

**Building Tools for the Analysis
of Cellular Heterogeneity**

**LATE-PCR and Allied Technologies for
Analysis of Genetic Heterogeneity in
Single Cells & Single DNA Molecules**

J. Aquiles Sanchez, Adam E. Osborne, and Lawrence J. Wangh
Brandeis University

October 16, 2009
NIH CEGS Grantees Meeting
Huntsville, Alabama



The LATE-PCR Technology Platform

Sample Preparation

Quantitative recovery of
nucleic acids from single cells

PurAmp
(DNA & RNA)

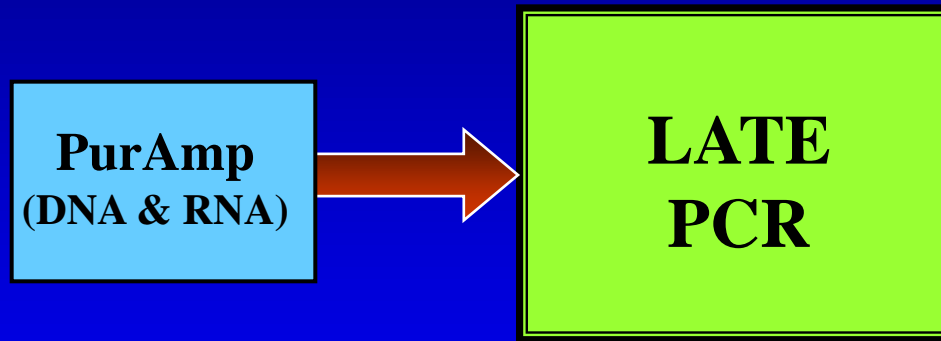


The LATE-PCR Technology Platform

**Sample
Preparation**

**Amplification
Method**

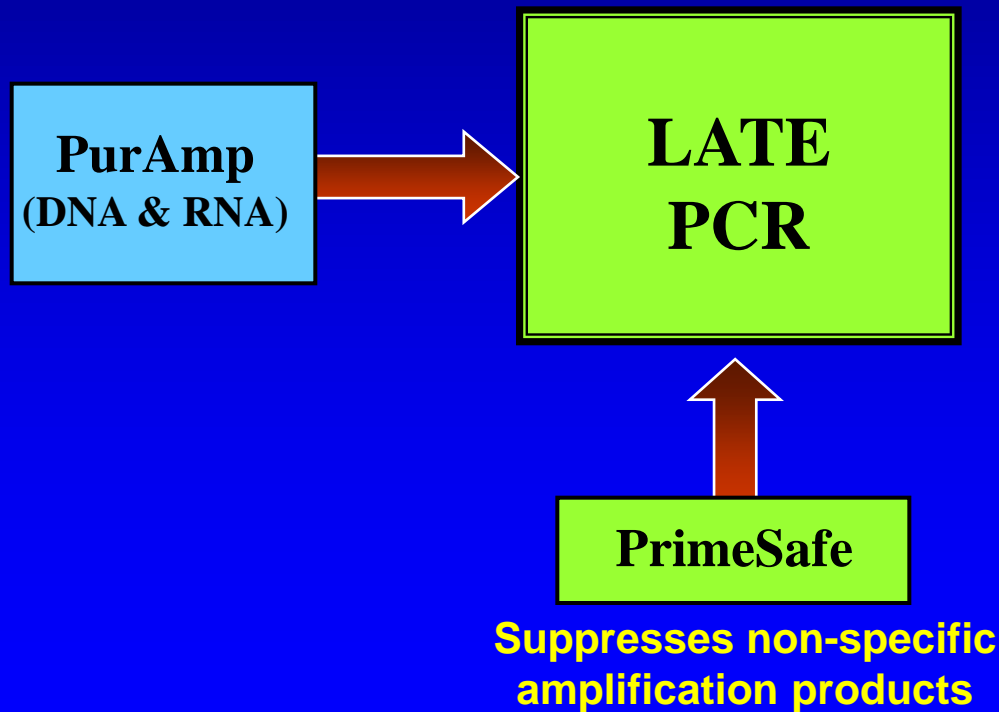
**Efficient amplification
of single-stranded DNA**



