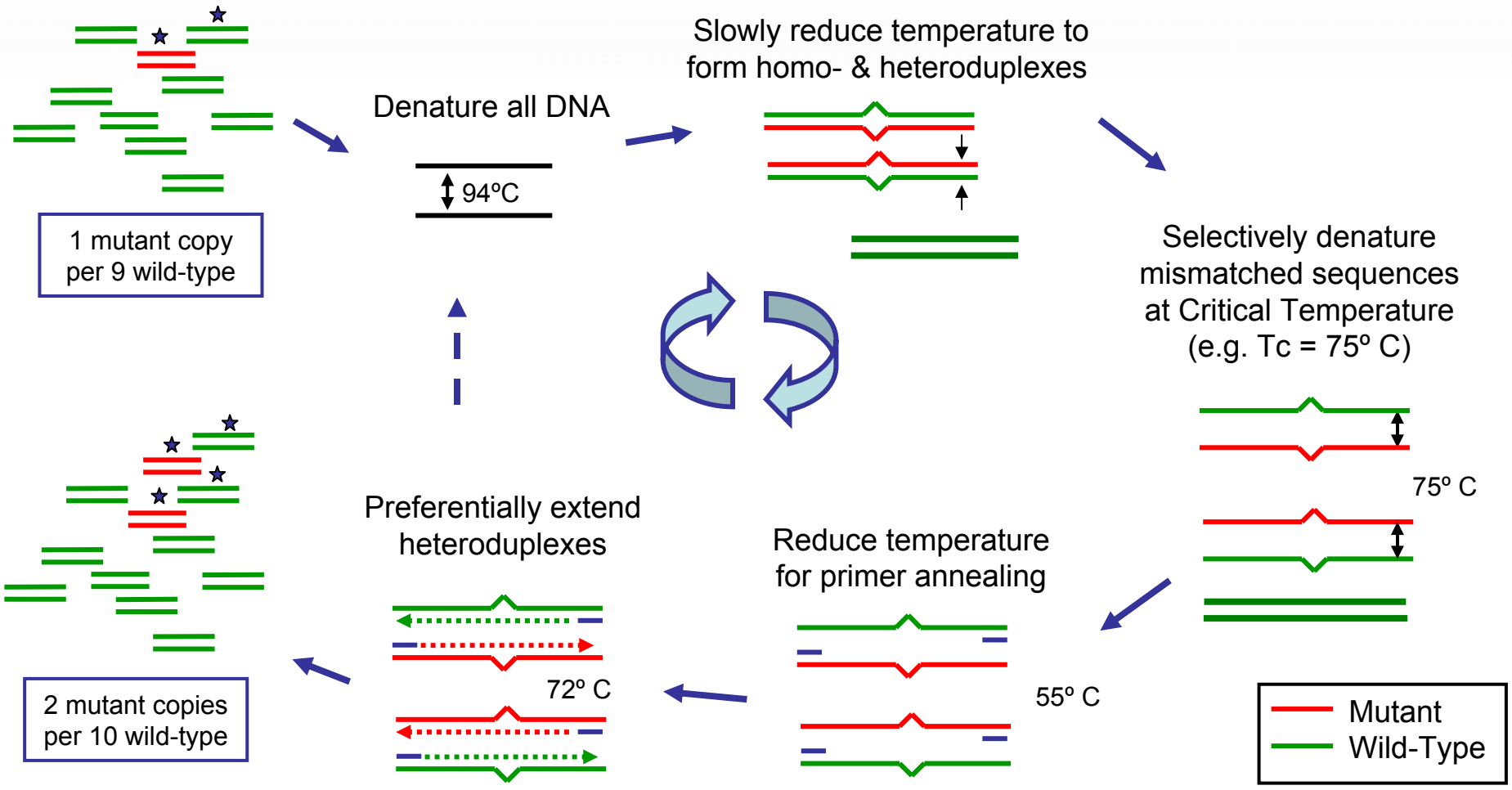
II. **Fast** COLD-PCR

- Amplifies by preferentially melting **homoduplexes** with lower Tm than wild type
- Amplifies **only** mutations that lower Tm (G/C ⟶ A/T)

