Deep Sequencing for DNA Diagnostics Using Next-Gen Sequencing and COLD-PCR

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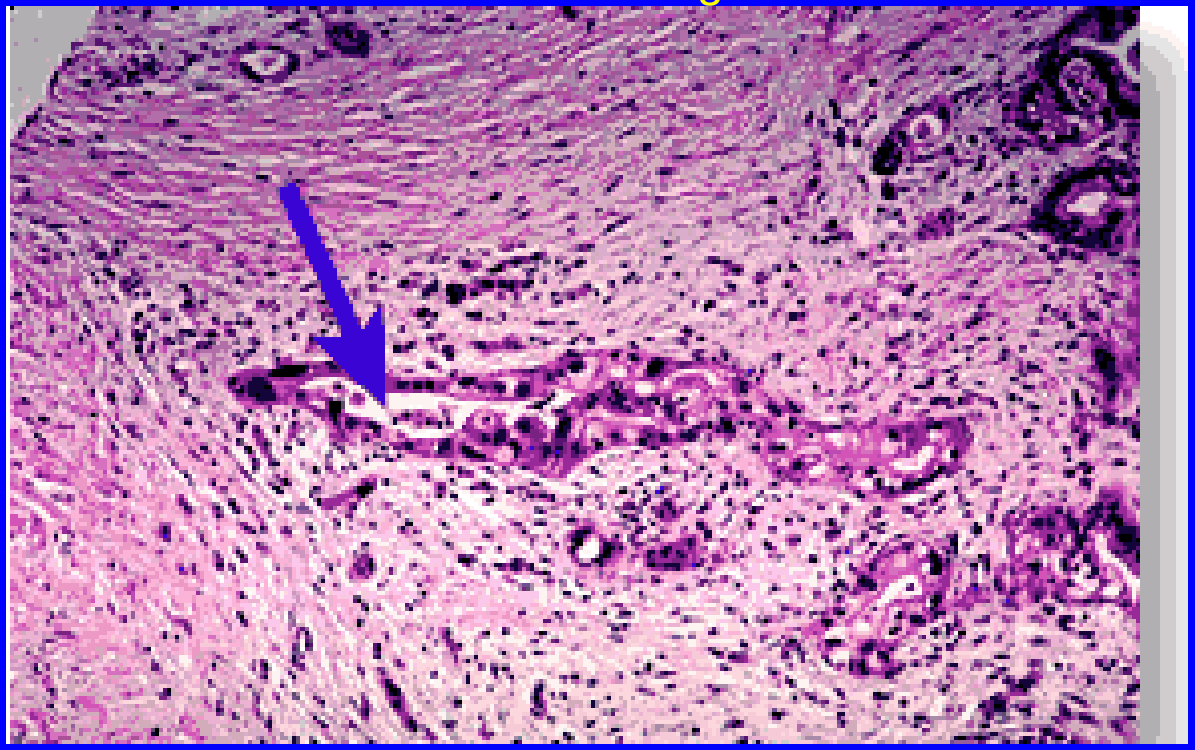
DNA mutation detection has numerous applications in Cancer Diagnostics and Personalized Medicine

LOW-LEVEL MUTATIONS ARE ALWAYS A POTENTIAL PROBLEM IN CLINICAL SAMPLES

LOW-LEVEL MUTATIONS ARE ENCOUNTERED:

In clinical cancer samples with frequent stromal contamination

Pancreatic infiltrating adeno-CA



- **LUNG CA (e.g. EGFR mutations); hematological CA (MDS, AML)**
- **SAMPLES OBTAINED from SURGICAL MARGINS**
- **BODILY FLUIDS (DNA biomarkers from plasma, CTCs sputum, feces)**
- **NON-MICRODISSECTED SAMPLES**
- **GENETIC MOSAICISM**

DO LOW-LEVEL MUTATIONS IN SOLID TUMORS MATTER?

The answer is case dependent

for example

- **Low-abundance TET2 mutant clones in chronic myelo-monocytic leukemia confer no prognostic value**
  (Smith et al, Blood 2010)

but..

- **Low-abundance Kras mutations in colorectal CA enhance the prediction of anti-EGFR MoAb resistance**
  (Molinari et al, Clin Cancer Res 2011)

DO LOW-LEVEL MUTATIONS IN SOLID TUMORS MATTER?

Traces of mutations may cause drug resistance..

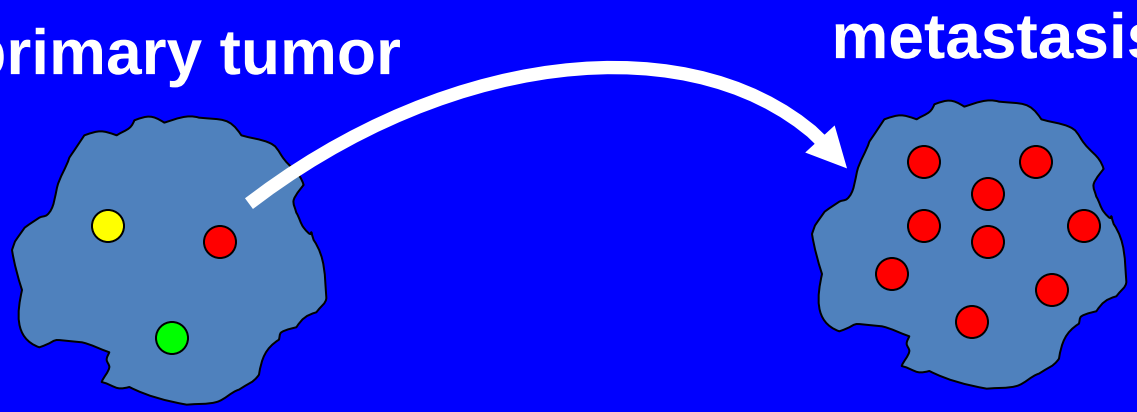
for example

- **traces of T790M mutation in EGFR cause resistance to small molecule inhibitors (erlotinib, imatinib) in lung CA (Kobayashi et al, NEJM 2005)**

- **traces of BCR-ABL mutations cause resistance to Gleevec in CML (Sawyers et al, Cancer Cell 2002)**

DO LOW-LEVEL MUTATIONS IN SOLID TUMORS MATTER?

Low-level mutations in primary CA may drive metastasis..



e.g. mutations in MORC1 AND KIF1C at the 1-4% level in primary breast CA
can become prevalent mutations in brain metastasis
(Shah et al, Nature 2010)

certain low-level mutations in primary tumor
may define the propensity of tumors to metastasize

LOW-LEVEL DNA VARIANTS ARE ALSO ENCOUNTERED:

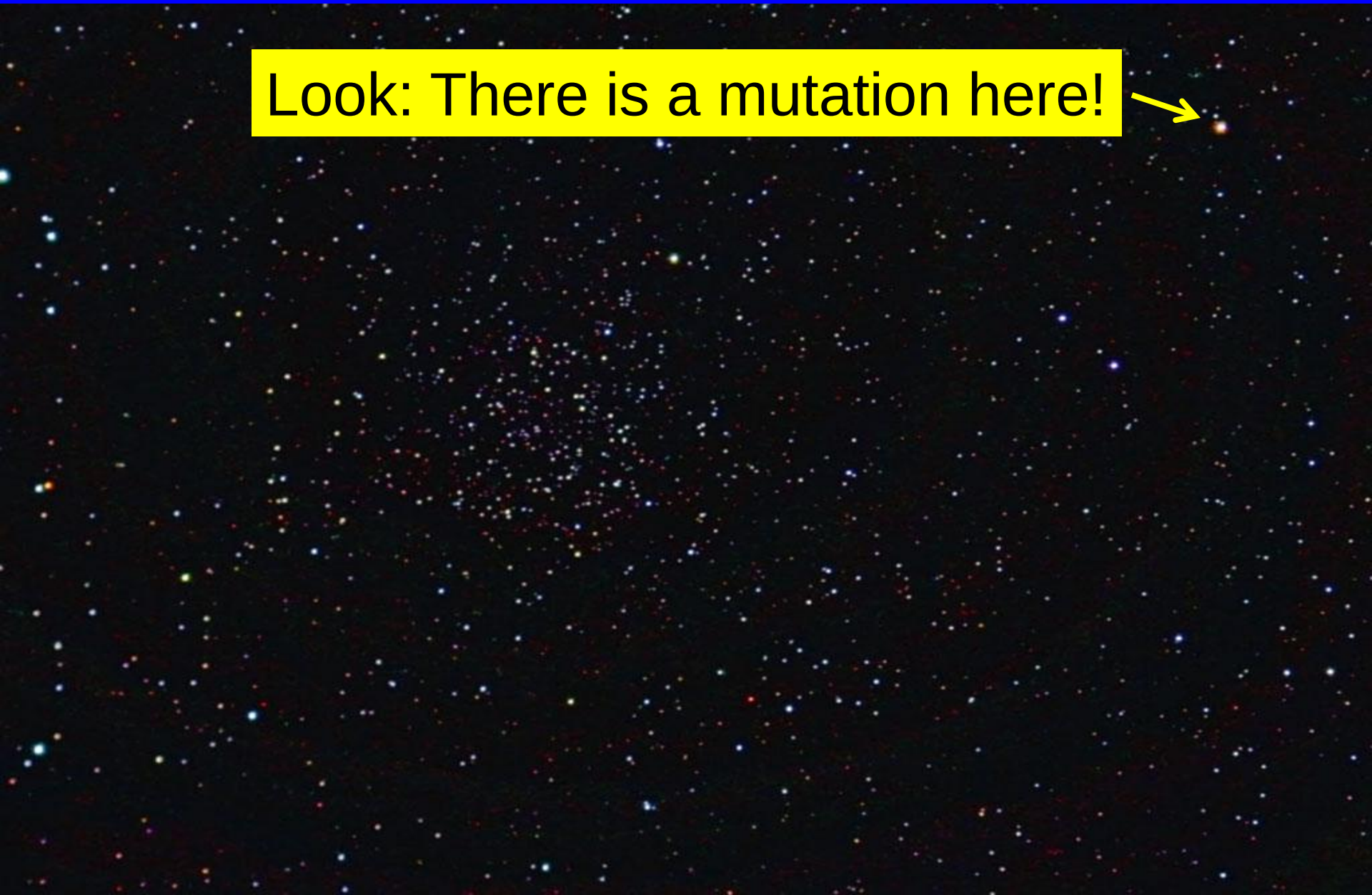
- In pre-natal diagnosis

(i.e. detection of small amounts fetal circulating-DNA in maternal blood)