The LATE-PCR Technology Platform

**Sample
Preparation**

**Amplification
Method**

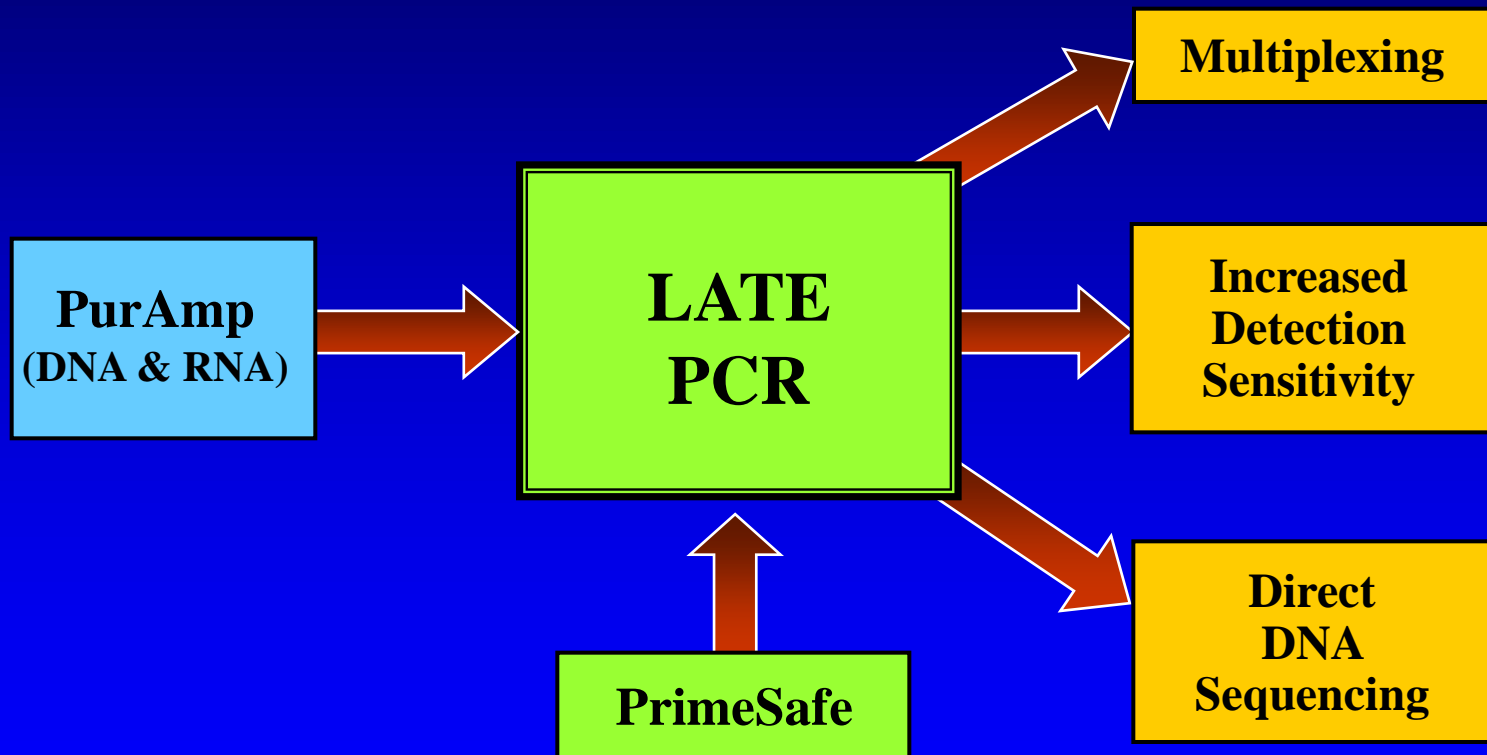


The LATE-PCR Technology Platform

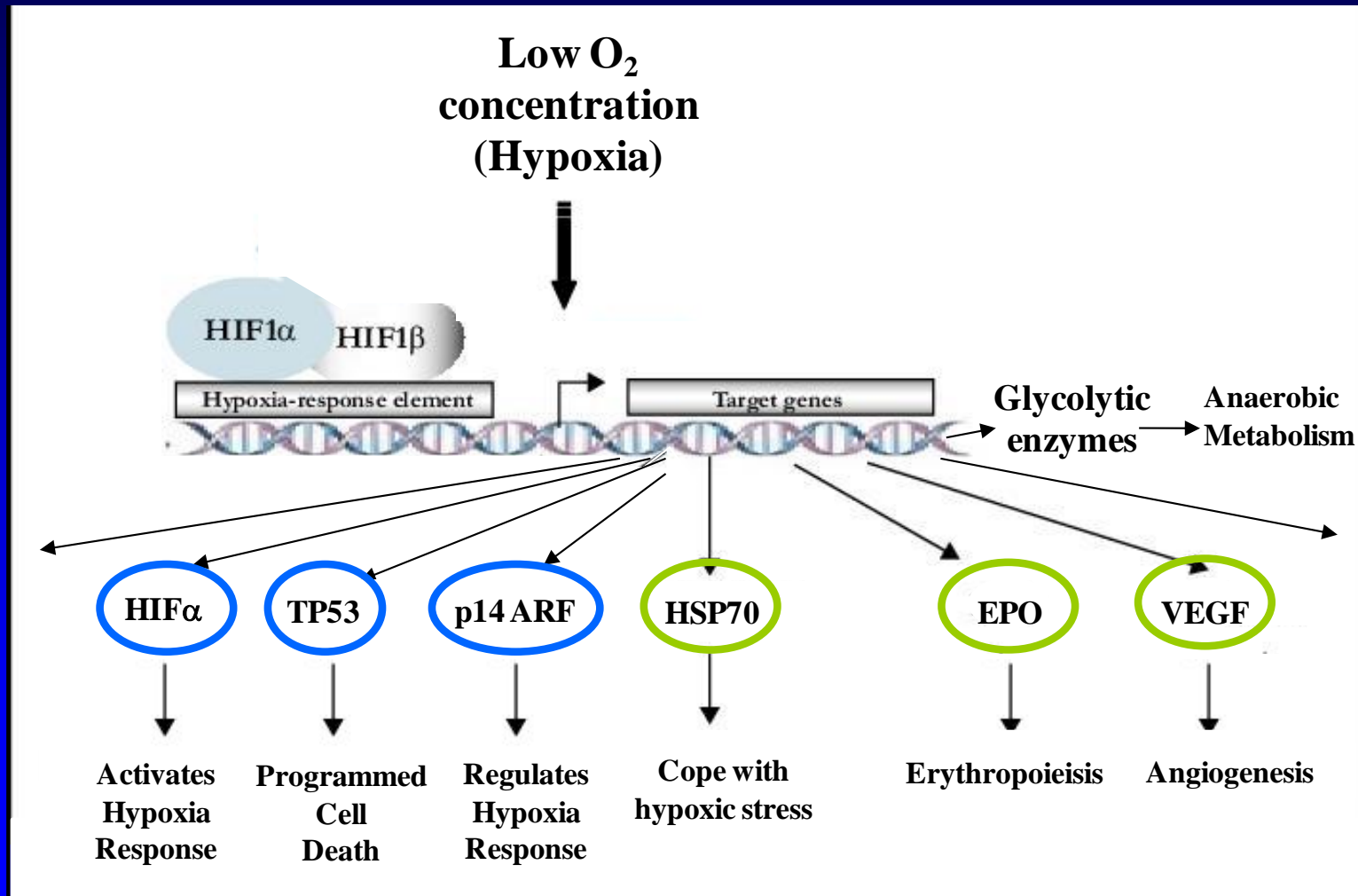
**Sample
Preparation**

**Amplification
Method**

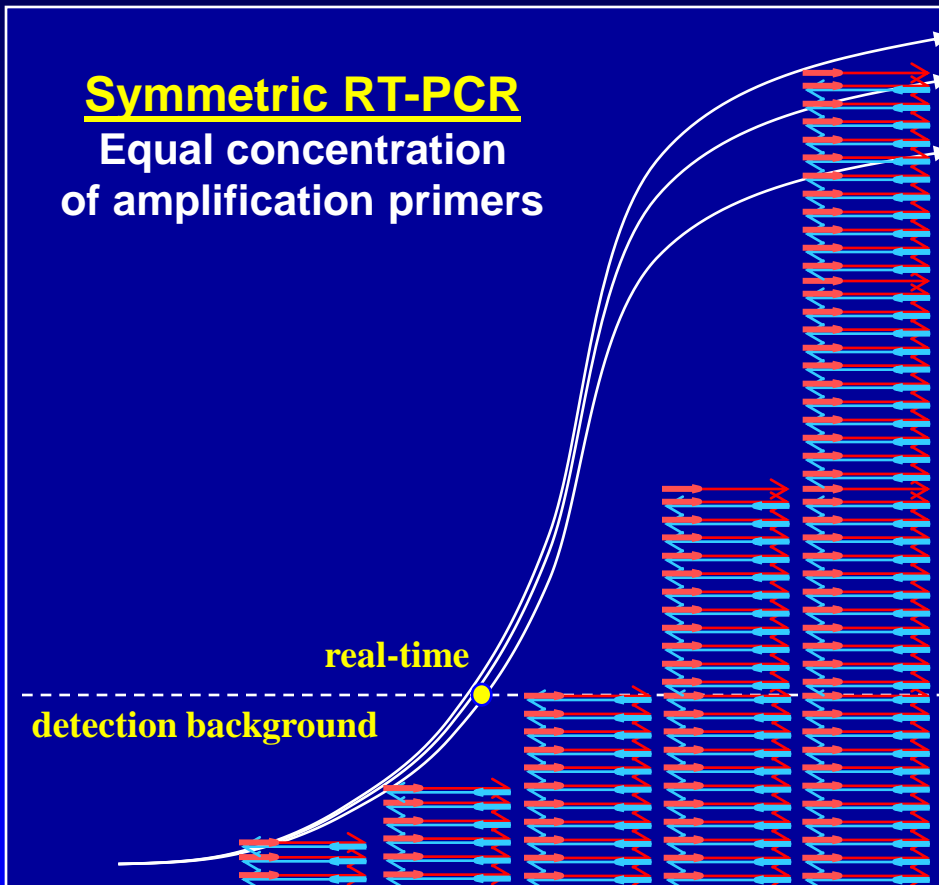
**Product
Analysis**



Genetic Heterogeneity at the Single Cell Level: The Hypoxia Transcriptional Response



Limitations of Conventional PCR



Limitations of Conventional PCR

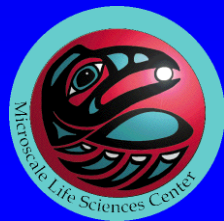
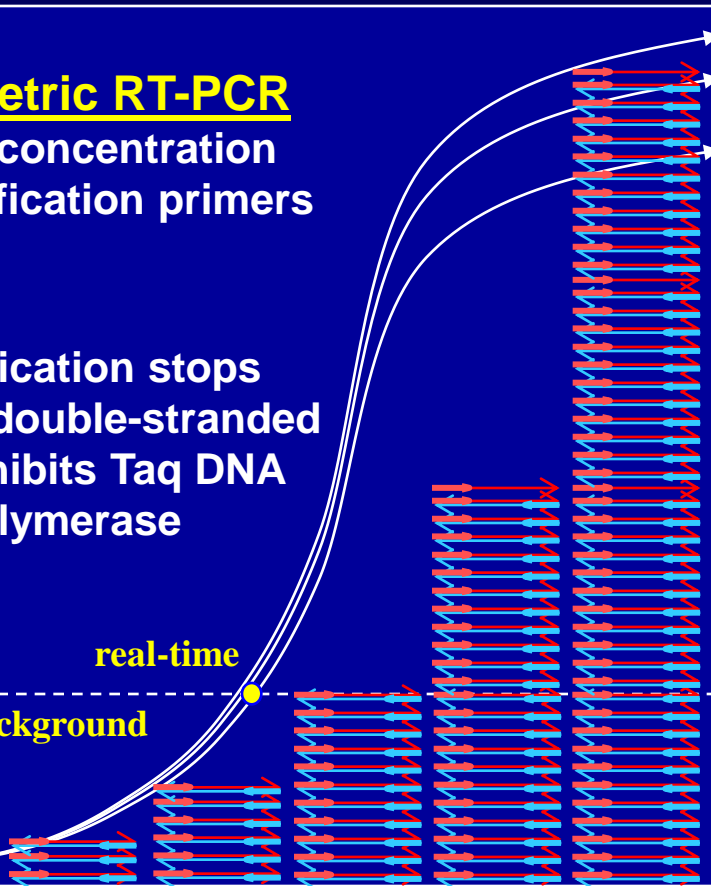
Symmetric RT-PCR