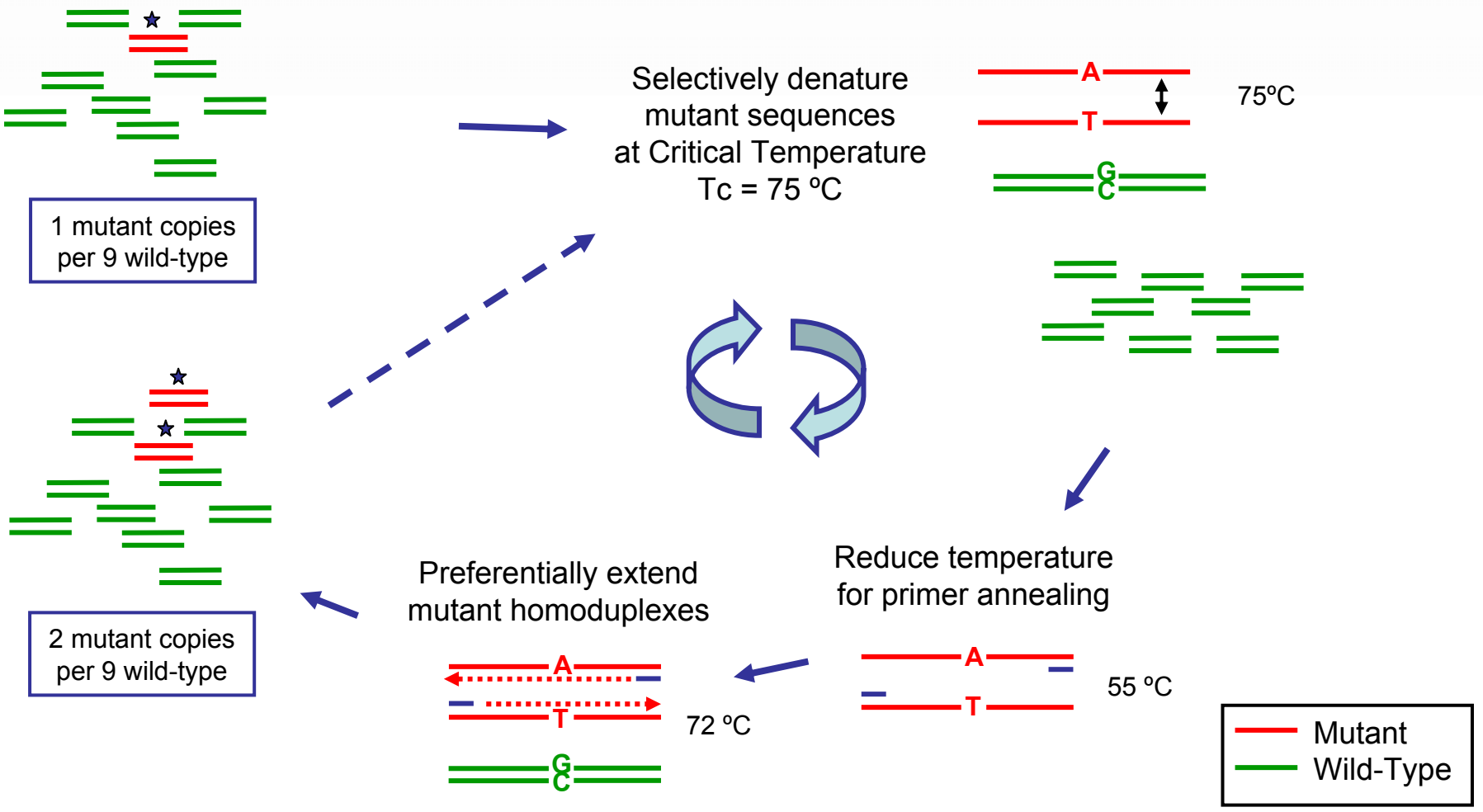
III. **Ice-**COLD-PCR

- As Full COLD-PCR above but utilises a third, reference sequence (RS) oligo
- RS promotes the efficiency of heteroduplex formation when % mutant is very low

“Full” COLD PCR



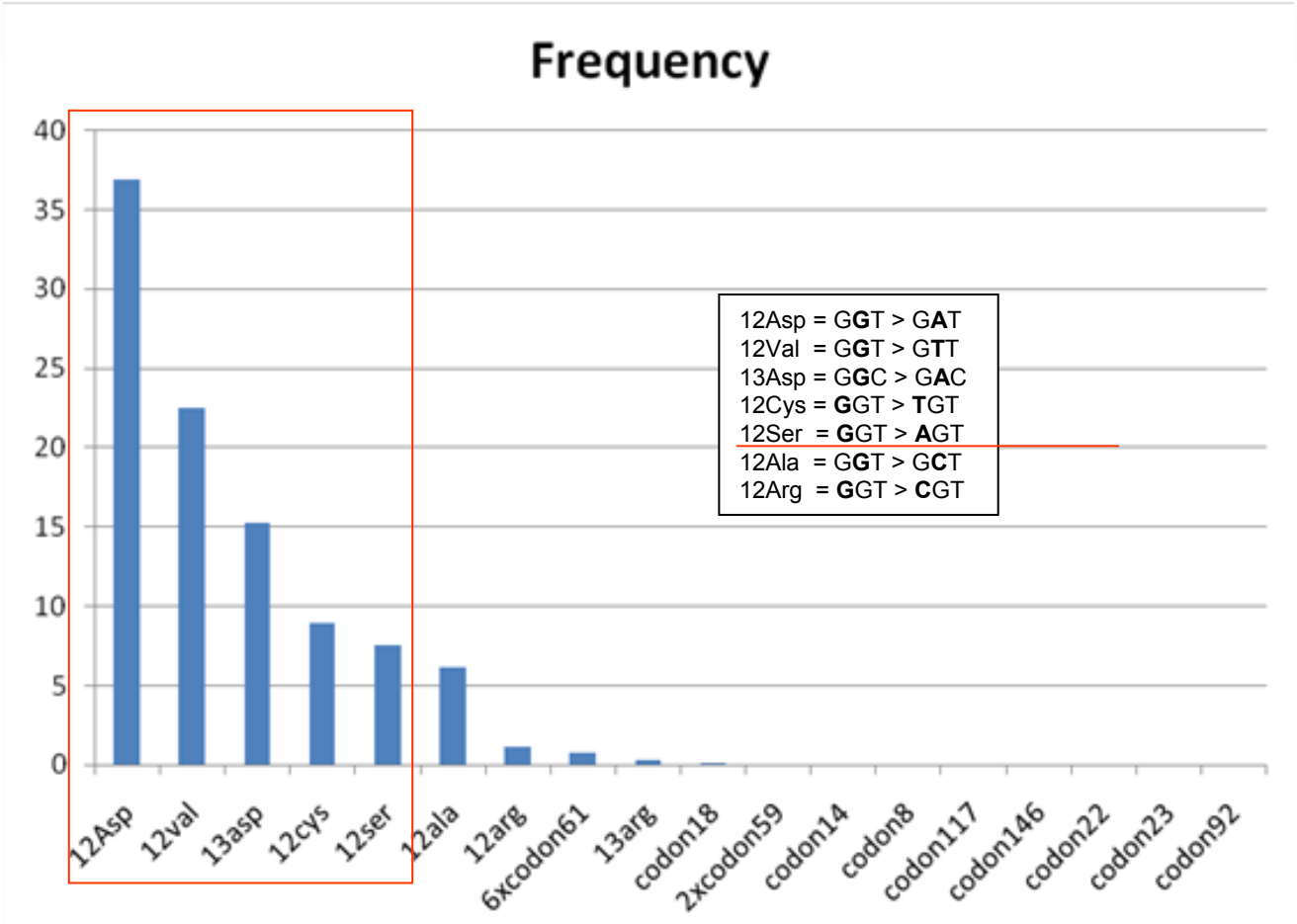
“Fast” COLD PCR



Theoretical limitations of COLD-PCR

- Amplicon size
  - As fragment size increases Δ(Tm-Tc) decreases
- Amplifies polymerase-induced errors
  - Preferentially use proof-reader
- Requires optimisation for each specific amplicon
- Well-to-well deviation from programmed temperature in many thermocyclers
  - Amplification is highly dependent on correct Tc
  - Addressed by modified protocol

K-RAS as model for Fast COLD-PCR



Fast COLD-PCR characterization

Fold enrichment			
Well	FCPCR 78.4°C	FCPCR 78.9°C	**Optimised FCPCR**
A1	No seq	No seq	10
B1	No seq	20	20
C1	No seq	20	50
D1	No seq	20	20
E1	No seq	30	30
F1	40	30	50
G1	30	20	50
H1	20	10	20
A3	No seq	[Not performed]	30
B3	No seq		30
C3	No seq		30
D3	No seq		No seq
E3	No seq		30
F3	20		30
G3	30		20
H3	20		20
A5	No seq	No seq	10
B5	No seq	No seq	30
C5	No seq	No seq	30
D5	No seq	No seq	30
E5	No seq	20	50
F5	No seq	40	50
G5	20	20	30
H5	No seq	20	20

K-RAS G12S ↓Tm mutation Tc = 78.9°C

FCPCR was run at and Tc and Tc – 0.5°C in the same thermocycler well positions

- 29% of wells produced sequence-able PCR products with FCPCR at Tc – 0.5°C
  - Average enrichment was **25.7-fold**
- 73% of wells produced sequence-able PCR products with FCPCR Tc
  - Average enrichment was **22.7-fold**