- In infectious diseases

(i.e. early detection of resistant strains emerging in a population of drug-responsive strains)

LOW-LEVEL MUTATIONS ARE DIFFICULT TO IDENTIFY ESPECIALLY WHEN THEIR POSITION IS UNKNOWN



LOW-LEVEL MUTATIONS ARE DIFFICULT TO IDENTIFY ESPECIALLY WHEN THEIR POSITION IS UNKNOWN

Next Task:
Find All Other Mutations in the Universe
- Without a Clue-

OUR SOLUTION

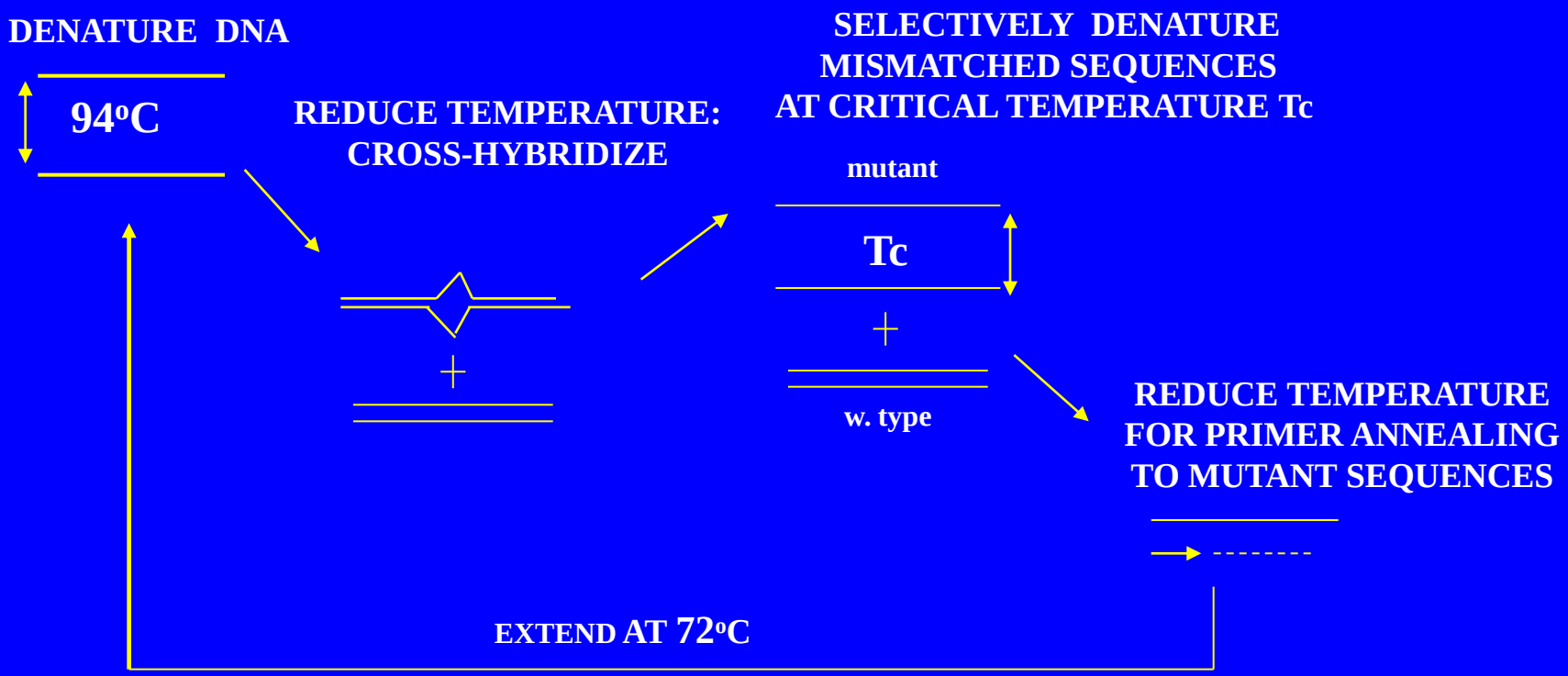
MAGNIFICATION OF UNKNOWN MUTATIONS via COLD-PCR

COLD-PCR (Nature Medicine, May 2008)
ice-COLD-PCR (Nucleic Acid Res, January 2011)
Temperature-Tolerant COLD-PCR (Clinical Chem 2012)

PRINCIPLE OF COLD PCR

Co-amplification at Lower Denaturation temperature

Li et al, Nature Medicine, May 2008



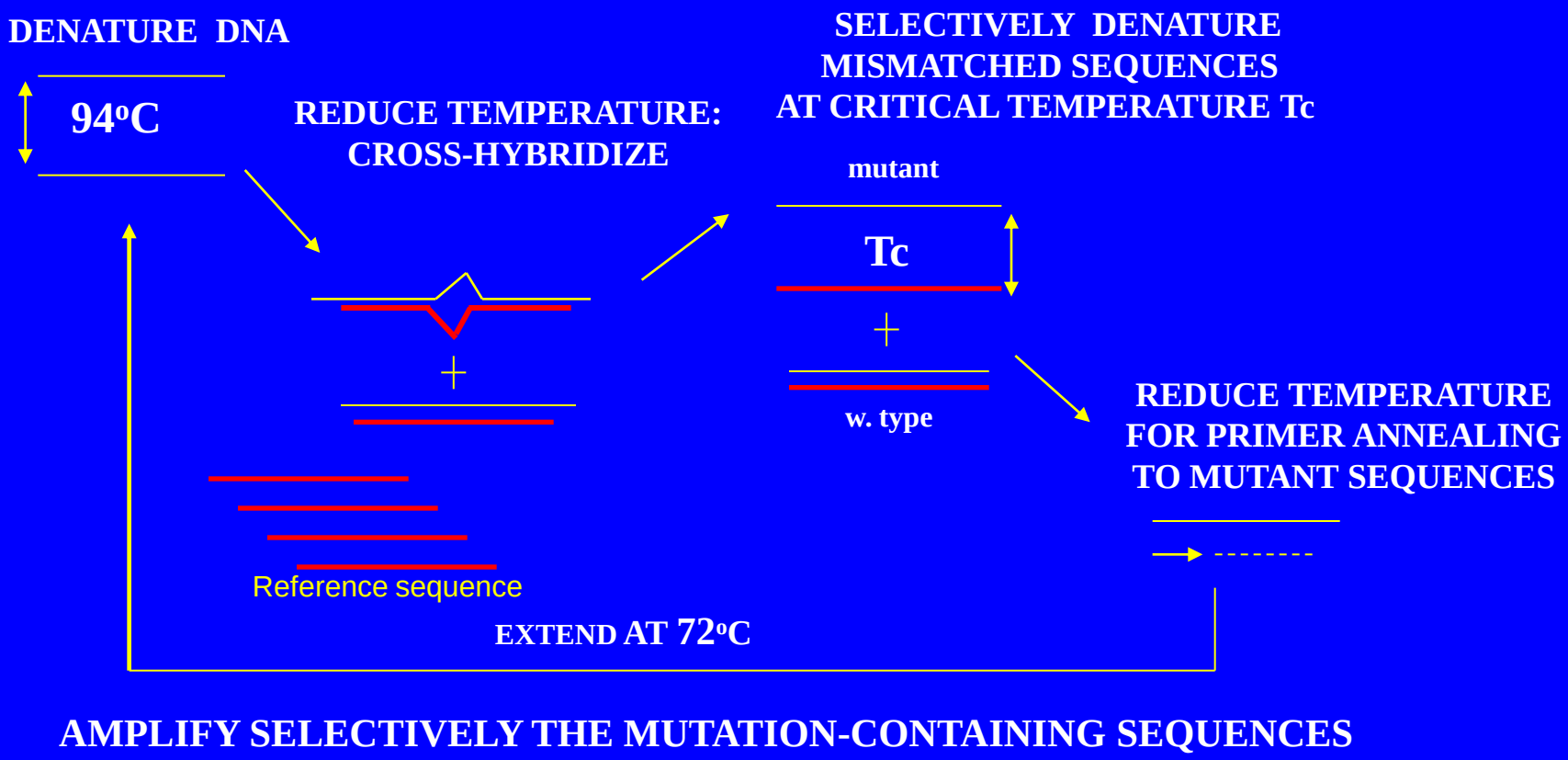
AMPLIFY SELECTIVELY THE MUTATION-CONTAINING SEQUENCES

Mutation enrichment occurs at ALL positions on the sequence

ice-COLD PCR

(improved and complete-enrichment COLD-PCR)