Equal concentration
of amplification primers

Amplification stops
because double-stranded
DNA inhibits Taq DNA
polymerase

real-time

detection background



Limitations of Conventional PCR

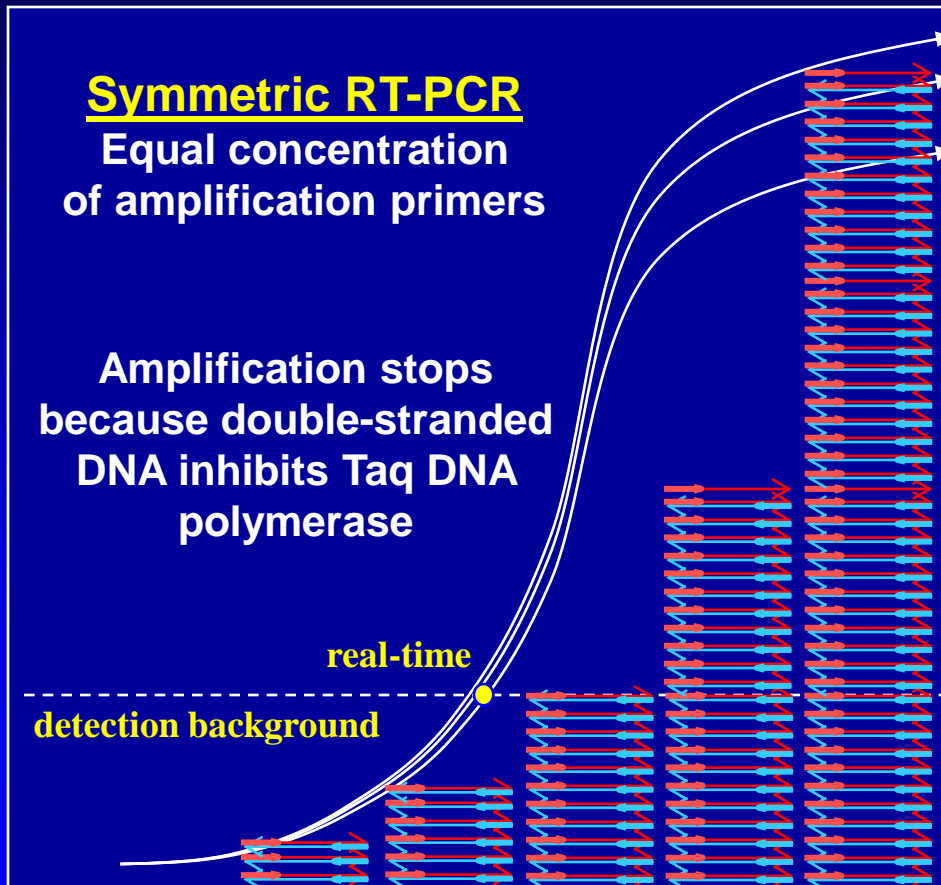
Symmetric RT-PCR

Equal concentration
of amplification primers

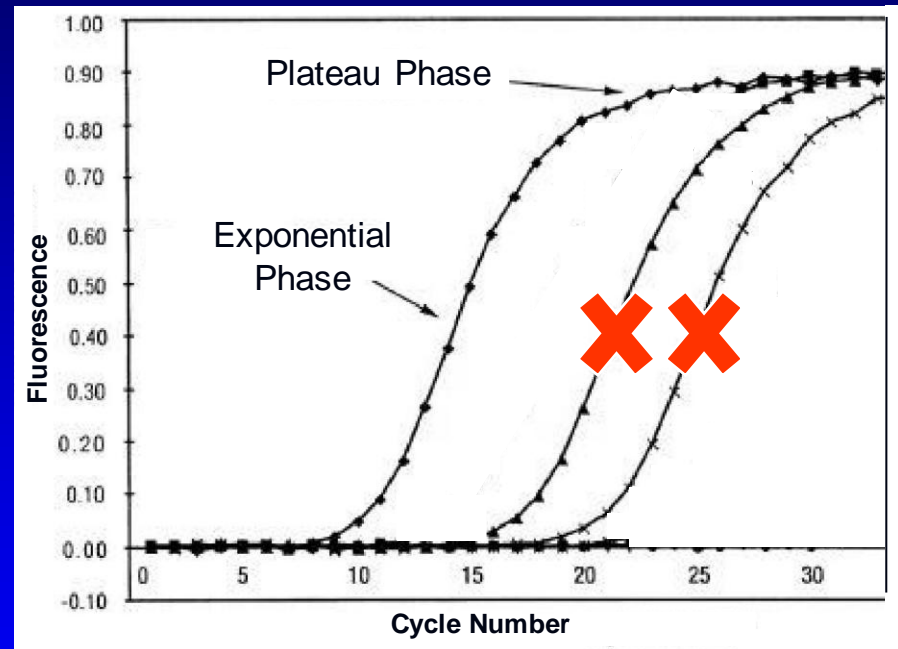
Amplification stops
because double-stranded
DNA inhibits Taq DNA
polymerase

real-time

detection background



Co-Amplification of Abundant & Rare Targets



Abundant targets prevent
amplification of rare targets

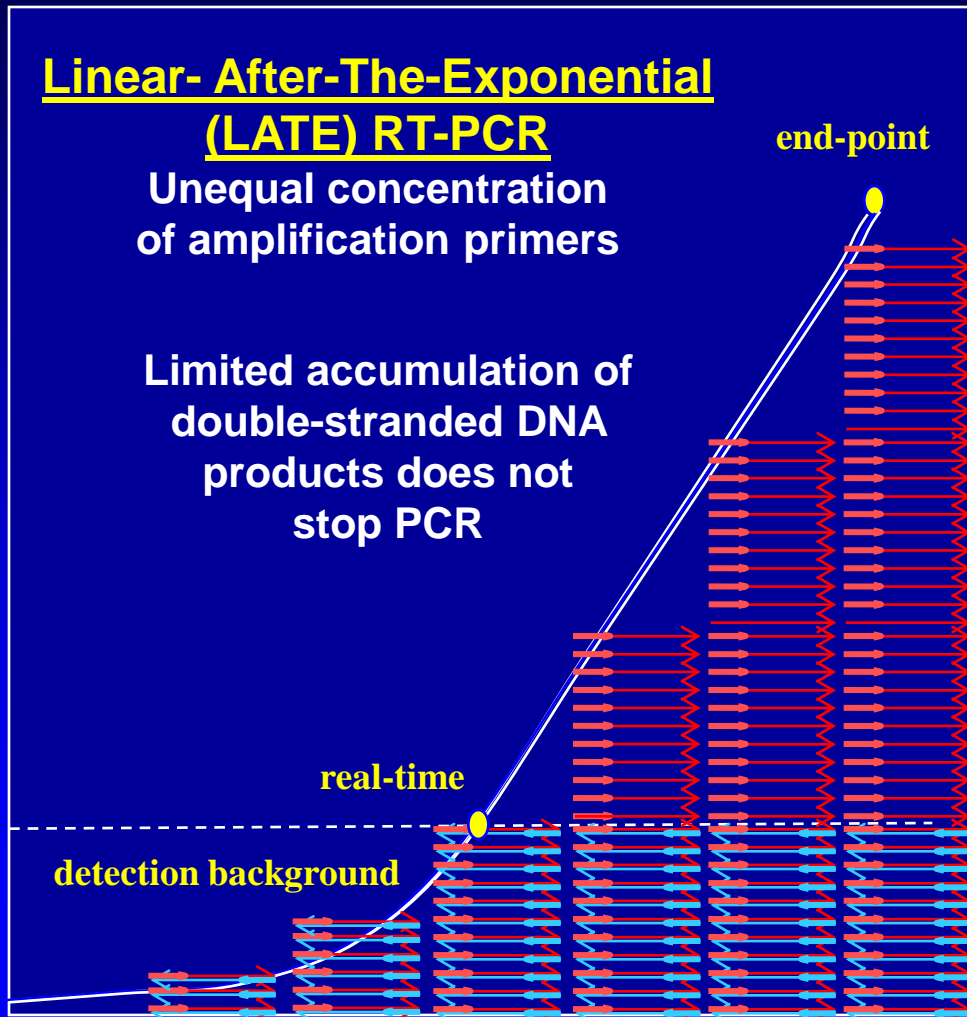


The LATE-PCR Solution

Linear- After-The-Exponential (LATE) RT-PCR

Unequal concentration
of amplification primers

Limited accumulation of
double-stranded DNA
products does not
stop PCR

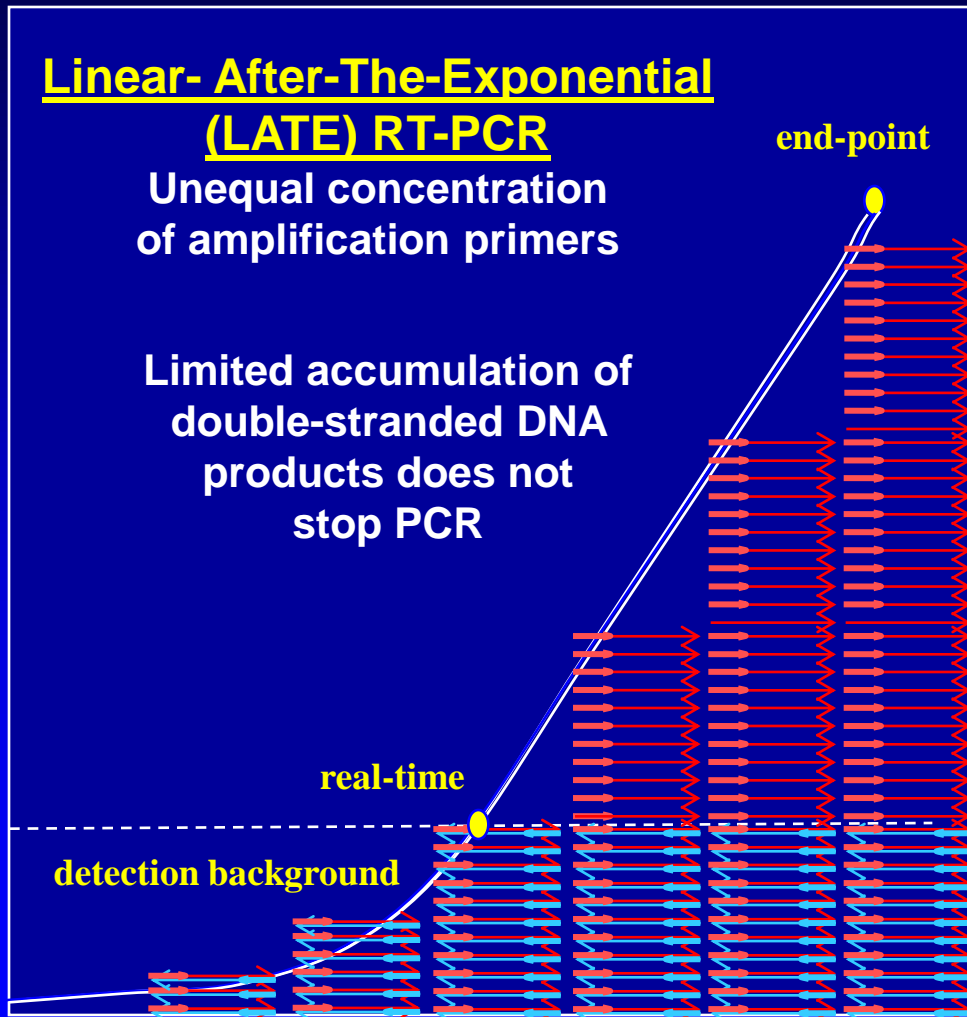


The LATE-PCR Solution

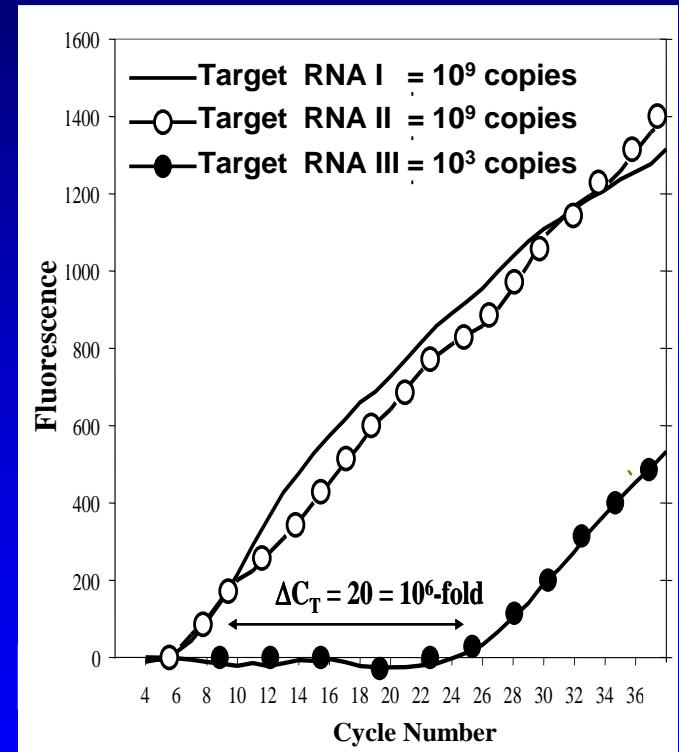
Linear- After-The-Exponential (LATE) RT-PCR

