

# LATE-PCR ASSAYS FOR MONITORING TRANSCRIPTIONAL RESPONSES TO HYPOXIA IN SINGLE CELLS RECOVERED FROM BARRETT'S ESOPHAGUS

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## 1. Abstract

### Abstract

Barrett's esophagus is a model system established by the Brian Reid laboratory at the Fred Hutchinson Cancer Research Center (FHRC) to study the impact of low oxygen tension (hypoxia) during neoplastic progression. We are using technologies developed at Brandeis University (LATE-PCR, PurAmp, PrimeSafe™) to construct assays to measure the transcriptional responses to hypoxia of single Barrett's esophagus cells.

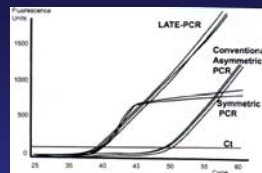
Quantitation of multiple mRNAs by conventional (symmetric) PCR requires real-time analysis and is hindered by amplification of highly abundant targets preventing amplification of rare targets. LATE-PCR is an advanced form of asymmetric PCR that enables sensitive and accurate measurement of multiple mRNA targets independently from their relative abundance in the cell in real-time or in a more convenient quantitative endpoint format. PurAmp allows quantitative mRNA preparation from single cells while PrimeSafe™ enhances the specificity and reliability of multiplexed LATE-PCR.

During hypoxia, the transcription factor HIF1 $\alpha$  up-regulates genes involved in alternative energy production (glycolysis; TP53 gene), enhanced blood supply (VEGF and Epo genes), and increased cell survival (HSP70 and p14ARF genes), among others. Two LATE-PCR assays under construction will monitor induction of these key hypoxia response genes in sets of three (set I: HIF1 $\alpha$ , TP53, p14ARF; set II: HSP70, VEGF, Epo).

We report here the successful design of the two triplex LATE-PCR assays for the above mRNAs and the on-going optimization of these assays for maximal amplification efficiency and detection sensitivity in a wide range of target copy numbers. The optimized assays will be transferred to FHRC for transcriptional analysis of the hypoxia response among single Barrett's esophagus cells.

## 2. Introduction to LATE-PCR

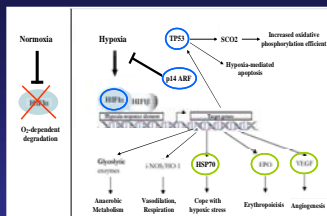
### Advantages of LATE-PCR I Improved Detection Sensitivity



- Conventional (symmetric) PCR is efficient but plateaus due to inhibition of Taq polymerase by the accumulating double-stranded amplification products.
- Conventional asymmetric PCR uses ratios of symmetric PCR primers to restrict the amount of amplified double-stranded DNA. Asymmetric PCR signals do not plateau but amplification is inefficient (delayed).
- LATE-PCR uses ratios of specially designed primers. LATE-PCR is initially as efficient as symmetric PCR but does not plateau yielding higher signals and improved detection sensitivity.

## 3. Hypoxia Assay Design

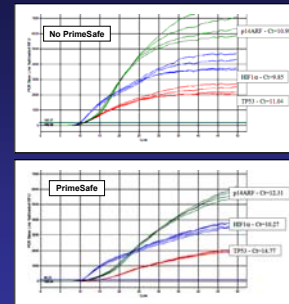
### The Hypoxia Response Targets for Multiplex LATE-PCR Assays



- Hypoxia stabilizes the alpha subunit of the HIF transcription factor. The stabilized alpha subunit joins the beta subunit and activates hypoxia-response genes by binding to hypoxia response promoter elements. The hypoxia response is negatively regulated by p14ARF.
- The two LATE-PCR assays under construction will monitor induction of the key hypoxia response genes shown here in sets of three (Set I - blue circles: HIF1 $\alpha$ , TP53, p14ARF; Set II - green circles: HSP70, VEGF, Epo).

## 4. Assay Optimization

### LATE-PCR Triplex Assay I PrimeSafe™ Optimization



PrimeSafe™ reduces signal scatter among replicates and improves linear amplification kinetics by suppressing background mis-priming events. All replicate samples are now reliable.

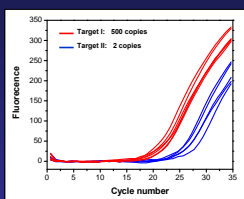
## Conclusions

- We have designed two triplex LATE-PCR assays to monitor the expression of six key hypoxia response genes (HIF1 $\alpha$ , TP53, p14ARF, HSP70, VEGF, Epo)
- The first triplex LATE-PCR hypoxia assay (HIF1 $\alpha$ , TP53, p14ARF) has been optimized for linear amplification kinetics, assay reproducibility, and maximum probe fluorescent signals.
- The components for the second triplex LATE-PCR assay (HSP70, VEGF, Epo) has been tested and the triplex reaction is currently being optimized.
- Triplex LATE-PCR hypoxia assays are currently performed on 1000 DNA targets. Future experiments will (1) test the assays in a range of target concentrations expected for mRNAs (10-10,000 copies) and (2) incorporate the reverse transcription steps prior to LATE-PCR amplification.

## Acknowledgements

- We thank Dr. Tom Paulson and Martin Suchorski from the Reid laboratory for their help in identifying the target genes for monitoring the hypoxia response.