Milbury et al, Nucleic Acid Res, January 2011



Reference sequence enables improved hybridization kinetics

EXAMPLES OF LOW-LEVEL MUTATIONS IN TUMOR CLINICAL SAMPLES, PREVIOUSLY 'INVISIBLE' VIA SANGER SEQUENCING, THAT BECOME DETECTABLE VIA COLD PCR

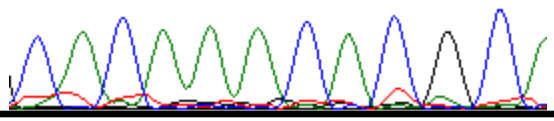
Sanger-di-deoxy-sequencing of CLINICAL tumor samples for p53 exon 8 mutations

REGULAR PCR
(94ºC denaturation)

↓

Lung tumor 64

reverse seq. NO mutation visible

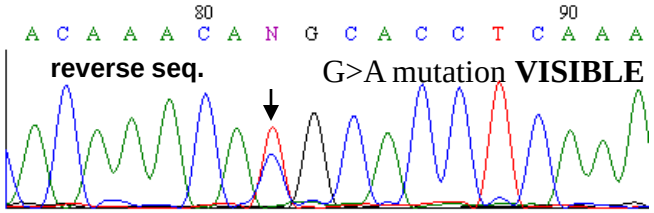


→

COLD PCR
(denaturation at Tc=86.5ºC)

↓

Lung tumor 64



EXAMPLES OF LOW-LEVEL MUTATIONS IN PLASMA AND FFPE SAMPLES, PREVIOUSLY 'INVISIBLE' VIA SANGER SEQUENCING, THAT NOW BECOME DETECTABLE VIA COLD PCR

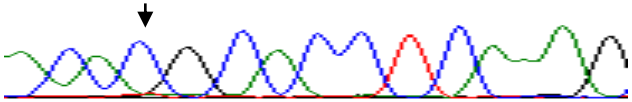
Detection of p53 mutations in **Plasma** of a radiation therapy patient

REGULAR PCR

↓ 87 bp fragment

Patient 6-87bp-reverse

Mutation is NOT visible



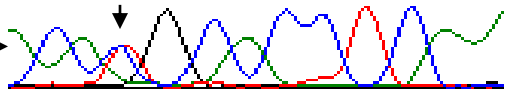
→

COLD PCR

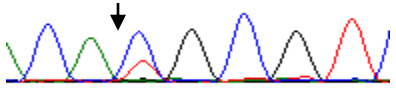
↓

Patient 6-87bp-reverse

G>A mutation **VISIBLE**



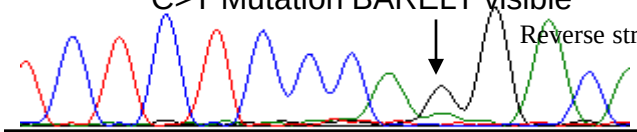
INDEPENDENT CONFIRMATION
(REMS/RFLP ASSAY)



Detection of p53 mutations in clinical **FFPE** samples from lung NSCLC patients

C>T Mutation BARELY visible

Reverse strand

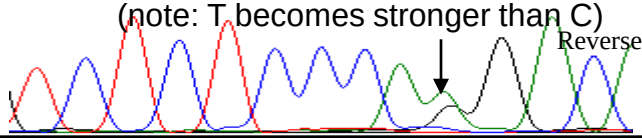


167 bp fragment

→

C>T Mutation CLEARLY visible
(note: T becomes stronger than C)

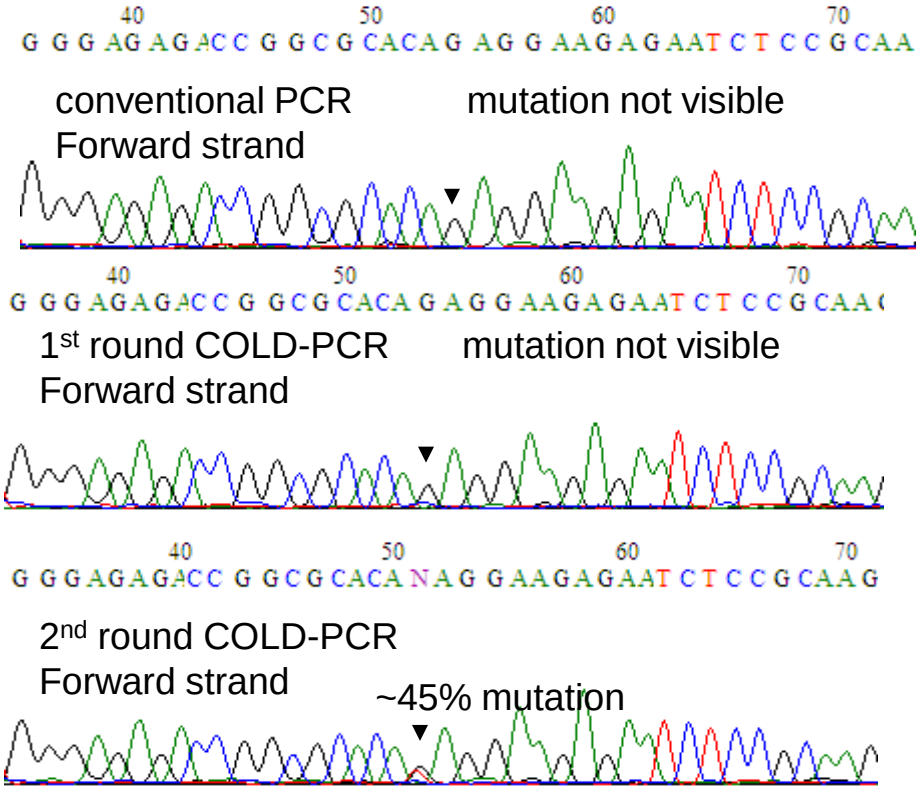
Reverse strand



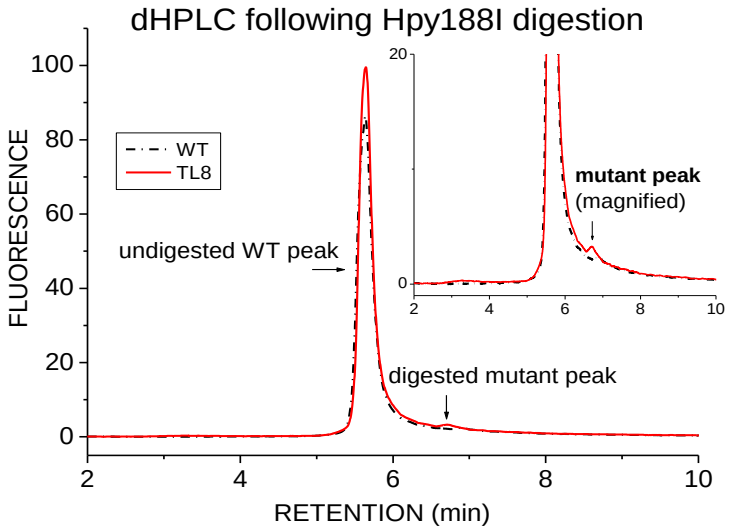
48 LUNG ADENOCARCINOMA SAMPLES SCREENED via 2-round COLD-PCR

TL8 (Glu 285 STOP; GAG > TAG)