Optimised FCPCR was run in the same thermocycler well positions

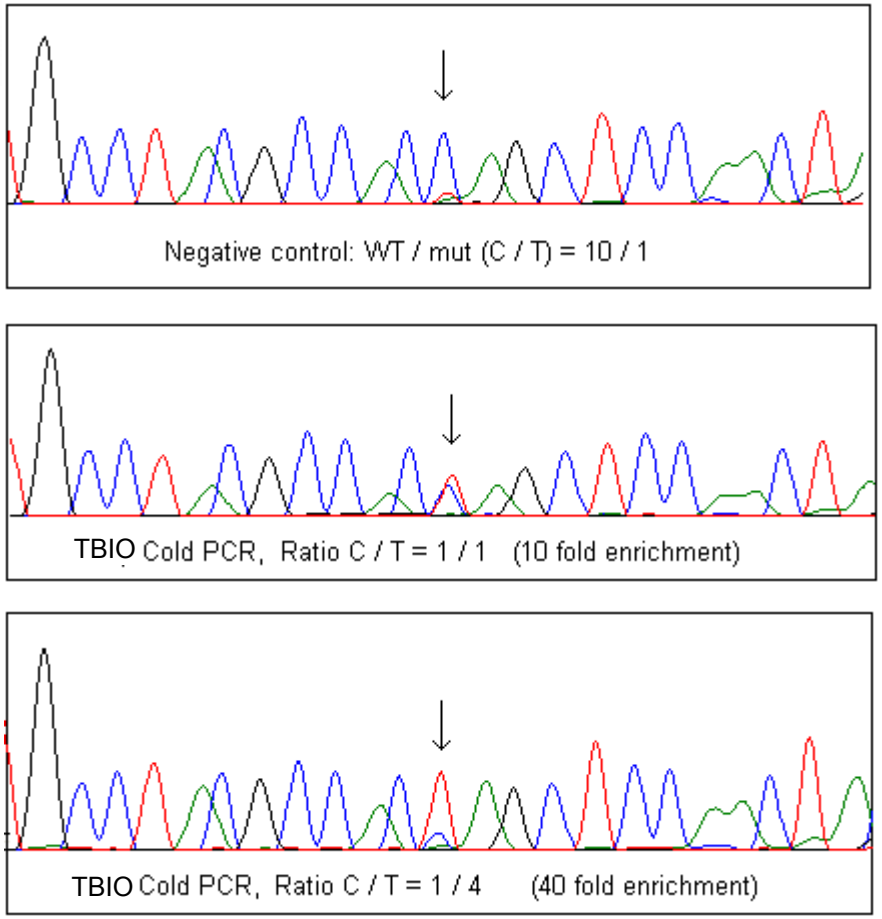
- 96% of wells produced sequence-able PCR products with optimised FCPCR
  - Average enrichment was **30-fold**

Conclusions:

- Well-to-well deviation from the programmed temperature is significant
- Applying the optimised protocol results in a more robust enrichment in the wells of the thermocycler

Modified Fast COLD-PCR sequencing results

- Samples containing a mixture of 10% G12S mutant and 90% wild-type K-RAS DNA were amplified by PCR or modified Fast Cold PCR and then sequenced.
- Tracings show change in ratio of C:T from 10:1 to 1:1 and to 1:4
- Represents enrichment of 10- and 40-fold
- Remaining sequence is unaffected



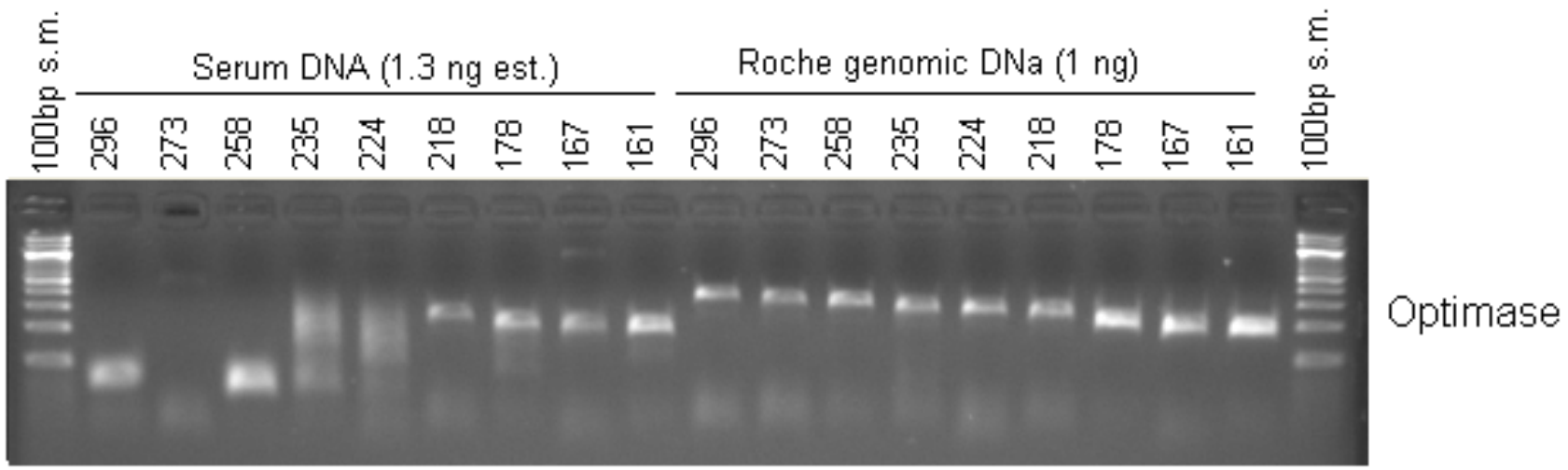
Cancer gene mutation detection in blood serum

- Tumour-derived, cell-free circulating DNA can be detected in plasma and serum of patients with various solid tumours
- To date, detection rates of tumour-associated mutations in plasma have been variable
- Improved analytical methods could offer the ability to screen for cancer and monitor its progression and response to therapy

Critical sensitivity issues:

1. Low total amount of circulating DNA
2. Low proportion of mutant to non-mutant DNA

Methods: DNA amplification from Serum



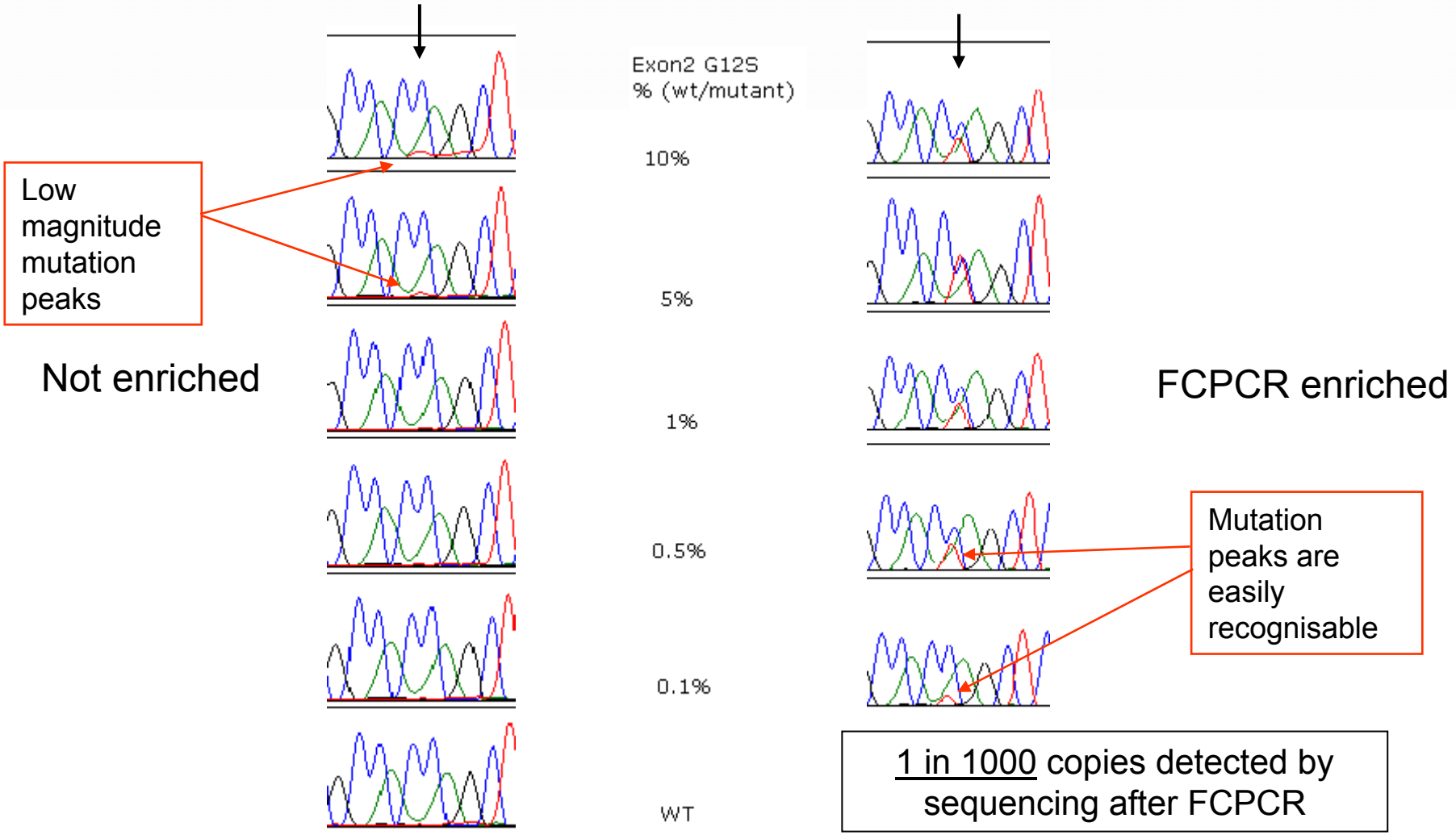
Different size PCR amplifications from healthy donor serum *vs*. genomic DNA using high-fidelity Optimase® polymerase to reduce replication error. (PCR with 15 touch down and 39 standard cycles.)

Amplification of segments greater than 218 bp is not possible from highly fragmented serum DNA, but is possible from genomic DNA.

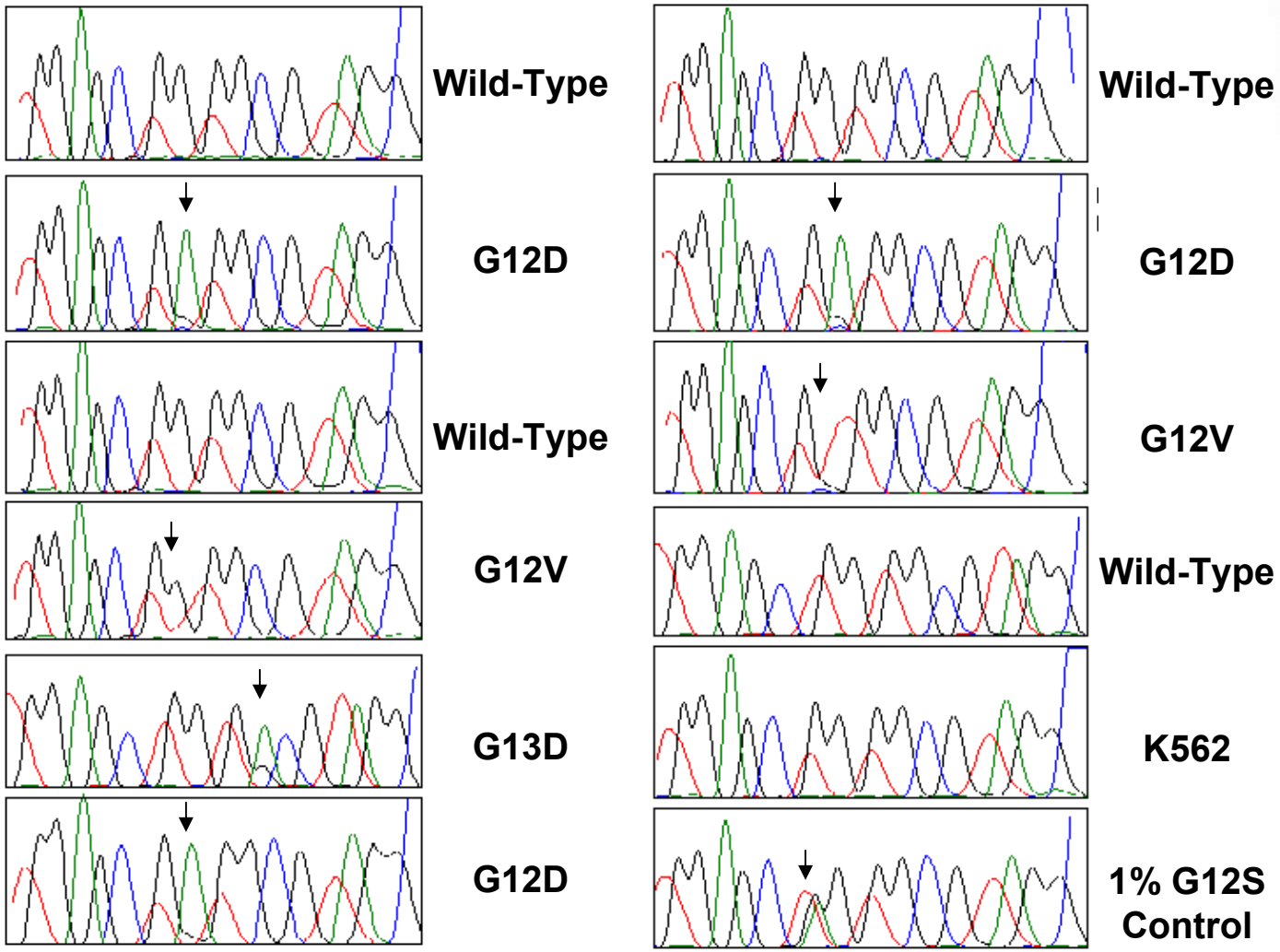
Fast COLD-PCR amplification LOD study

- 10,000 copies of plasmid DNA at varying mutant/wild-type ratios
- K-RAS was amplified using standard PCR on the 218-bp fragment
- Subsequent “enrichment” FCPCR used nested primers on the 218-bp product to create a second round 161-bp PCR product
- PCR products were sequenced and also digested with SURVEYOR Nuclease and separated on a WAVE System