Unequal concentration of amplification primers

Limited accumulation of double-stranded DNA products does not stop PCR



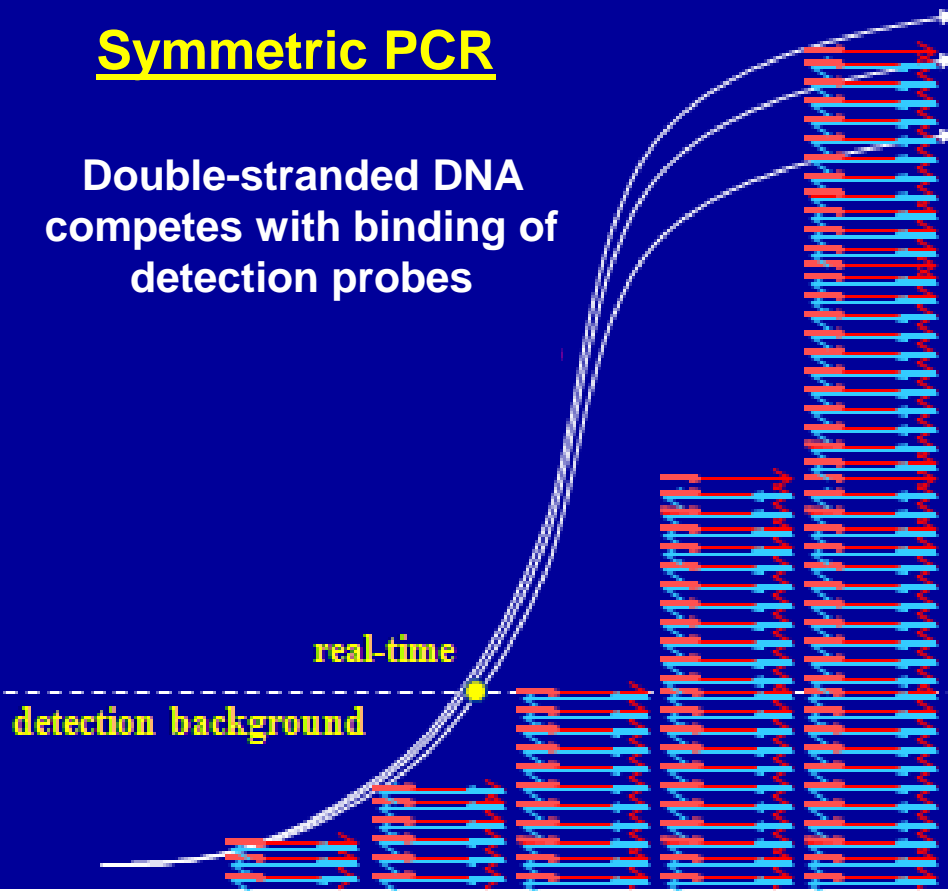
Co-Amplification of Abundant & Rare Targets



Hartshorn & Wangh (2009) One-step RT-LATE-PCR for mRNA and Viral RNA Detection and Quantification (Methods in Molecular Biology series, The Humana Press)

Symmetric PCR

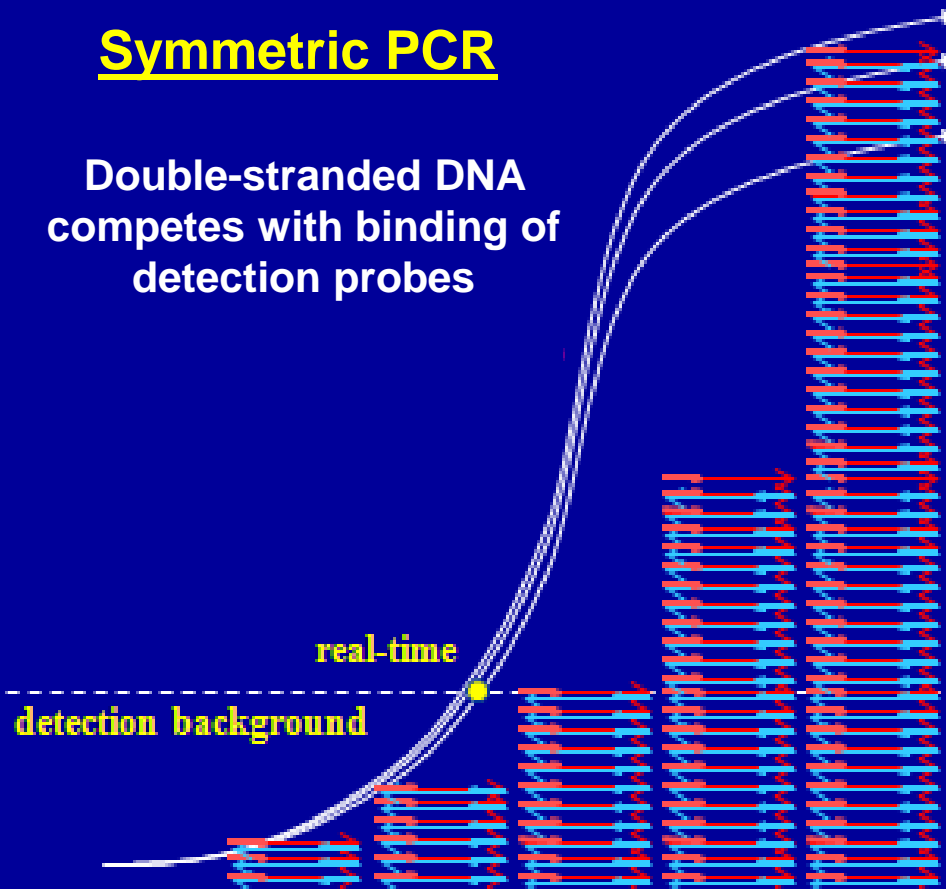
Double-stranded DNA
competes with binding of
detection probes



LATE-PCR Provides Increased Detection Sensitivity

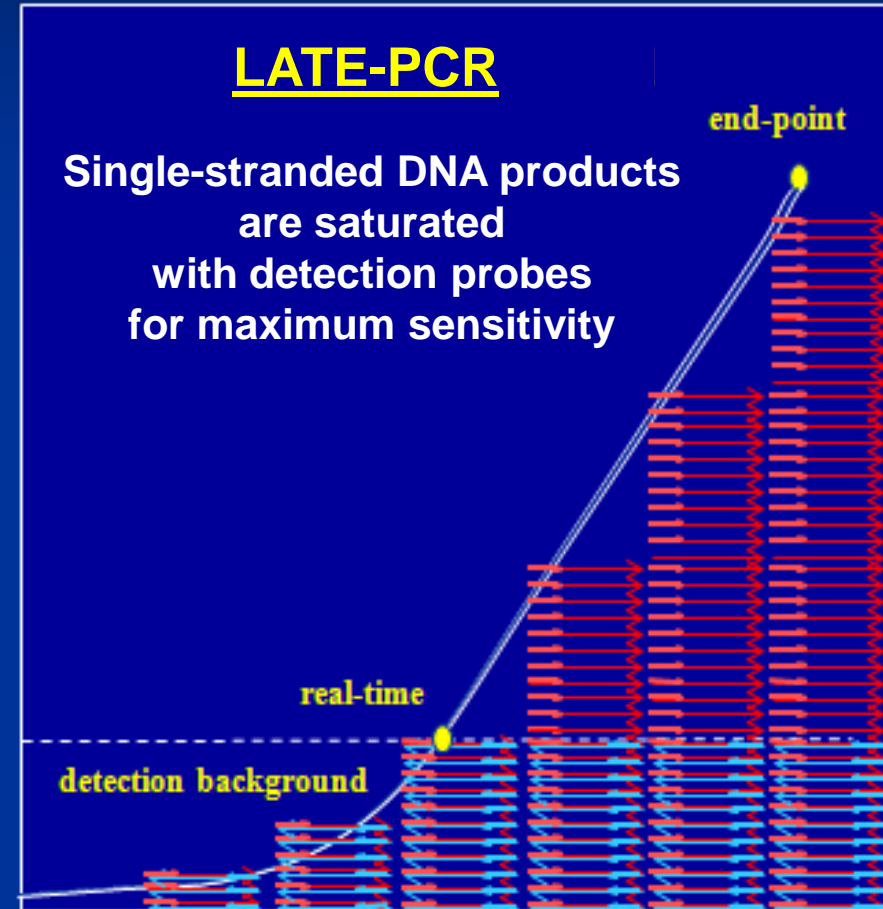
Symmetric PCR

Double-stranded DNA competes with binding of detection probes

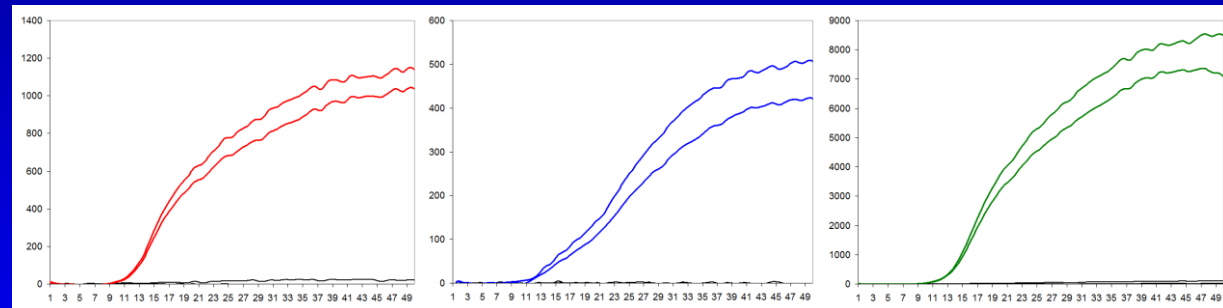
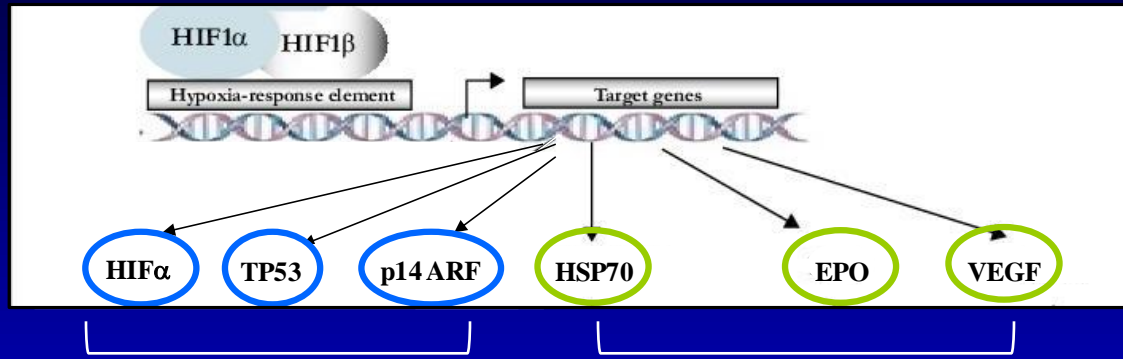