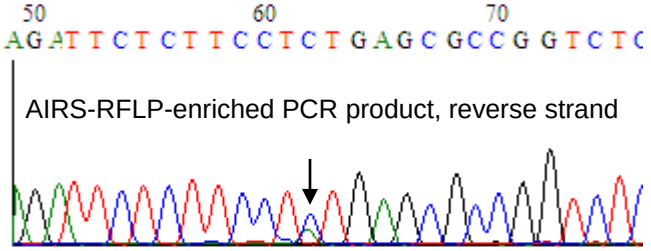
A **COLD-PCR**



B **independent confirmation (AIRS-RFLP)**



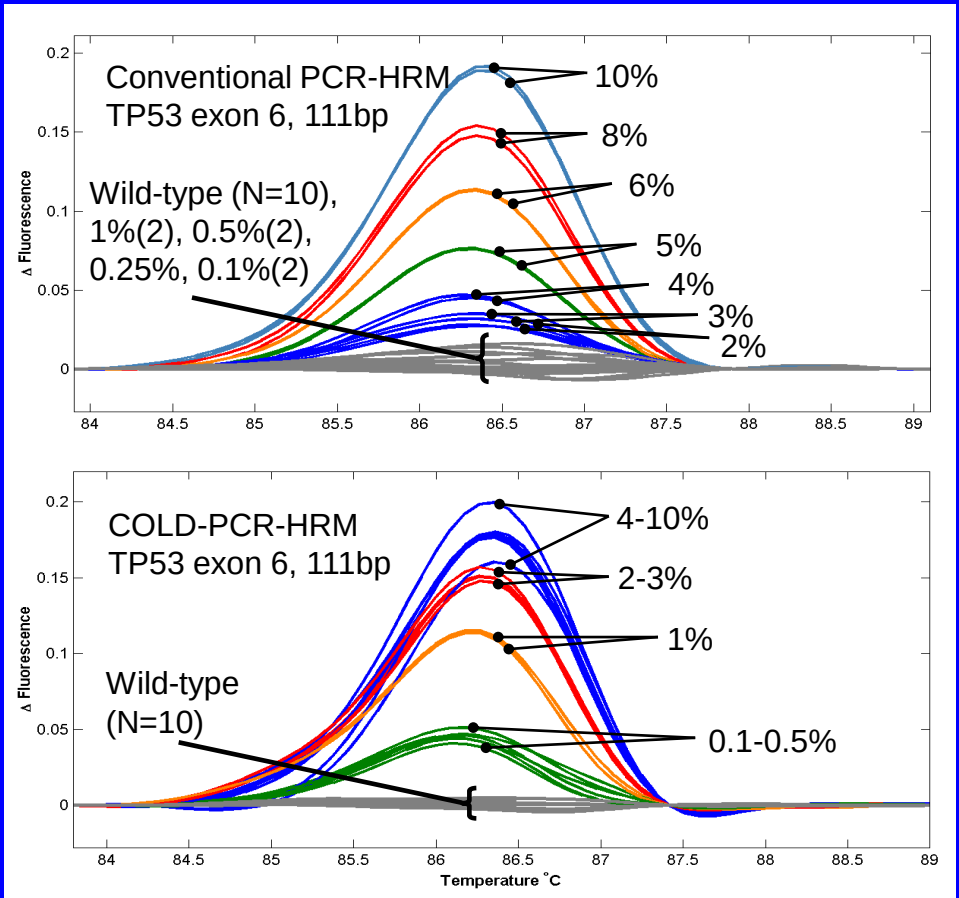
C



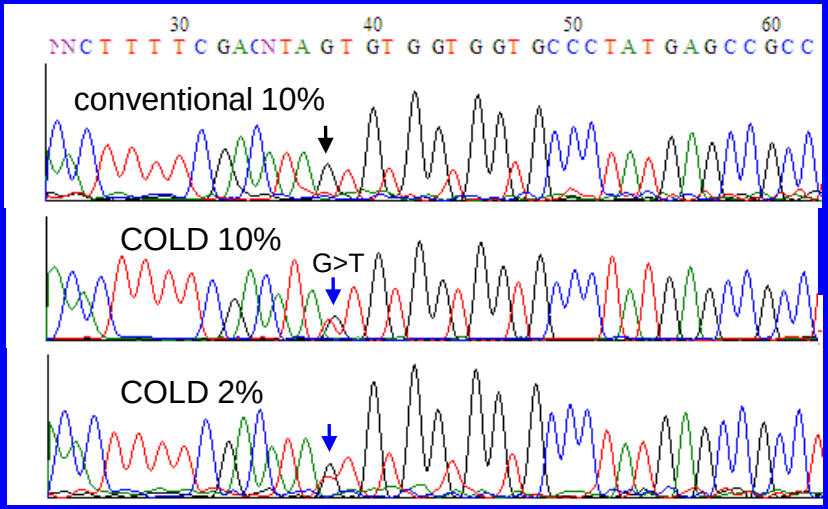
27 mutations discovered, including 8 mutations at the 1-17% abundance and 3 below 1% abundance

IMPROVEMENT OF HIGH RESOLUTION MELTING (HRM) BY REPLACING PCR WITH COLD-PCR

A



B



COLD-PCR RESULTS TO A 10-20-FOLD INCREASE IN HRM SENSITIVITY AND THE ABILITY TO SEQUENCE THE LOW-LEVEL VARIANTS

Milbury et al, Clinical Chemistry, 2009; 55:2130-2143

recent COLD-PCR reports by other groups

mostly using HRM or sequencing

- Boisselier et al, Human Mutation 2010: COLD-PCR-HRM for IDH1 mutations in brain tumors (application: testing of **TUMOR MARGINS**)

- Kristensen LS, et al, Human Mutation 2010: COLD-PCR-HRM for Kras mutations (application: **CRC TREATMENT ASSESSMENT**)

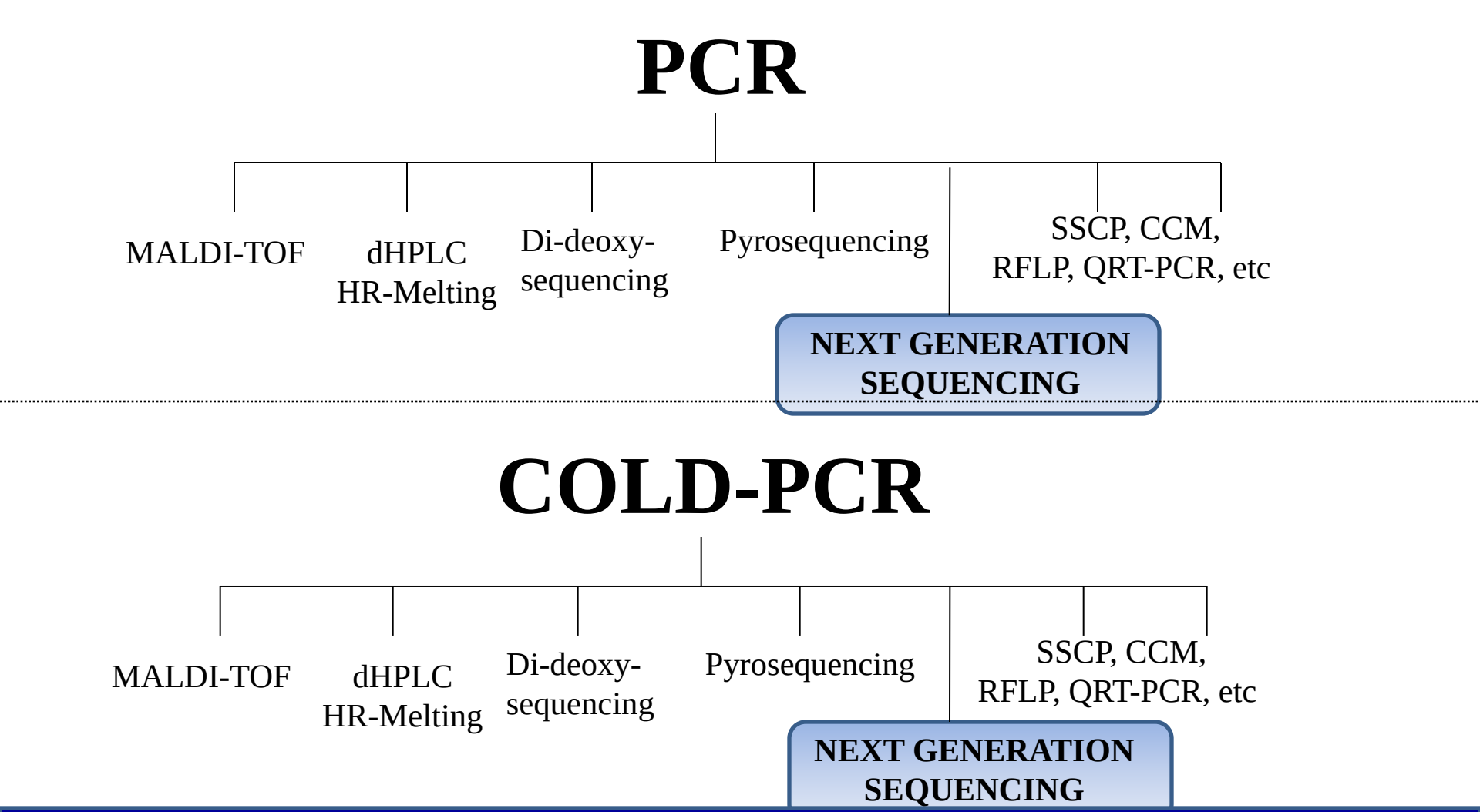
- Distel B, et al, AACR 2011, application of COLD-PCR in sequencing EGFR from **CIRCULATING TUMOR CELLS**

- Galbiati S et al, Clinical Chemistry, 2011 Laboratory of Laura Cremonesi, Milano, Italy (application: **PRENATAL DIAGNOSIS**)

- Application of COLD-PCR sequencing for the early detection of HBV antiviral **DRUG RESISTANT MUTATIONS**

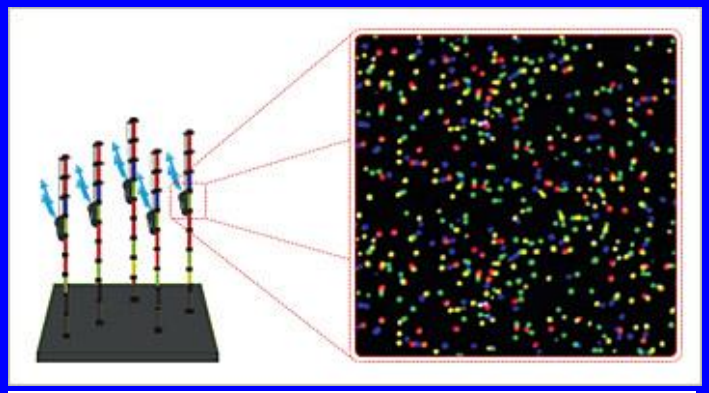
- Chen et al, BMC Plant Biology 2011: COLD-PCR-based mutation scanning in peach floral genes (**CROP IMPROVEMENT**)

PCR IS PERFORMED PRIOR TO ALMOST ALL PCR-BASED METHODS FOR MUTATION DETECTION



THEREFORE THE SENSITIVITY OF ALL THESE METHODS INCREASES BY REPLACING PCR WITH COLD-PCR

Next Generation Sequencing Technology 2011: revolutionizing personalized medicine and tumor biology



but…. good enough for detecting low-level mutations in heterogeneous tumors or mixed clinical samples??