## Advantages of LATE-PCR II Multiplex Detection of Rare and Abundant mRNAs



- In conventional multiplex PCR, double stranded amplification products from abundant mRNAs overwhelm the amplification reaction and prevent amplification of rare mRNAs targets in the same sample.
- By limiting the concentration of one member of each primer pair in the reaction, LATE-PCR enables multiplex amplification of both abundant and rare mRNA transcripts.

## Multiplex LATE-PCR Assay Design Specifications

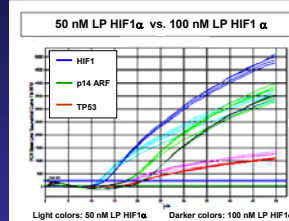
Gene	Epo	VEGF	HSP70	TP53	HIF1 $\alpha$	p14ARF	Length (bp)	Fluor
p14ARF	69.6°C	74°C	65.9°C	4.4°C	82.1°C	17.5°C	65	Quasar 670
TP53	70.3°C	73.9°C	65.4°C	3.2°C	84.3°C	14.7°C	70	Cal Red 680
HIF1 $\alpha$	76.9°C	73.9°C	64.9°C	3.8°C	82.7°C	13.8°C	76	Quasar 670
HSP70	69.8°C	73.3°C	65.6°C	3.5°C	87.6°C	17.6°C	79	Cal Red 680
VEGF	71.6°C	73.9°C	63.8°C	3.1°C	84.6°C	13.8°C	57	Quasar 670
Epo	70.6°C	73.5°C	66.6°C	2.9°C	81.4°C	18.8°C	58	Cal Orange 560

EP: Excess primer; LP: Limiting primer; Tm: melting temperature; A: Amplicon

### LATE-PCR Primer and Probe Design Criteria

- 1  $\mu$ M Excess primer; 50 nM Limiting Primer
- $Tm_{Limiting\ Primer} - Tm_{Excess\ Primer} > 3^\circ C$
- $Tm_{probe} - Tm_{Limiting\ Primer} > 5^\circ C$
- $Tm_{Amplicon} - Tm_{Excess\ Primer} < 18^\circ C$

## LATE-PCR Triplex Assay I Limiting Primer Optimization



Raising the HIF1 $\alpha$  limiting primer concentration from 50 nM to 100 nM improves the linearity of the amplification kinetics. The amplification kinetic plots of all the amplicons are now linear.

## References

### About LATE-PCR

- Rice, J.E., Sanchez, J.A., Pierce, K.E., et al. (2007) Monoplex/multiplex linear-after-the-exponential-PCR assays combined with PrimeSafe and Dilute-N-Go sequencing. *Nature Protocols*, 2:10.
- Pierce, K.E. and Wangh, L.J. (2007) "LATE-PCR and allied techniques: Real-time detection strategies for rapid, reliable diagnosis from single cells." In: *Single-Cell Diagnostics, Methods in Molecular Medicine Series* (ed. Alan Thornhill), Series Editor John Walker, Humana Press, UK.
- Pierce, K.E., Sanchez, J.A., Rice, J.E., and Wangh, L.J. (2005) Linear-After-The-Exponential (LATE)-PCR: Primer design criteria for high yields of specific single-stranded DNA and improved real-time detection. *Proc Natl Acad Sci USA*, 102:8609-8614.
- Sanchez, J.A., Pierce, K.E., Rice, J.E., and Wangh, L.J. (2004) Linear-After-The-Exponential (LATE)-PCR: An advanced method of asymmetric PCR and its uses in quantitative real-time analysis. *Proc Natl Acad Sci USA*, 101(7):1933-1938.

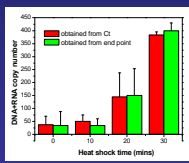
### About PurAmp and its uses

- Hartshorn, C., Eckert, J.J., Hartung, O., and Wangh, L.J. (2007) Single-cell duplex RT-LATE-PCR reveals Oct4 and Xist RNA gradients in 8-cell embryos. *BMC Biotechnol.* 7:57.
- Hartshorn, C., Anshelevich, A., and Wangh, L.J. (2005) Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. *BMC Biotechnology*, 5:2.

### About single cell genomics using conventional PCR

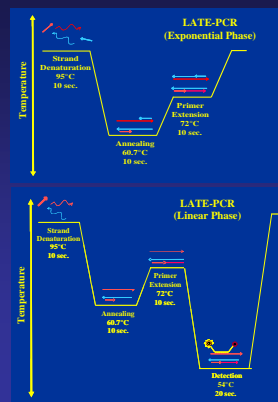
- Pierce, K., Rice, J.E., Sanchez, J.A., and Wangh, L.J. (2002) QuantLyse: Reliable DNA amplification from single cells. *BioTechniques* 32(5):1106-1111.

## Advantages of LATE-PCR III Quantitative Endpoint Analysis

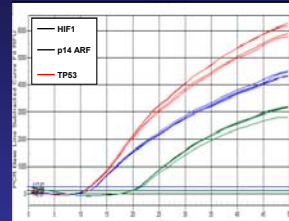


- Quantitation of heat shock mRNA in single mouse embryos
- LATE PCR exhibits linear kinetics of amplicon accumulation. As a result, endpoint fluorescent signals yield the same quantitative information on initial target numbers as C<sub>v</sub> values.

## Multiplex LATE-PCR Amplification Conditions



## LATE-PCR Triplex Assay I Probe Optimization



Redesigning the probe for the TP53 amplicon resulted in higher fluorescent signals. For that amplicon, the fluorescent signals for all amplicons are now strong.