K-RAS Sequencing - / + FCPCR enrichment

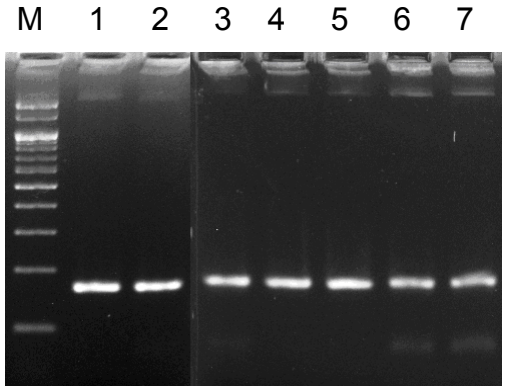


Fast COLD-PCR of K-RAS in Matched Tumour-Plasma Samples



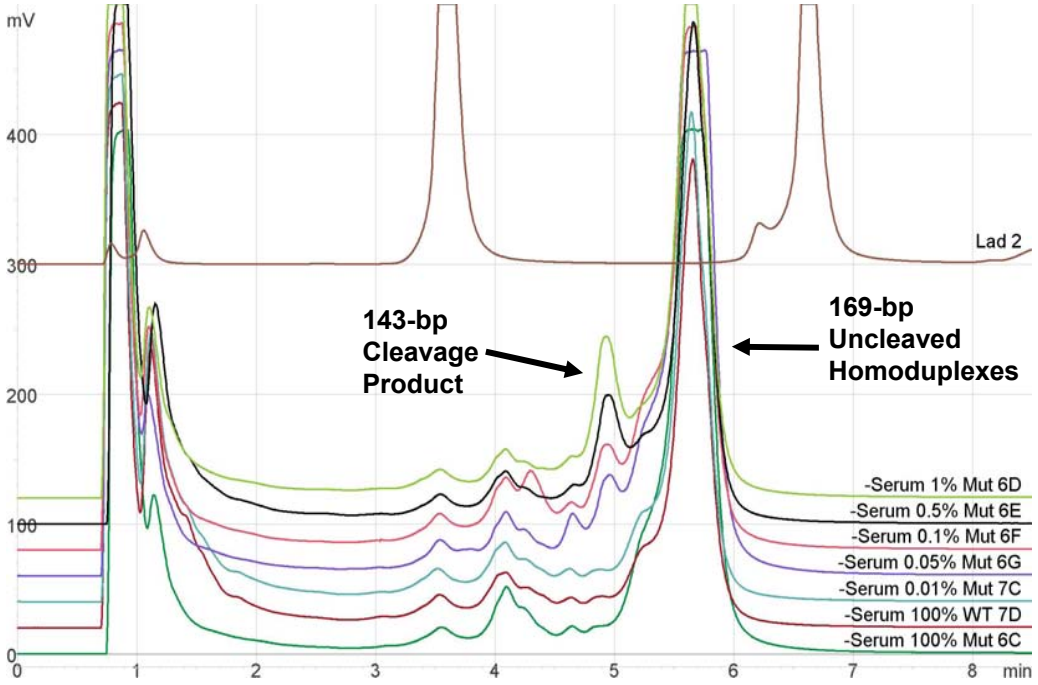
EGFR exon 20 T790 wild-type : mutant C>T mixtures enriched by Fast COLD-PCR

A

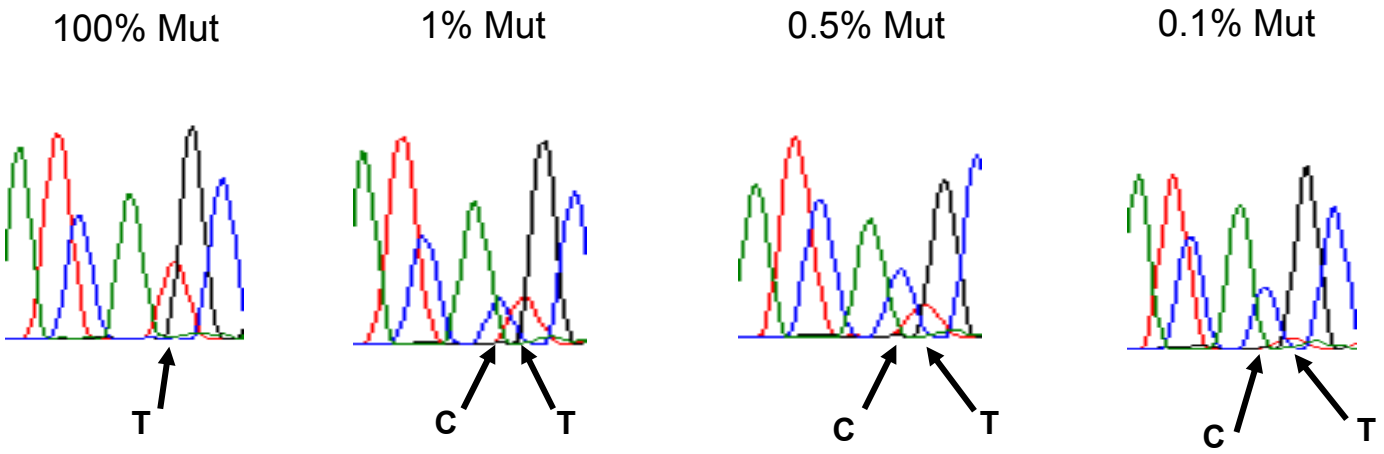


Lane	Sample
1	100% Mut
2	1% Mut
3	0.5% Mut
4	0.1% Mut
5	0.05% Mut
6	0.01% Mut
7	100% WT
M	100bp ladder