Primary PCR amplification

Nested PCR amplification

Library preparation

NGS Sequencing

Conventional PCR

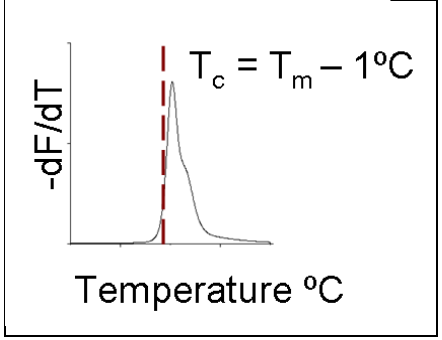
COLD-PCR

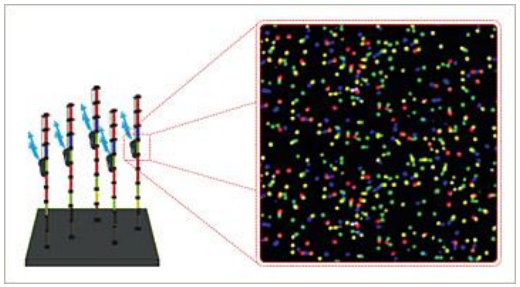
Conventional PCR

COLD-PCR

- Pool purified PCR products across mutant serial dilution
- Prepare indexed (multiplexed) library with Illumina TrueSeq kit

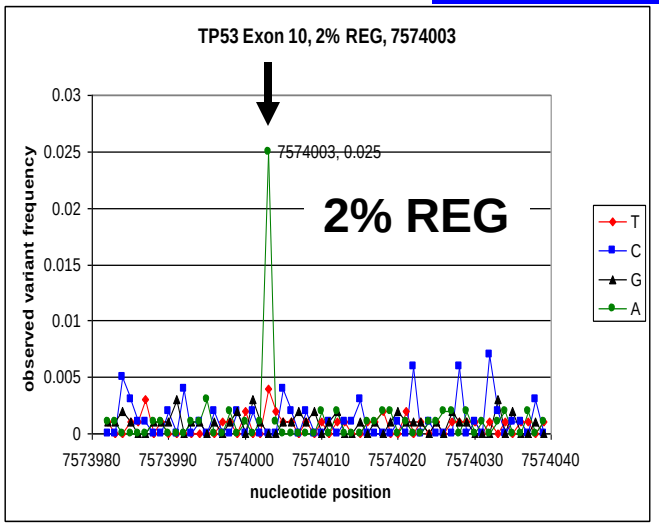
- Paired-end sequence analysis on the Illumina HiSeq 2000



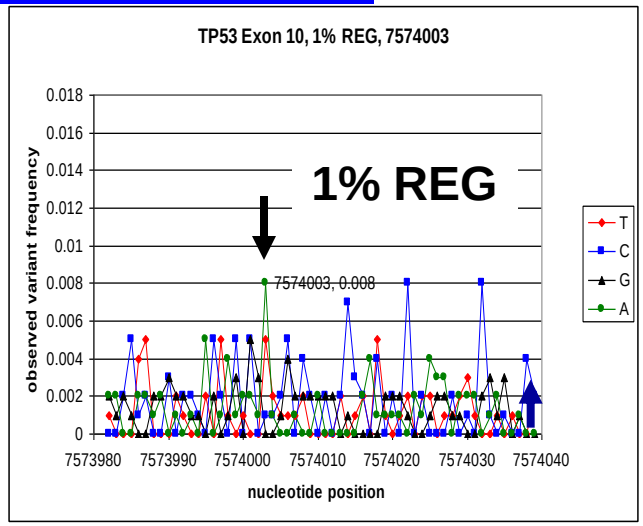


CENTER FOR CANCER COMPUTATIONAL BIOLOGY
DANA-FARBER CANCER INSTITUTE

CONVENTIONAL PCR



seq depth = 1629

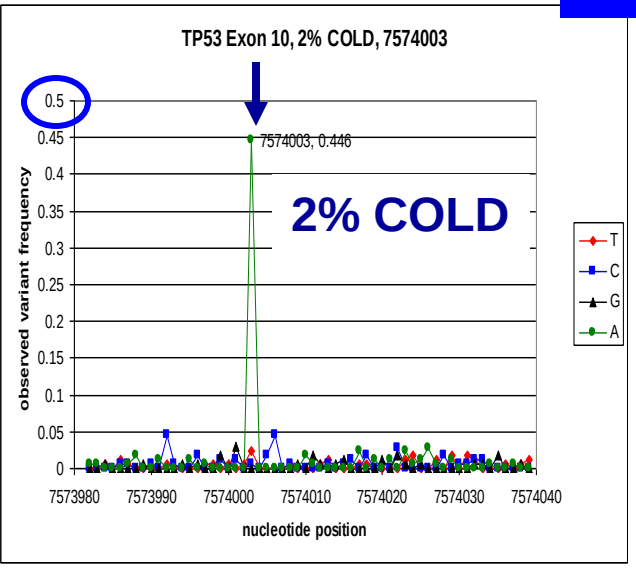


seq depth = 1300

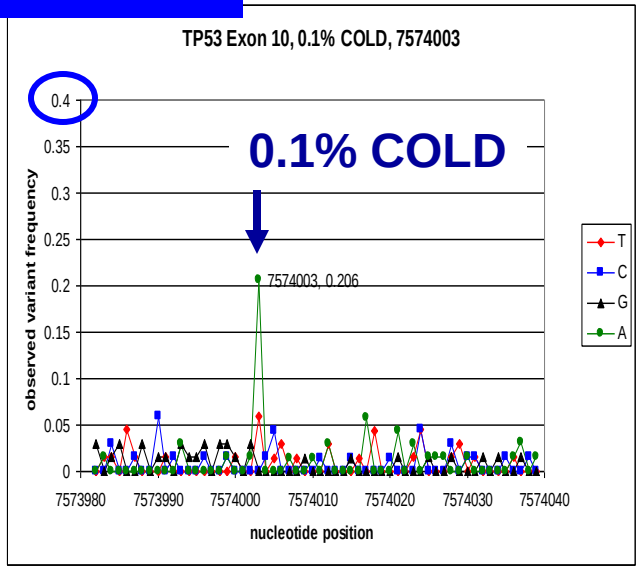
In the end it comes down to:

mutation vs. noise, not depth

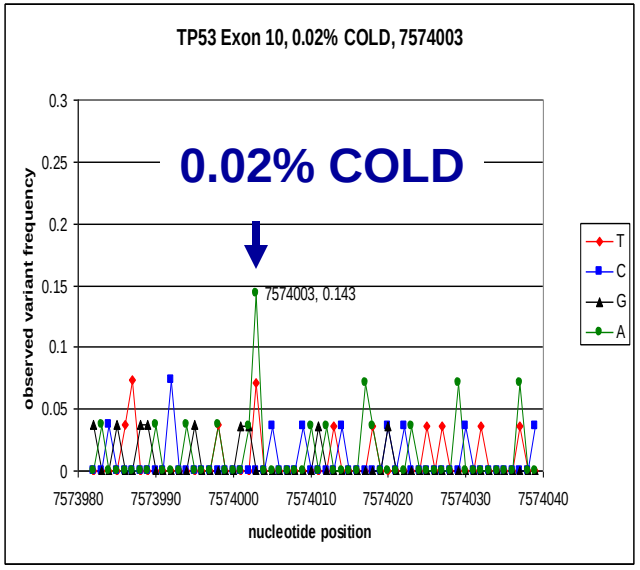
COLD PCR



seq depth = 175



seq depth = 68



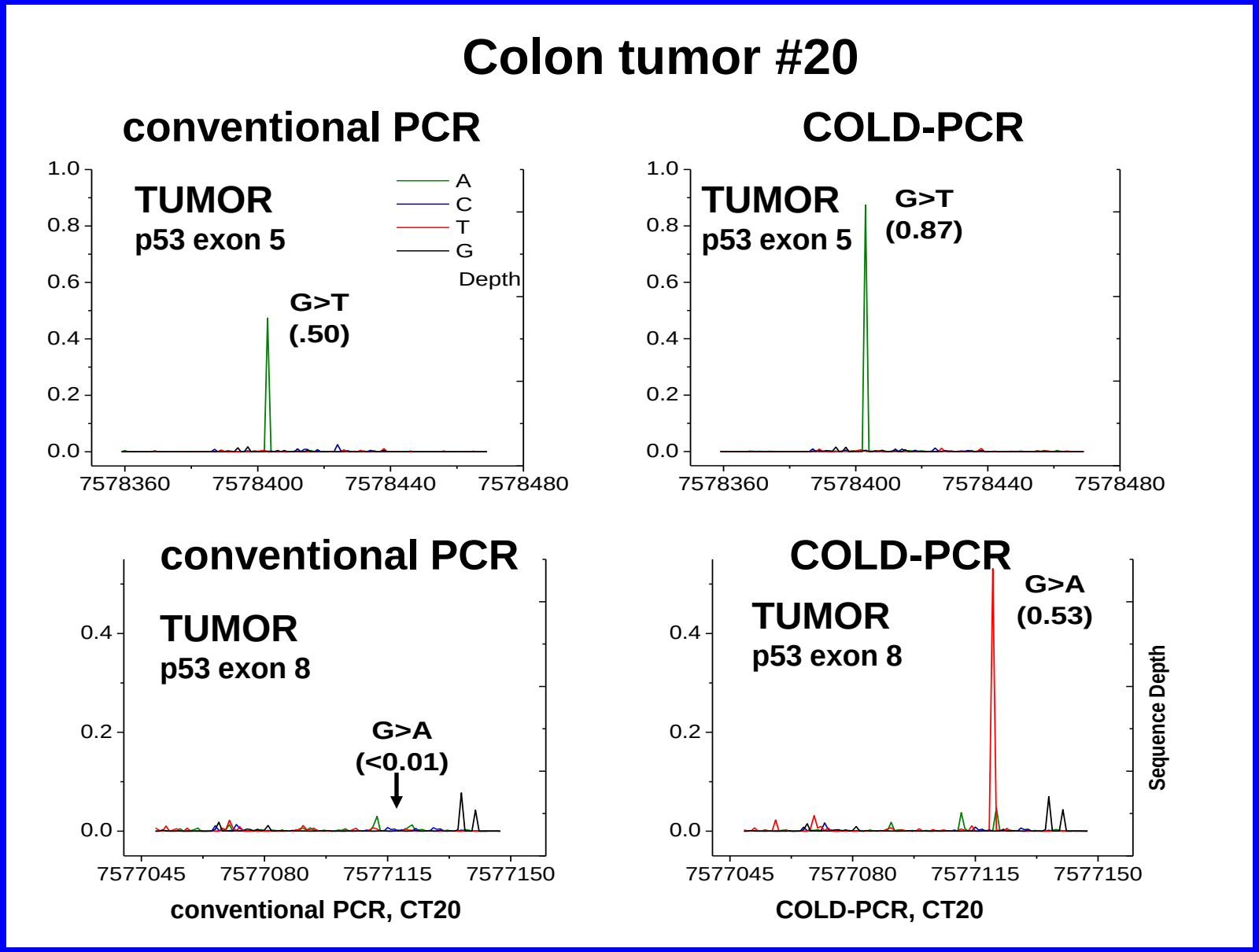
seq depth = 28

CONVENTIONAL PCR-NGS vs. COLD-PCR-NGS

- Clinical specimens
  - Lung, colon adeno-carcinomas
  - Some contain low-abundance mutations ranging from <1% to ~17% (independently verified)
  - Including putatively normal match
- Sequenced on the Illumina HiSeq2000 sequencer