LATE-PCR

Single-stranded DNA products are saturated with detection probes for maximum sensitivity



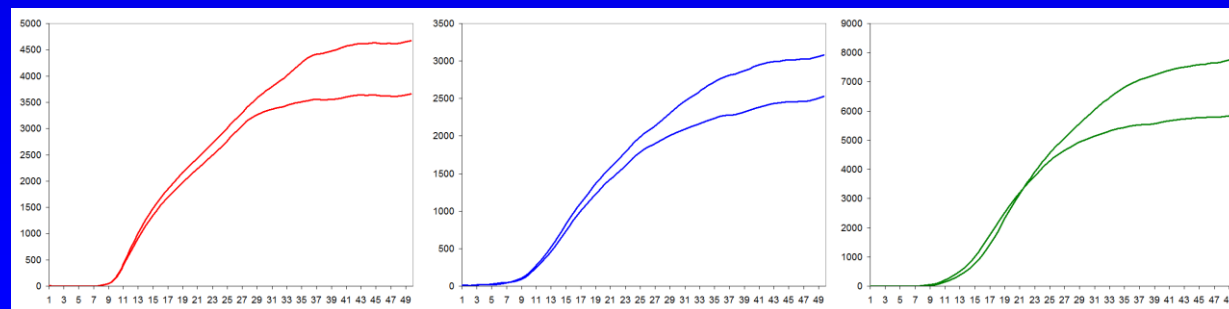
Multiplex LATE-PCR Assays for Analysis of the Hypoxia Response in Single Cells



HSP70

EPO

VEGF



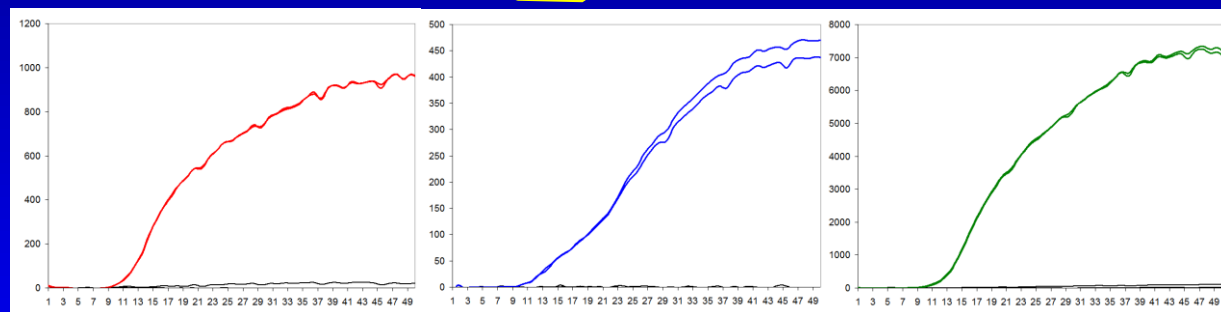
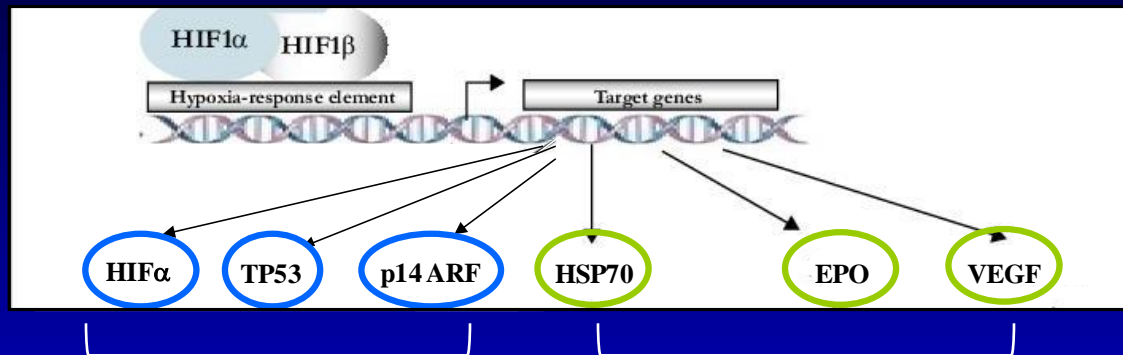
HIF1 α

TP53

p14ARF



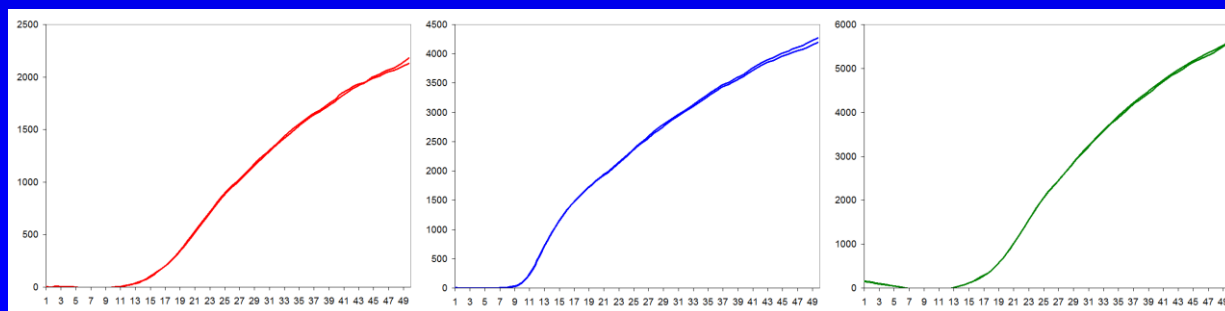
Optimized Multiplexed LATE-PCR Assays Using PrimeSafe



HSP70

EPO

VEGF



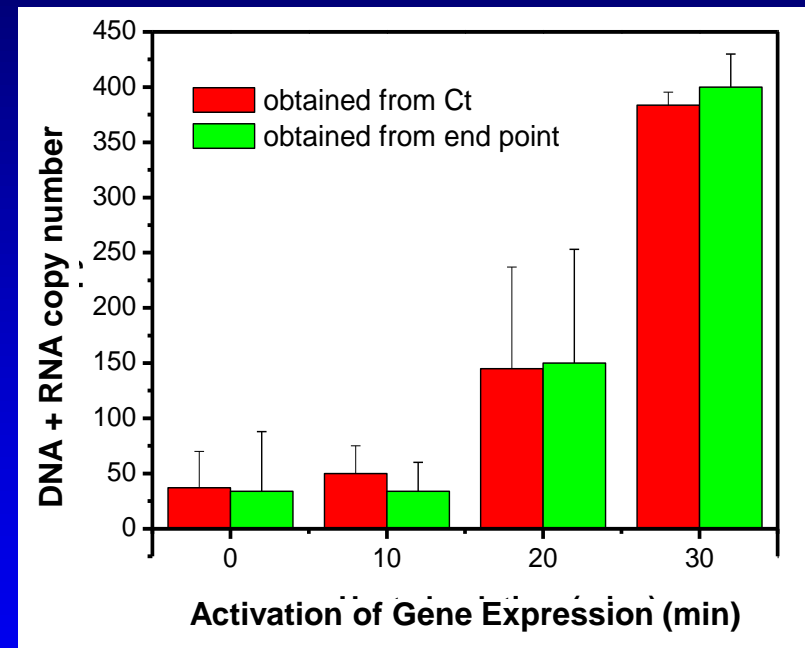
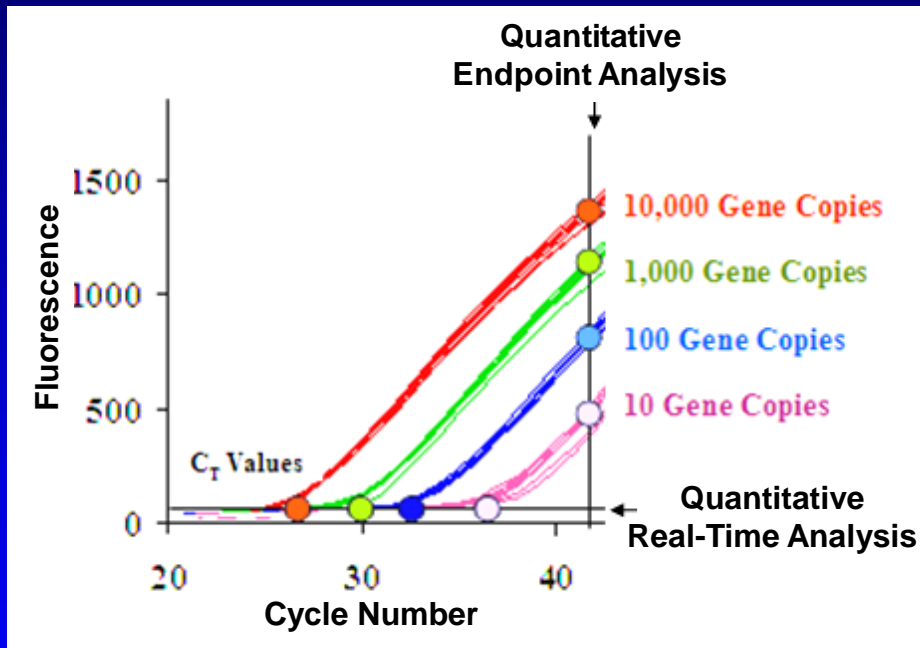
HIF1 α