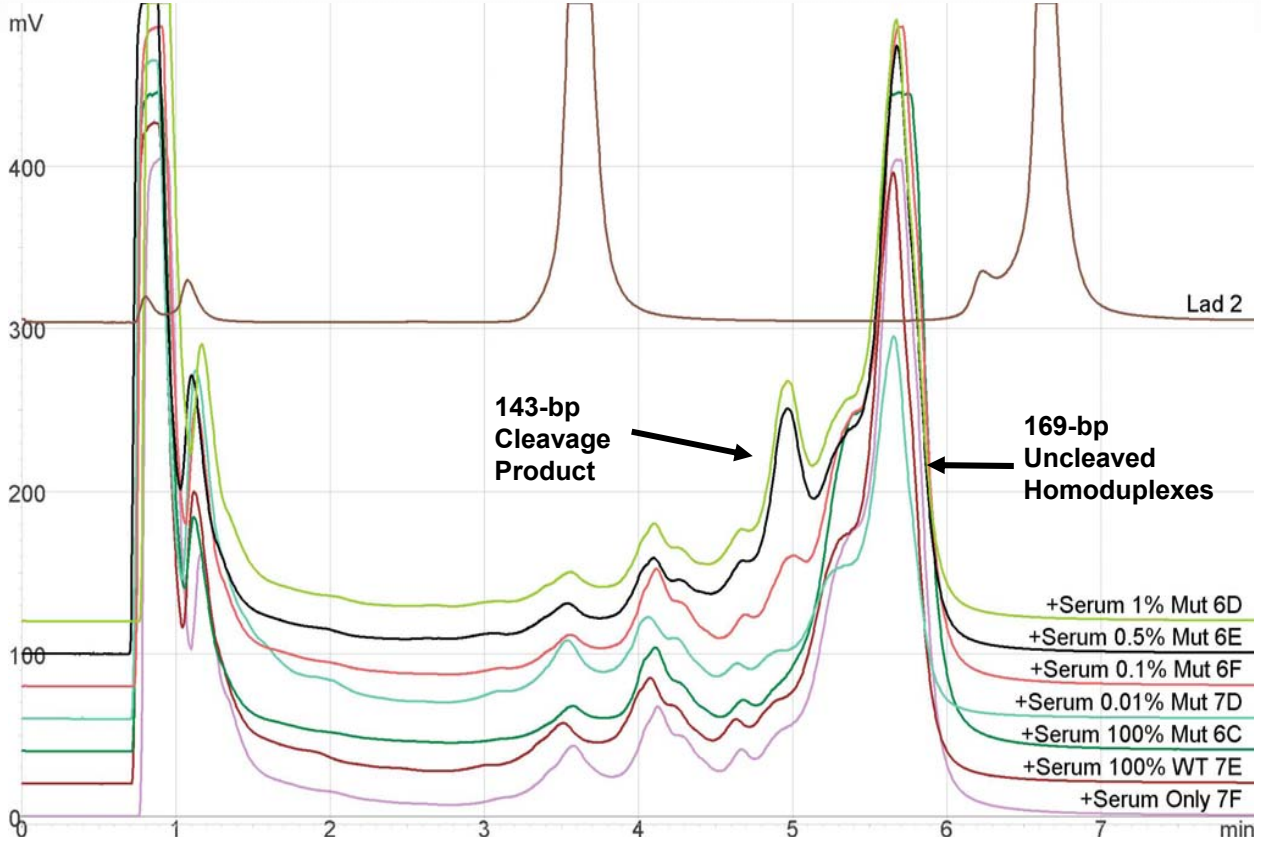
B



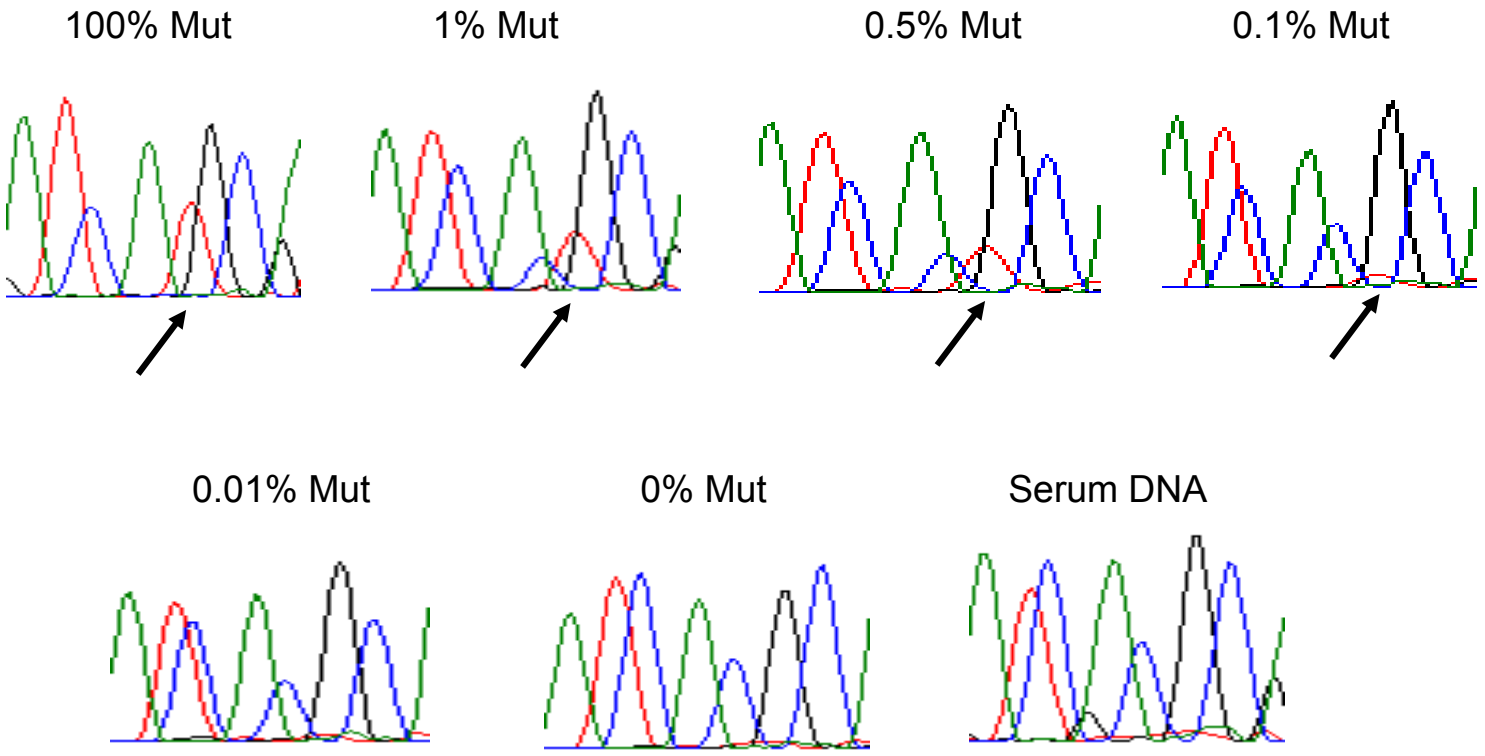
EGFR exon 20 T790 wild-type : mutant plasmid mixtures enriched by Fast COLD-PCR



EGFR exon 20 T790 wild-type : mutant mixtures in serum enriched by Fast COLD-PCR

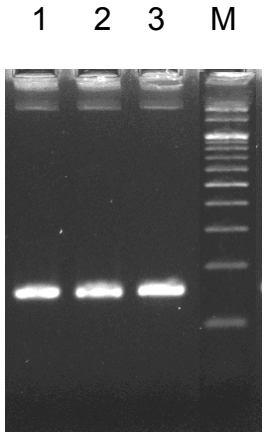


EGFR exon 20 T790 wild-type : mutant plasmid mixtures in serum enriched by Fast COLD-PCR