Milbury et al, Clinical Chemistry, March 2012

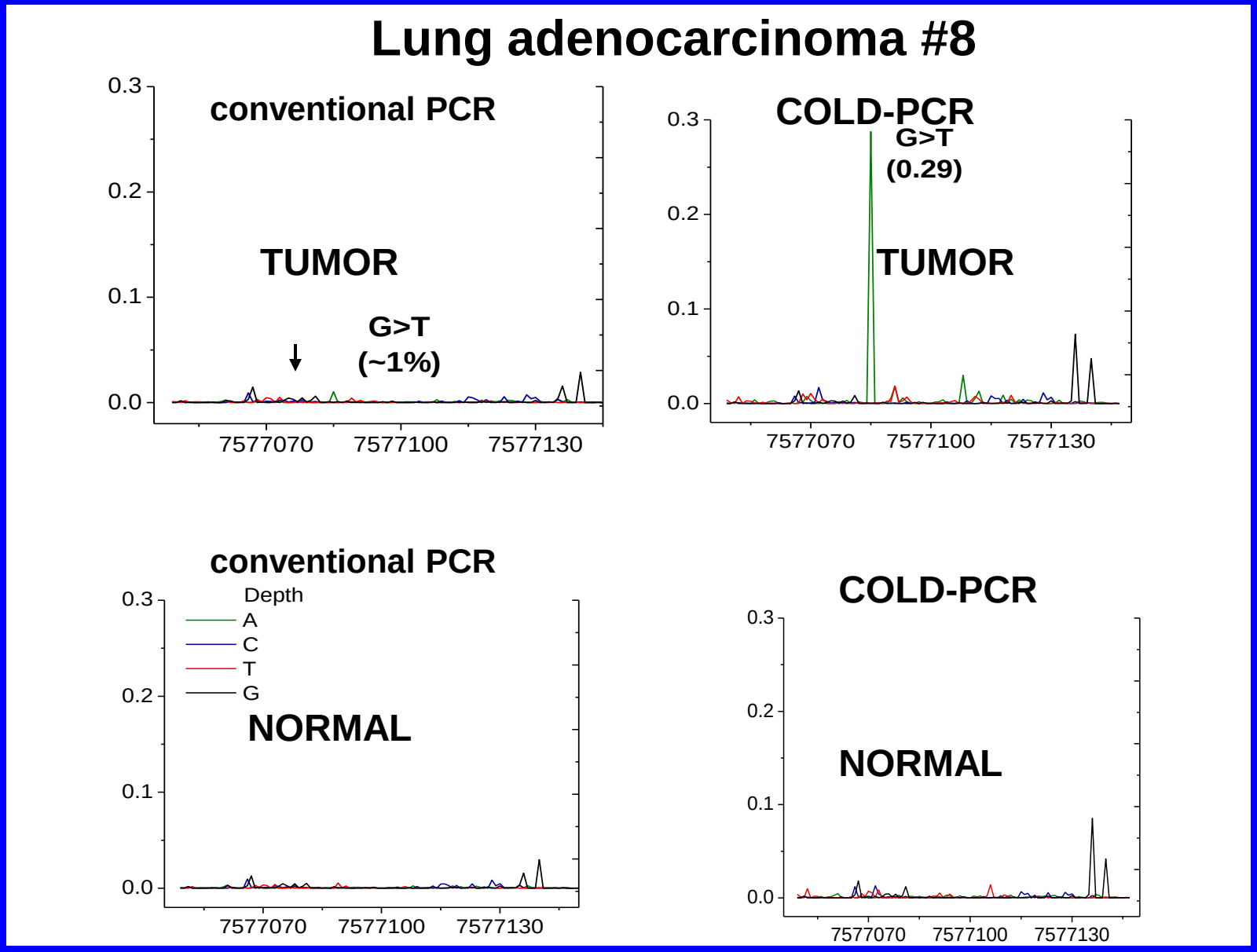
Illumina variant and noise plots



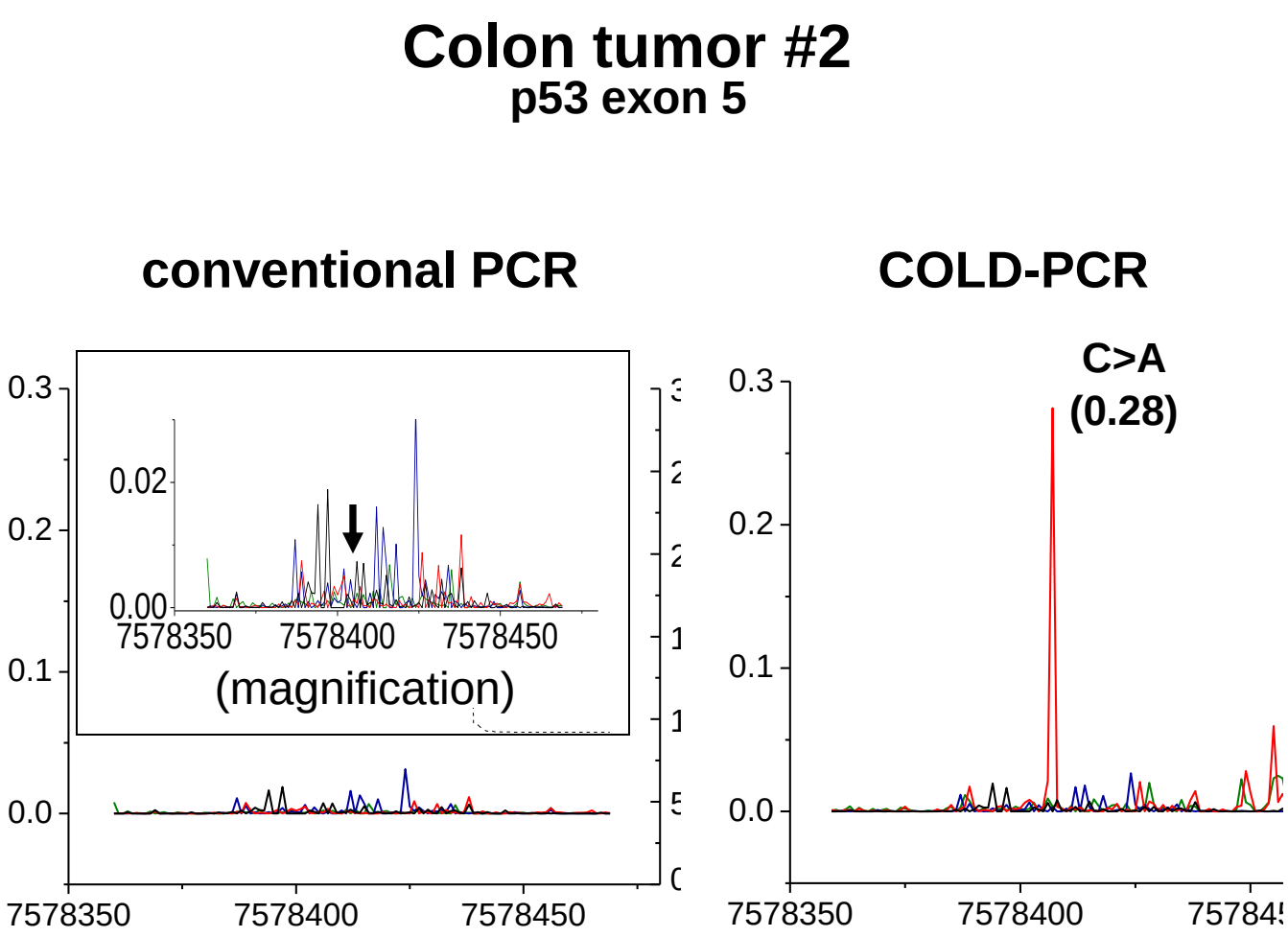
Nucleotide Frequency

Nucleotide Position

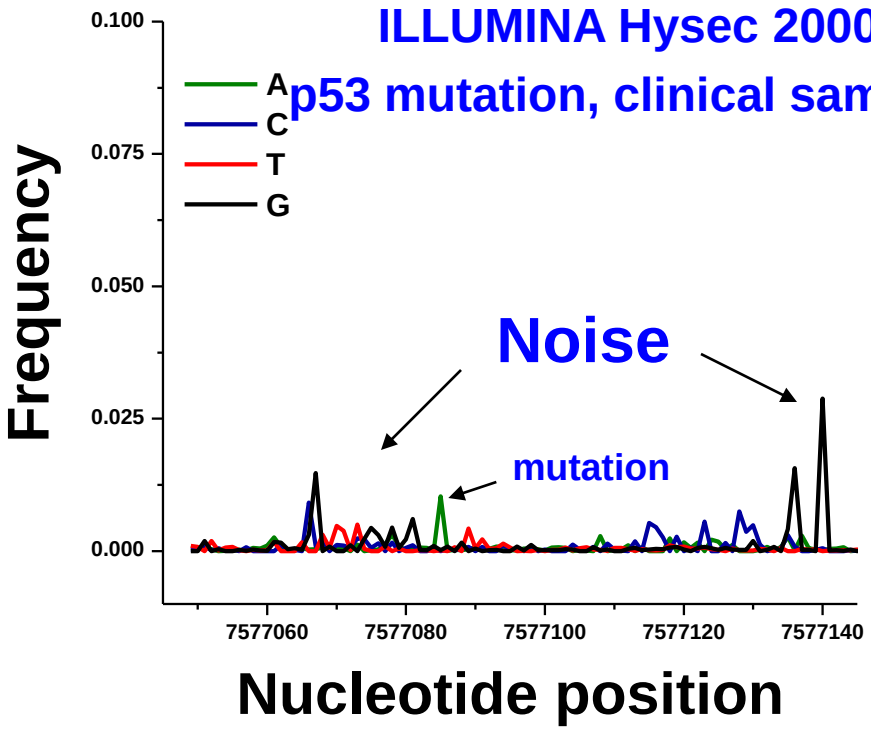
Illumina variant and noise plots



Illumina variant and noise plots



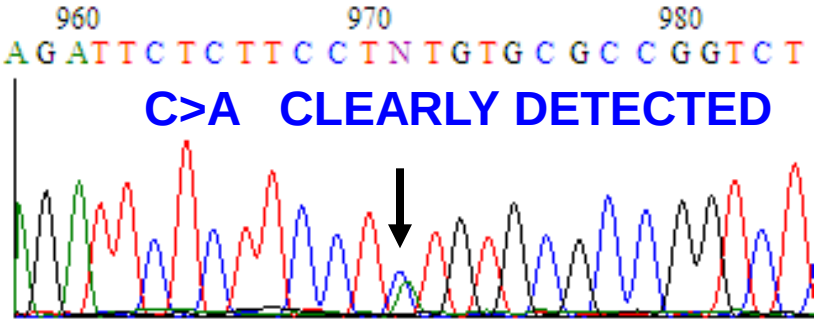
Validating ambiguous Next Gen Sequencing data



EXAMPLE: Lung CA sample with a low-level mutation: A fairly common situation in molecular diagnostics

validation via

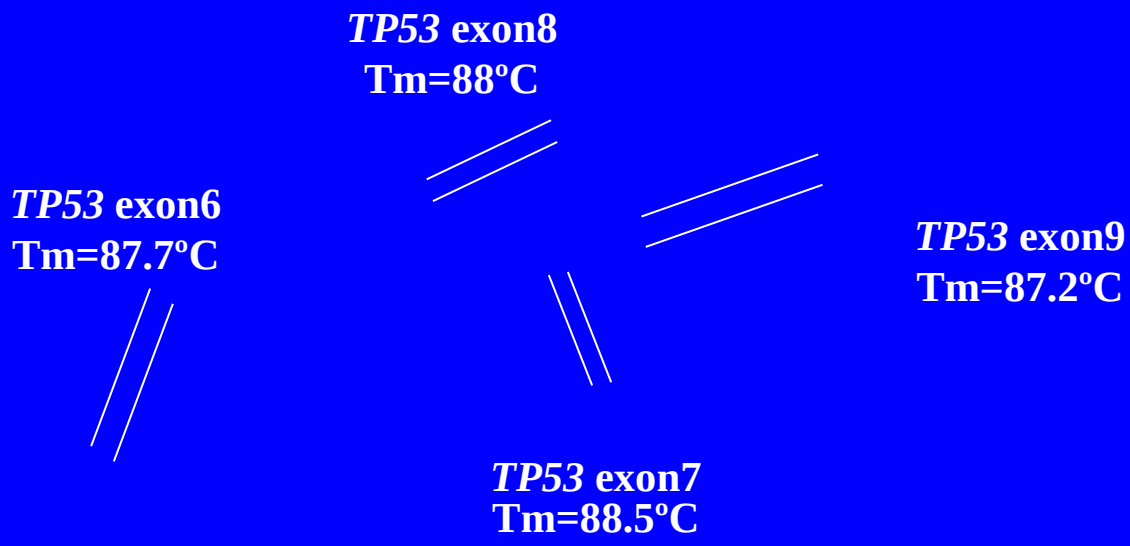
COLD-PCR plus Sanger Sequencing



New development in COLD-PCR:

Temperature-tolerant COLD-PCR

Sequences with diverse Tm



Castellanos et al

Clinical Chem 2012

Temperature-tolerant COLD-PCR

Enriching diverse mutant sequences (single PCR protocol across all PCR wells)

Castellanos et al (In Press)