TP53

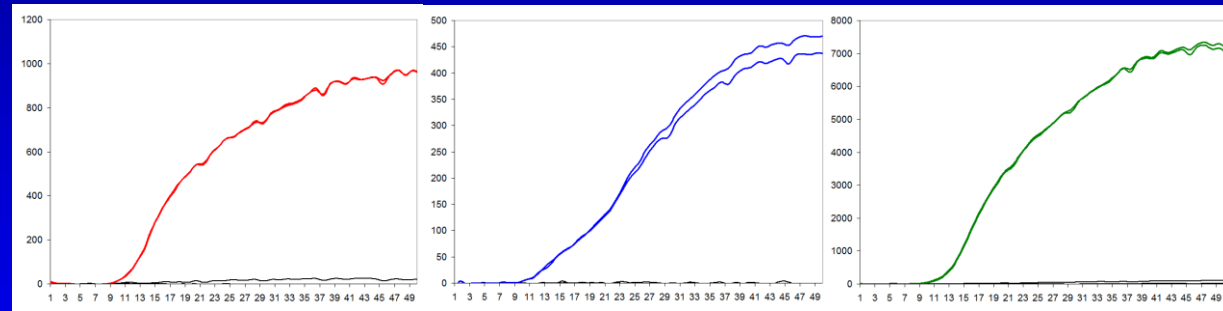
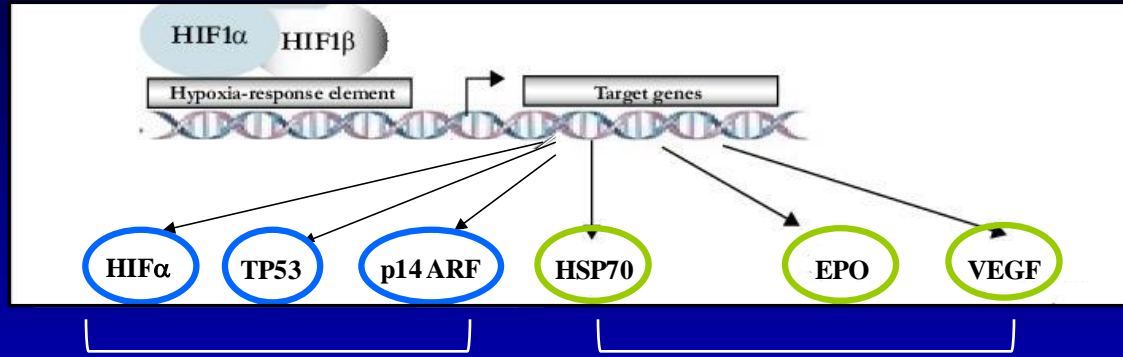
p14ARF



Quantitative Endpoint Analysis



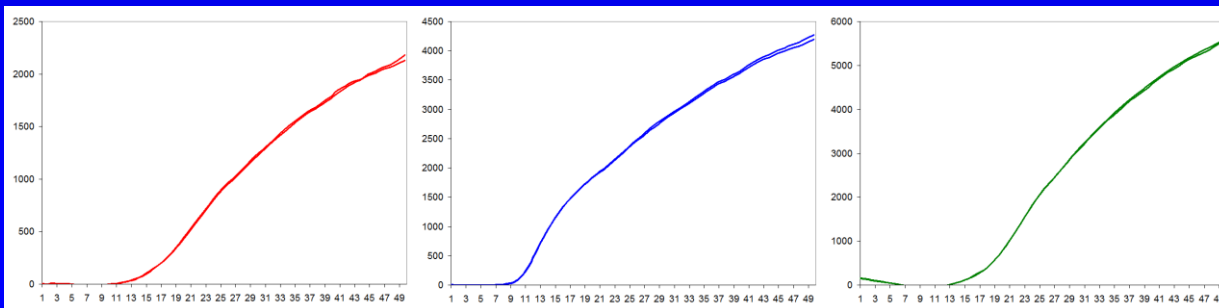
Multiplex LATE-PCR Assays for Analysis of the Hypoxia Response in Single Cells



HSP70

EPO

VEGF



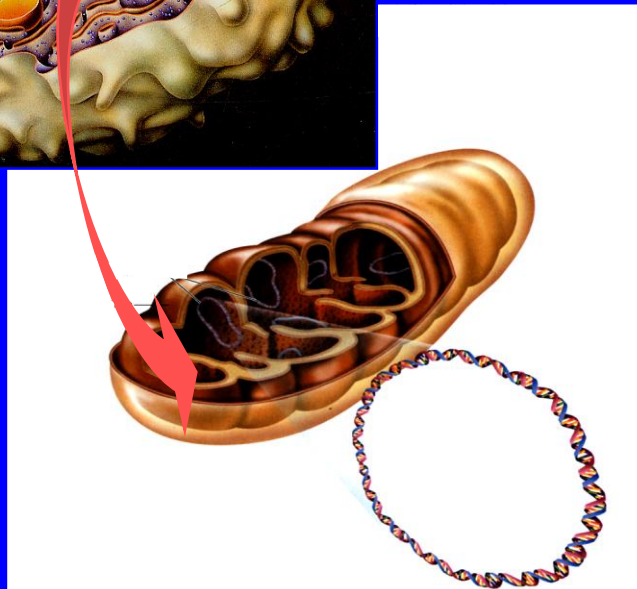
HIF1 α

TP53

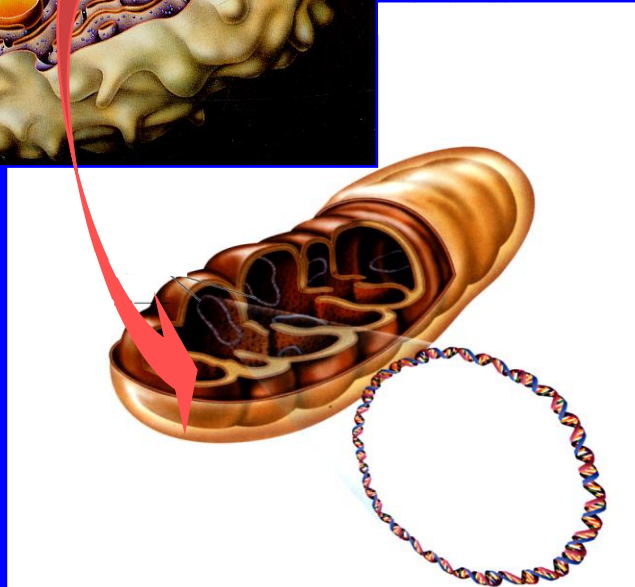
p14ARF



Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes

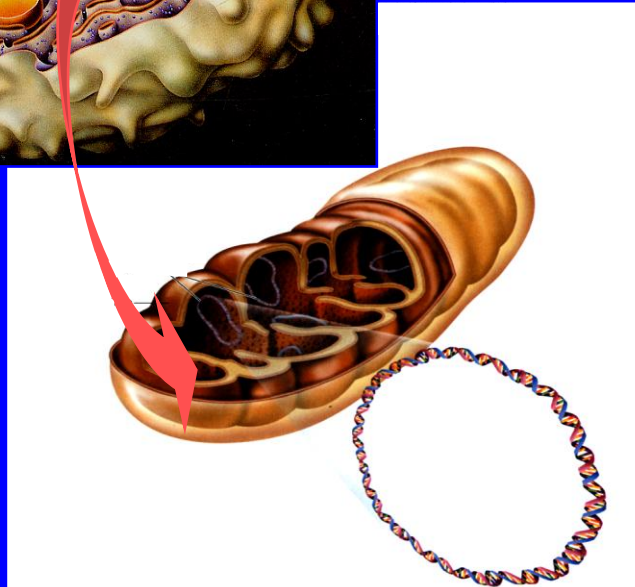


Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes



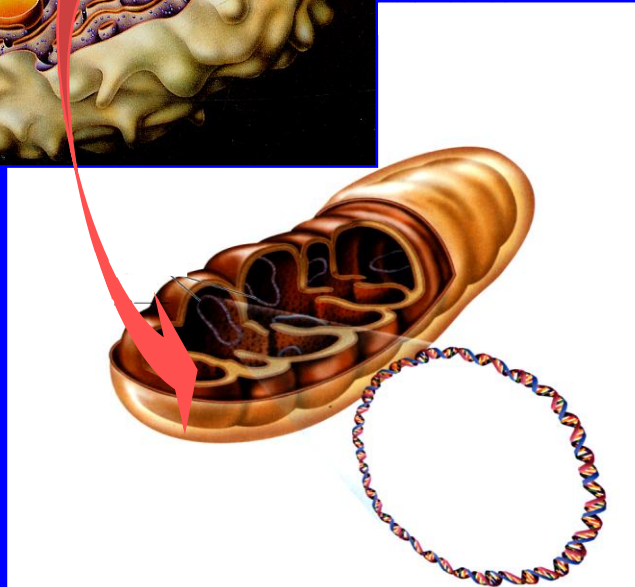
- Neoplasia is driven by the accumulation of genetic changes

Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes



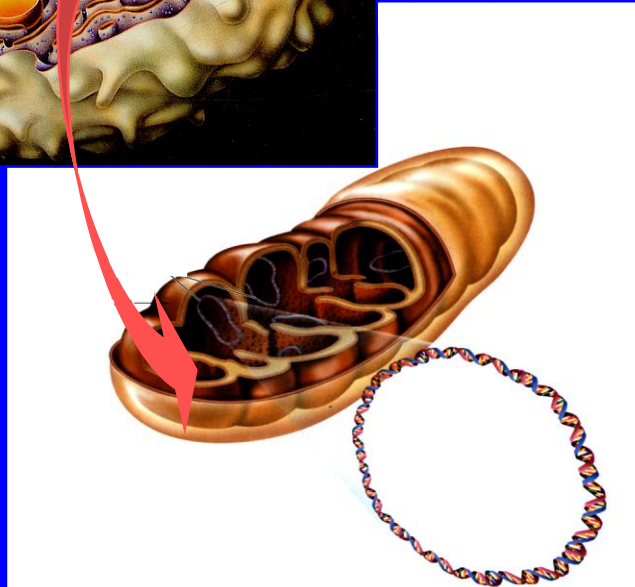
- Neoplasia is driven by the accumulation of genetic changes
- Does mitochondria undergoes random genetic changes during neoplastic progression?

Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes



- Neoplasia is driven by the accumulation of genetic changes
- Does mitochondria undergoes random genetic changes during neoplastic progression?
- A single cell has ~ 1000 mitochondrial DNAs

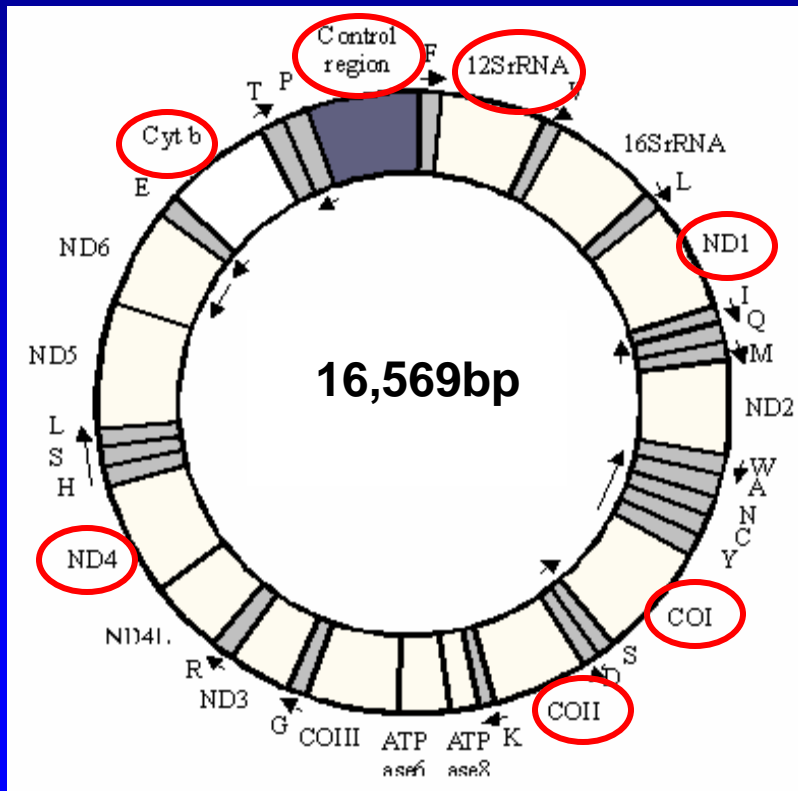
Genetic Heterogeneity at the Level of Single DNA Molecules: Mutational Load of Mitochondrial Genomes



- Neoplasia is driven by the accumulation of genetic changes
- Does mitochondria undergoes random genetic changes during neoplastic progression?
- A single cell has ~ 1000 mitochondrial DNAs
- Detection of random, low frequency mutations requires analysis of single mitochondrial DNAs

A 10-plex LATE-PCR Assay for Analysis of Single Mitochondrial DNA

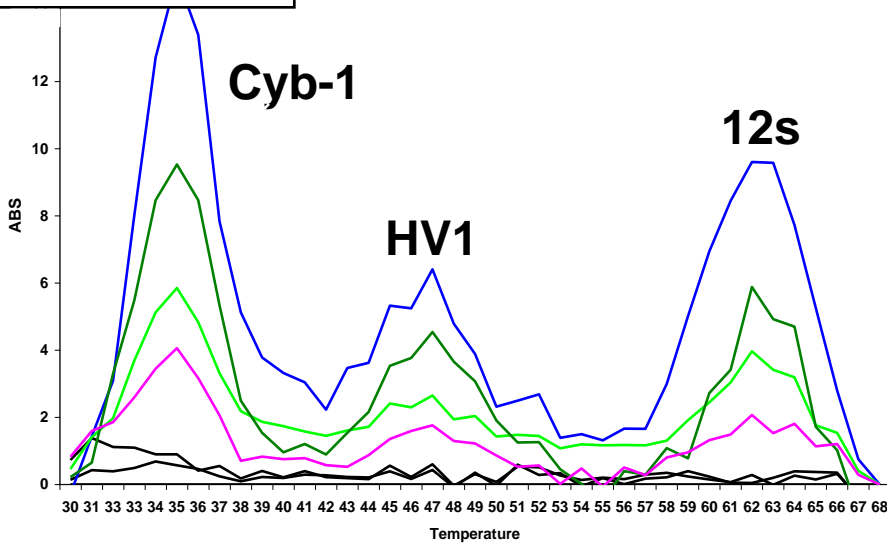
- 10 primers sets for 8 genes/regions: HV1, HV2, 12srRNA, ND1, ND4, CO1, CO2, and CytB
- Covering 30% of the mitochondrial genome



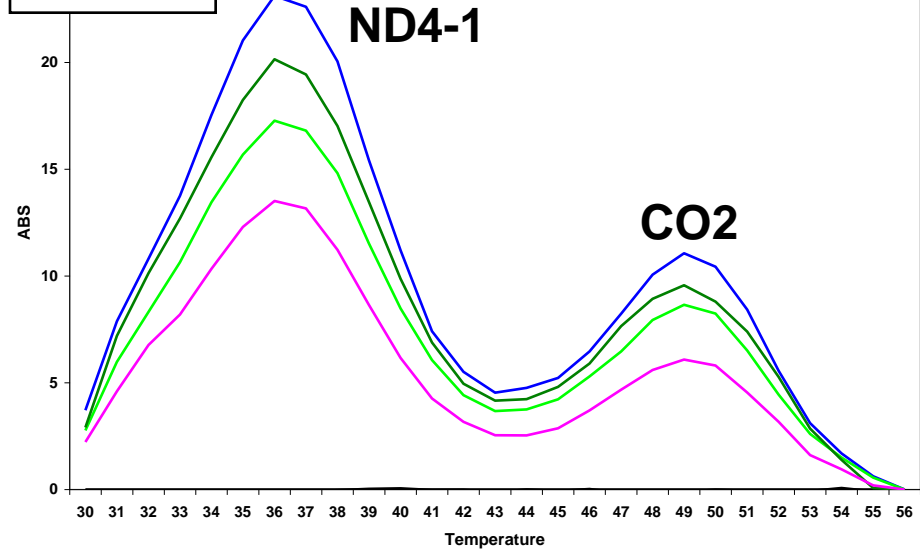
Gene	Length (bp)	Amplicon Length (bp)	% Gene Covered
HV1	420	96-590 (495)	100
HV2	300	33-440 (408)	100
12srRNA	954	106-899 (794)	83
ND1	957	197-780 (584)	61
ND4	1378	105-539 (434)	63
		804-1240 (436)	
CO1	1542	36-608 (573)	37
CO2	684	123-644 (522)	76
Cytb	1135	105-498 (394)	68
		606-983 (378)	