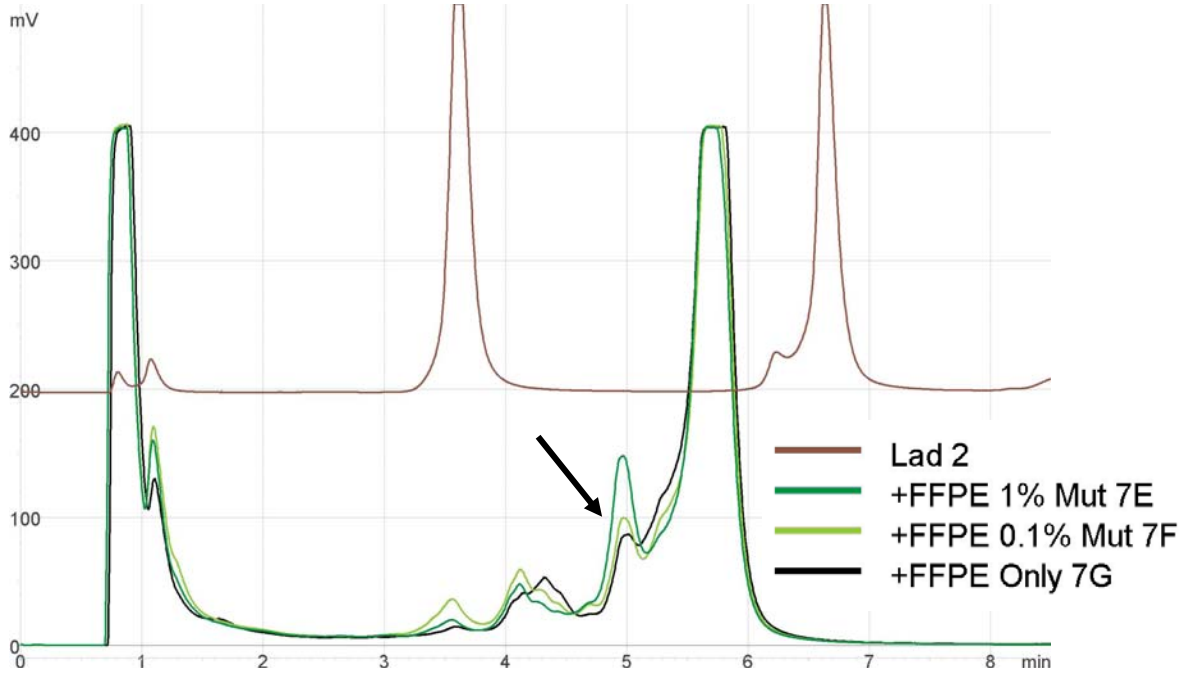
EGFR exon 20 T790 wild-type : mutant mixtures in FFPE DNA enriched by Fast COLD-PCR

A

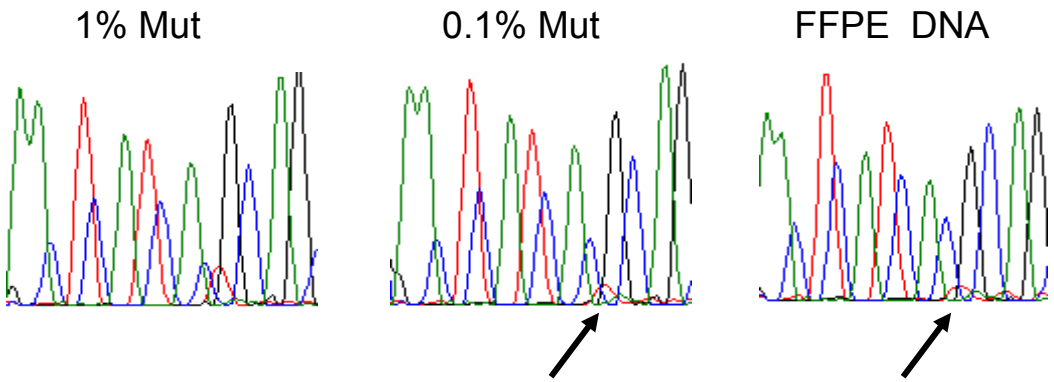


Lane	Sample
1	1% Mut+FFPE DNA
2	0.1% Mut+FFPE DNA
3	FFPE DNA Only
M	100bp ladder

B



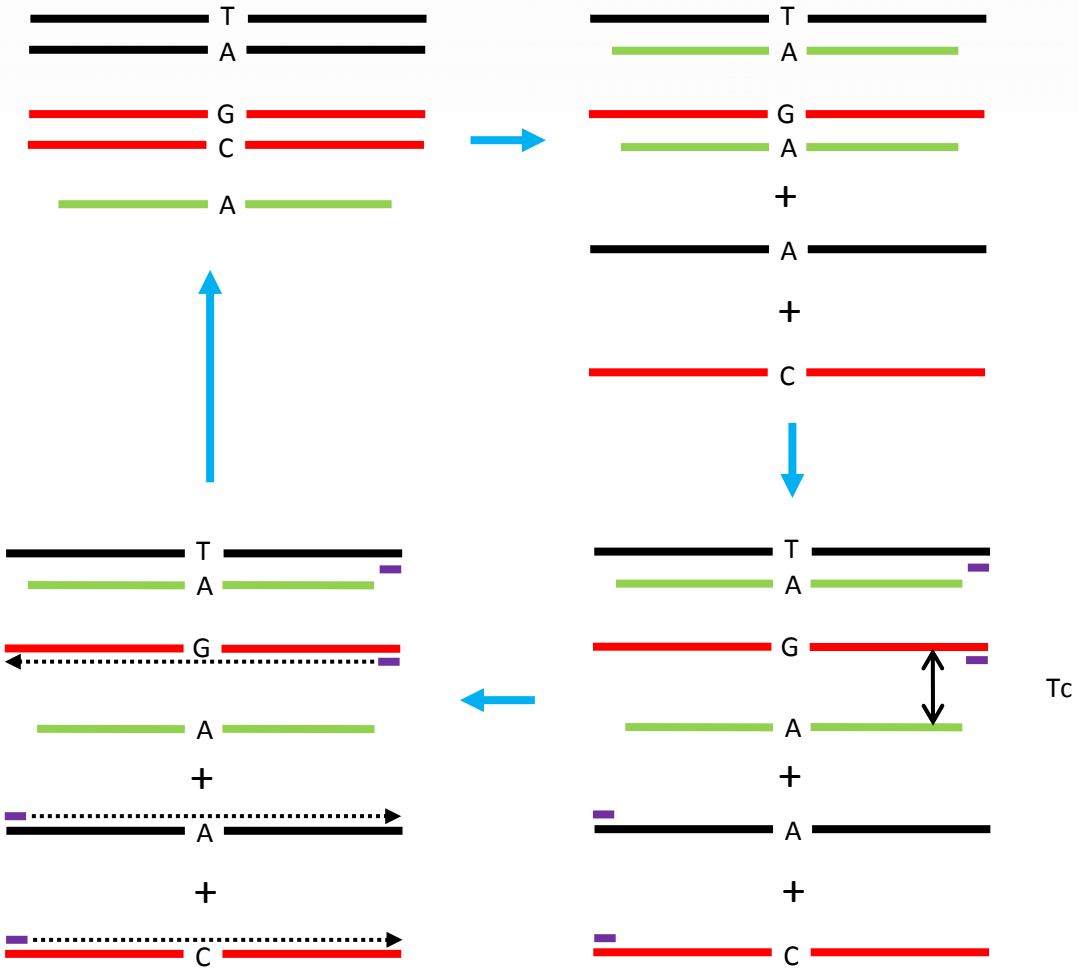
EGFR exon 20 T790 wild-type : mutant plasmid mixtures in FFPE DNA enriched by Fast COLD-PCR