Repeat at $Tc_{max} = Tc_1 + 2.5^oC$

Repeat X times
Enrich mutant seq N

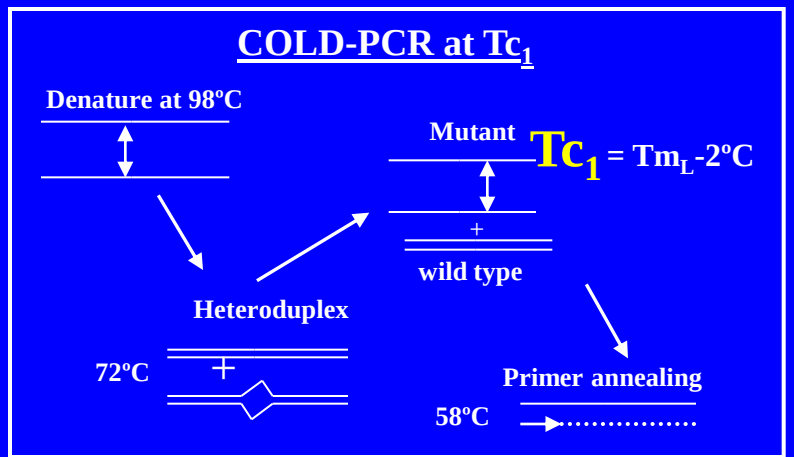
etc

Repeat at $Tc_3 = Tc_2 + 0.5^oC$

Repeat X times
Enrich mutant seq 3

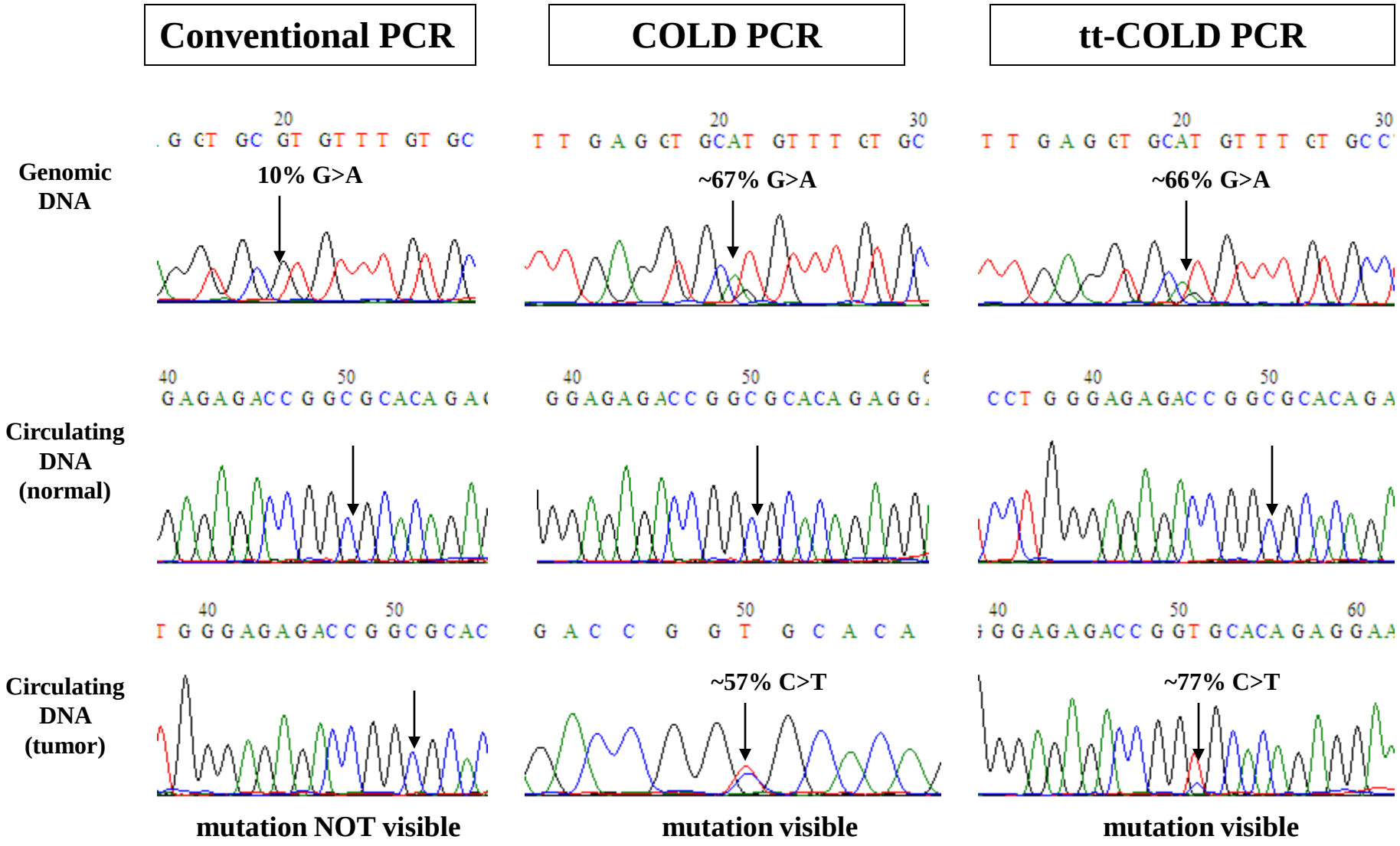
Repeat at $Tc_2 = Tc_1 + 0.5^oC$

Repeat X times
Enrich mutant seq 2

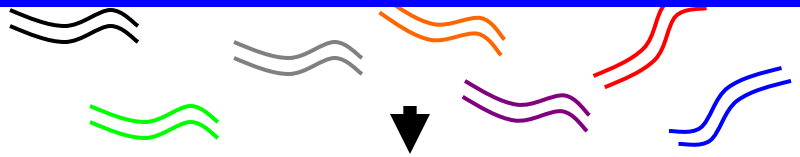


Repeat X times
Enrich mutant seq 1

Temperature-tolerant tt-COLD-PCR:
enrichment of diverse mutant sequences using a single PCR protocol

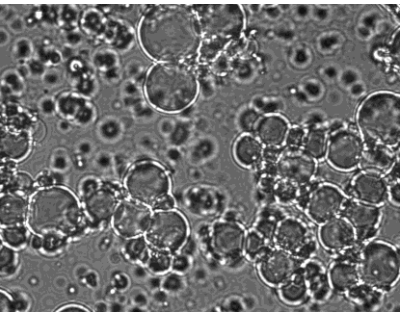


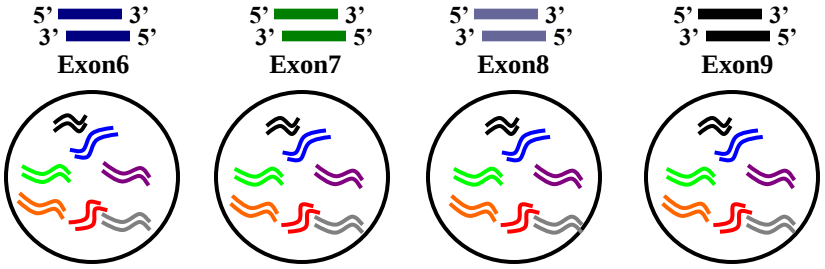
TEMPERATURE-TOLERANT COLD-PCR IN EMULSION: MULTIPLEXED, SINGLE TUBE METHOD



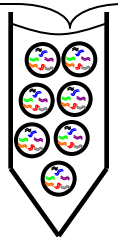
emulsify

Emulsion

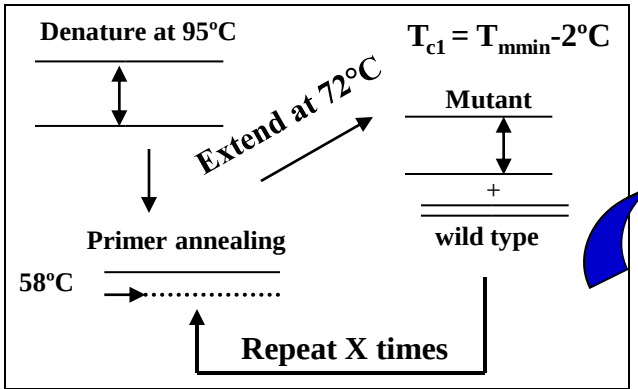




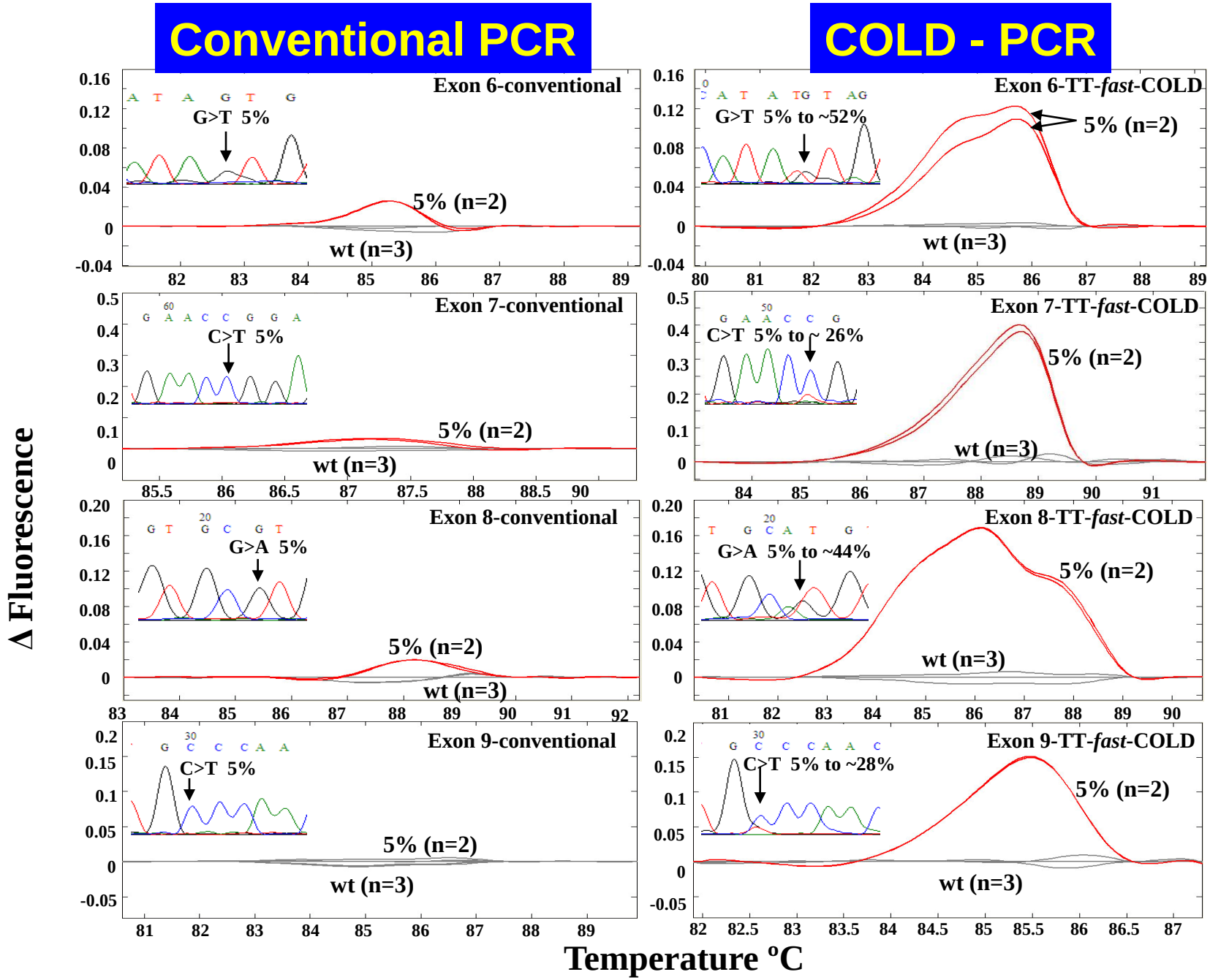
… + N

Mix in a  **single tube**

TT-COLD-PCR



TEMPERATURE-TOLERANT COLD-PCR IN EMULSION: p53 exons 6-9



SUMMARY

COLD-PCR technology:
enables sensitive and reliable sequencing
for personalized medicine

infiltrating, diffuse-type tumor specimens
sub-optimally micro-dissected
or heterogeneous tumor samples
DNA from circulating DNA, circulating cells,
sputum or other bodily fluids
tumor margins, stromal cells, and others

PCR is best served COLD!

THANK YOU

Contributors and Collaborators

LAB

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