Amplification Products from the 10-Plex Assay

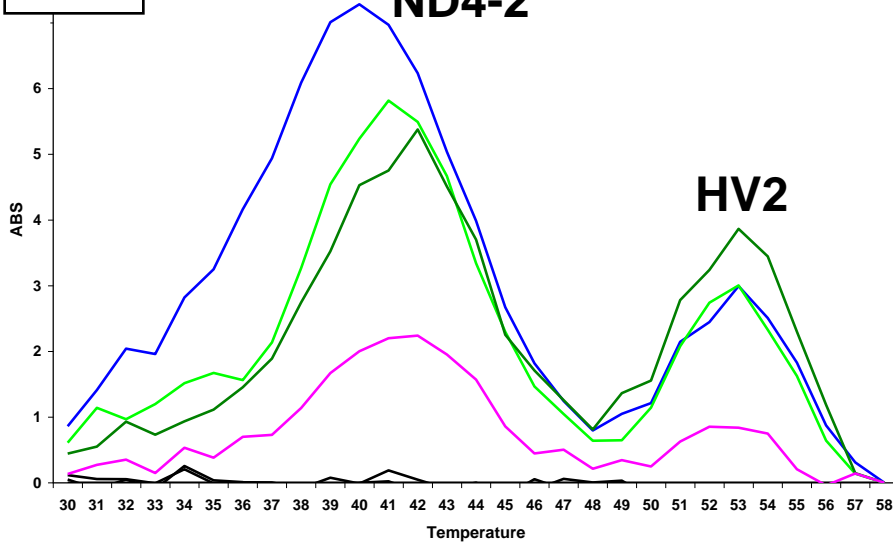
Cal Orange



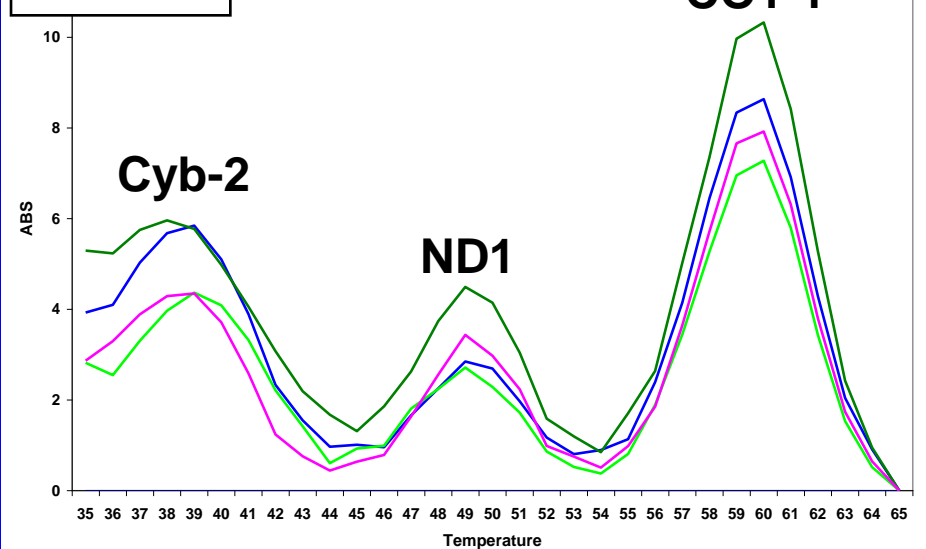
Quasar



Fam

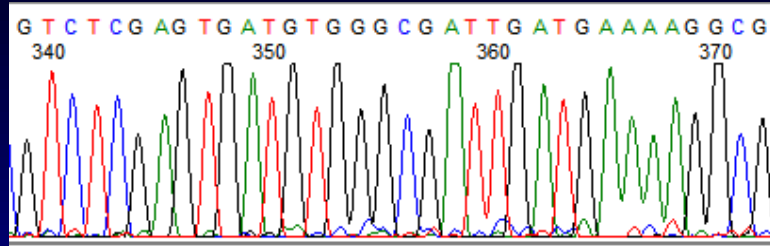
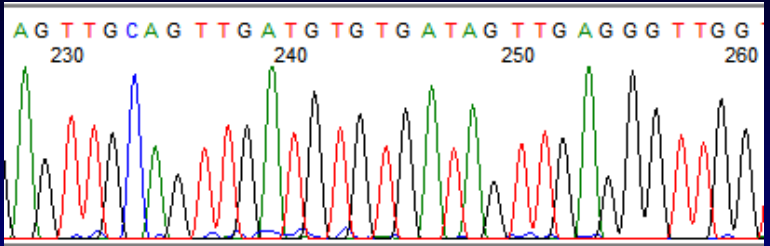


Cal Red



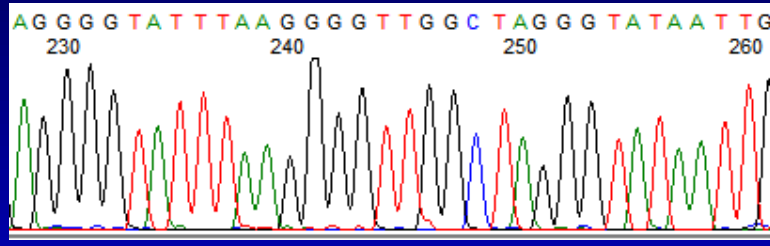
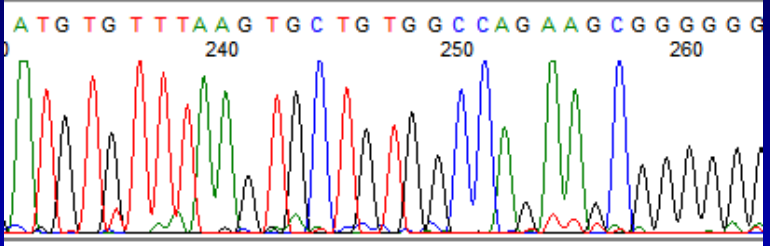
Sequencing of Individual Products from the 10-Plex

HV1



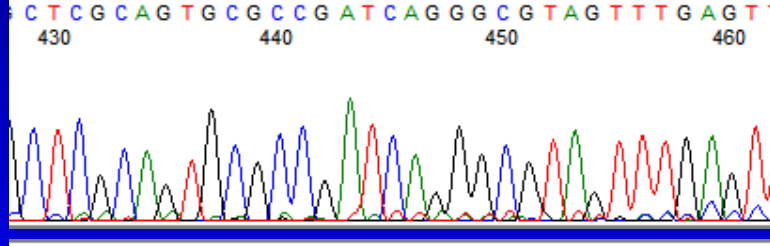
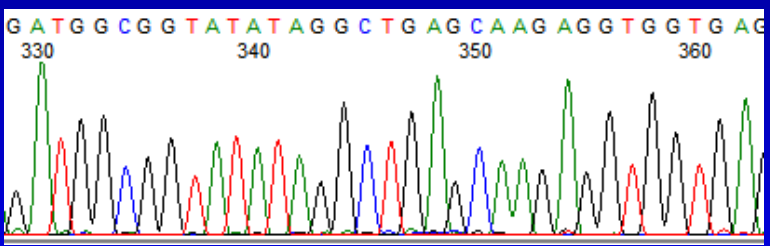
Cyb1

HV2



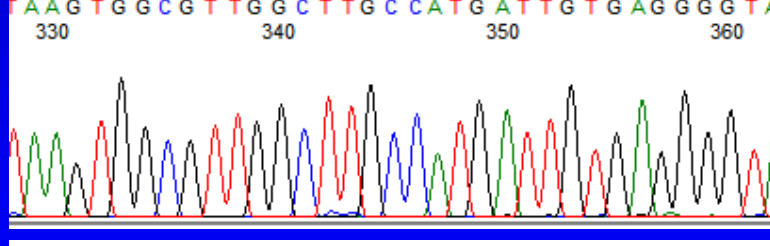
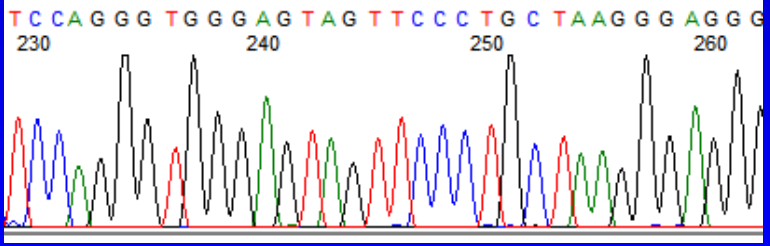
Cyb2

12s



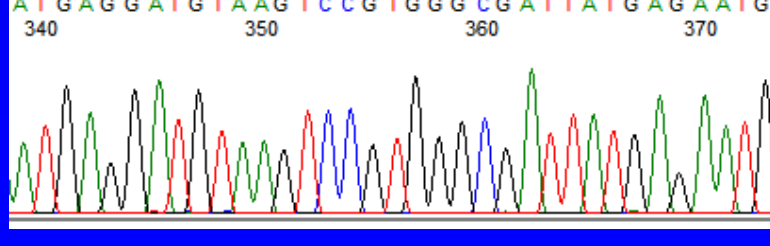
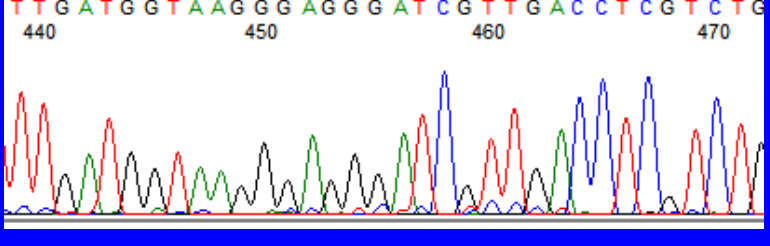
ND1

CO1-1



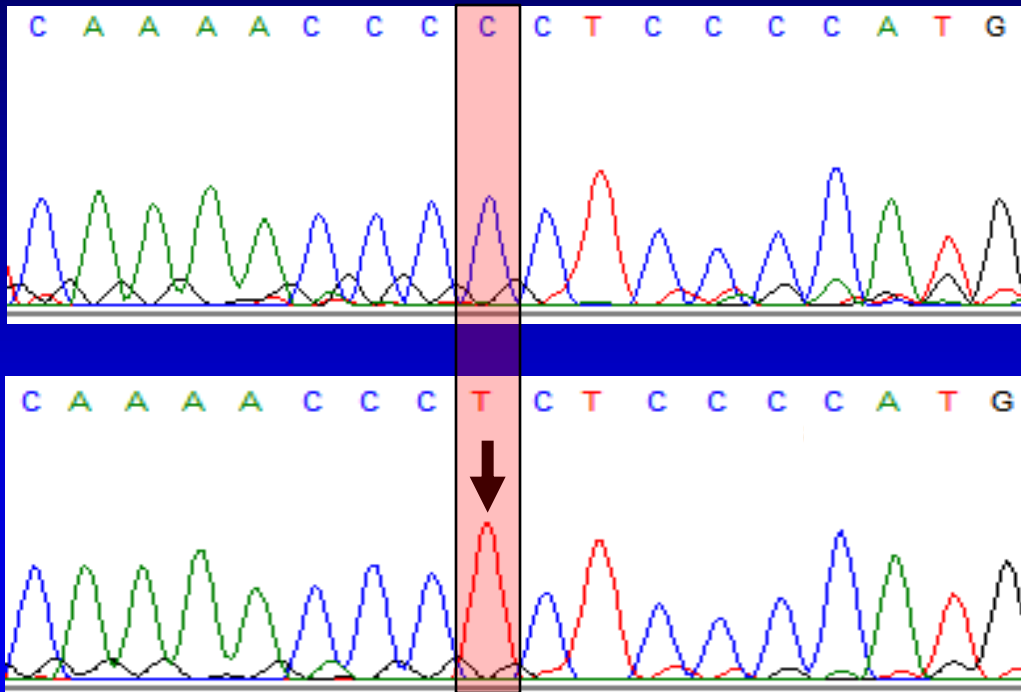
ND4-1

CO2



ND4-2

Analysis of Single Mitochondrial DNAs Identifies Random Mutations Not Seen in Bulk DNA



Total Cell Sample
1000 mtDNA copies
HV1 Gene

Single Molecule Sample
1 mtDNA copy
HV1 Gene

Osborne *et al.*, (2009) Single-Molecule LATE-PCR Analysis of Human Mitochondrial Genomic Sequence Variations, PLoS ONE 4: e5636, 2009