Ice-COLD PCR

- Improvement of mutation enrichment in Tm-neutral and Tm-raising mutations
  - Making “Full” equivalent to “Fast”
- Single assay to allow very high sensitivity mutation detection at all locations within an amplicon

Ice-COLD PCR



Wild-Type strand

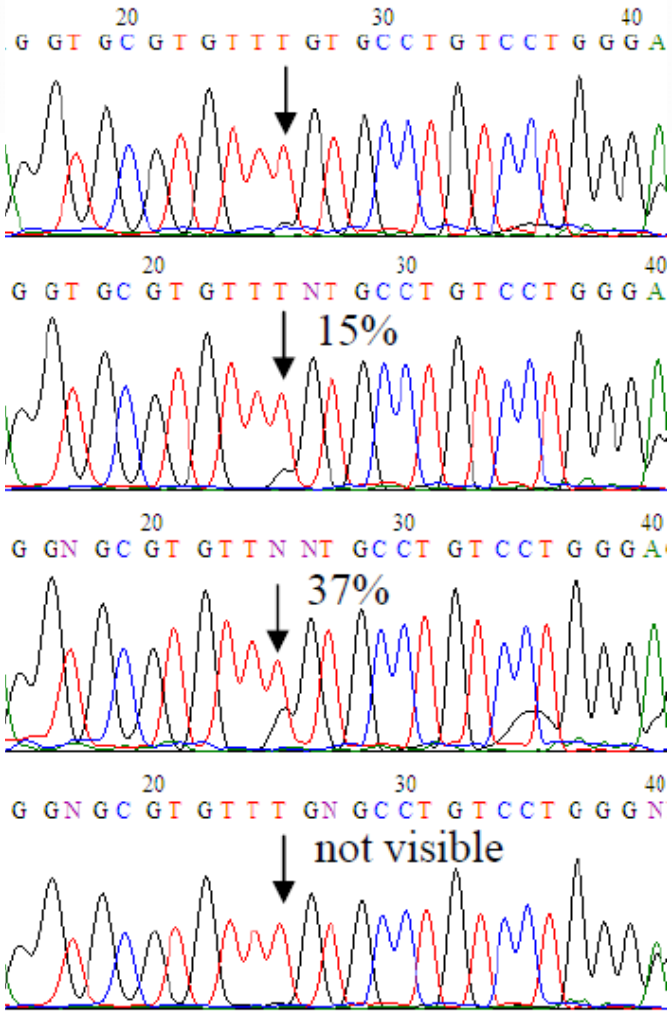
Mutant strand

RS Oligo

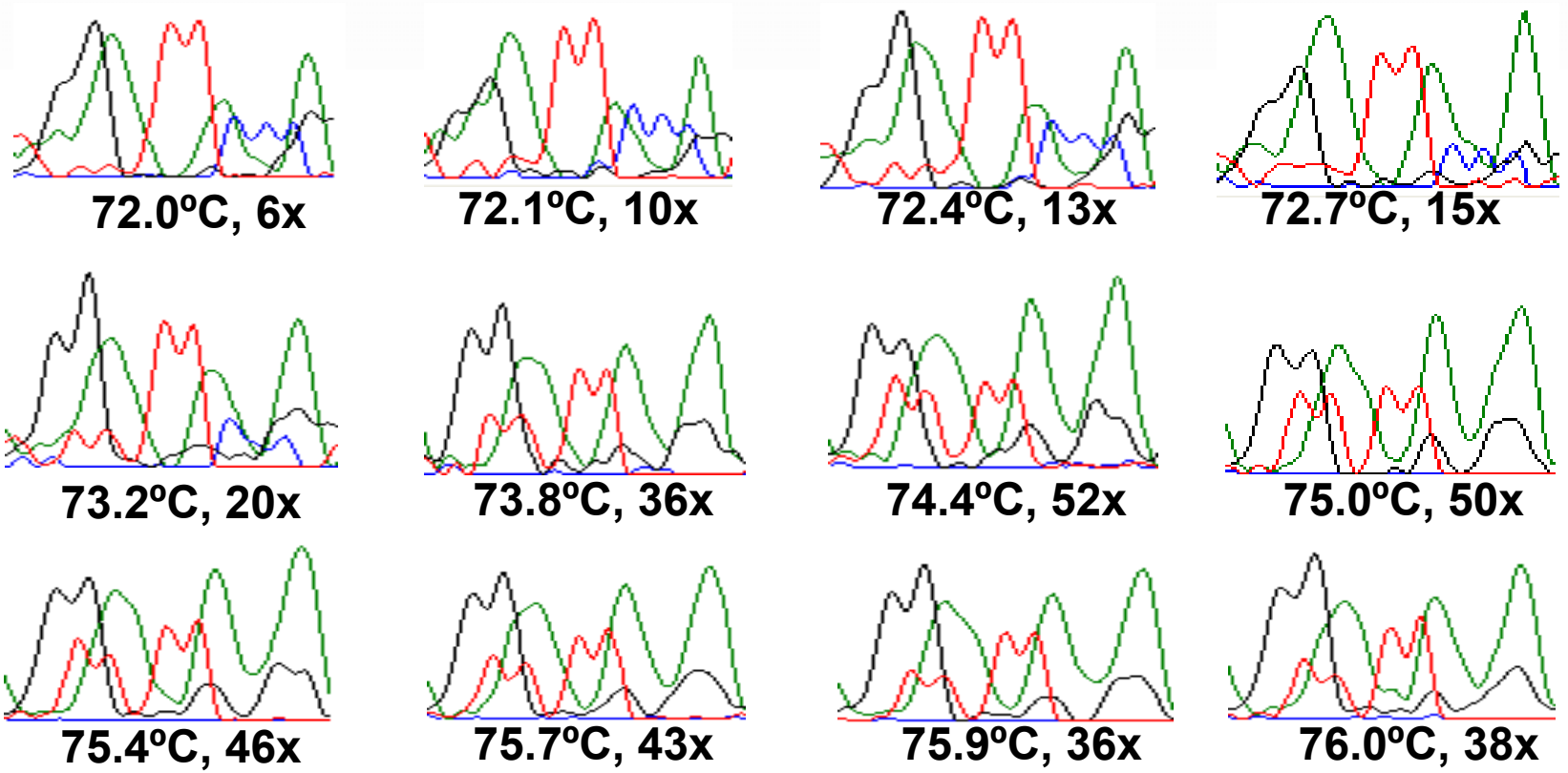
Primers

Tm-increasing T>G p53 Exon 8 mutation (3%)

- Standard PCR
- Full COLD-PCR
- Ice-COLD-PCR
- Fast COLD-PCR



EGFR19ΔE746 GGAATT>GTT Critical Temperature



Acknowledgements

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  - Mike Makrigiorgos
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