

MEASUREMENTS OF mRNA LEVELS IN SINGLE CELLS AND SMALL GROUPS OF NORMAL AND NEOPLASTIC CELLS USING NOVEL TECHNOLOGIES

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Abstract

We are using three novel technologies invented in our laboratory, LATE-PCR, PrimeSafe, and PurAmp to construct multiplexed assays for quantitative analysis of specific mRNAs in single cells and small groups of cells. LATE-PCR is an advanced form of asymmetric PCR in which efficient exponential amplification of double-stranded DNA is followed by cycles of linear amplification of single-stranded DNA. The resulting single strands are detected using fluorescent probes.

Using these technologies we have already constructed reactions for measurement of Xist RNA, Oct 4 mRNA, Cdx2 mRNA, and heatshock protein (hsp) 70i mRNA. Duplex assays in various combinations have been used to quantify the levels of these gene transcripts in single blastomeres recovered from mouse embryos at the 4 cell and 8 cell stages of early development, as wells as from intact embryos. These assays can also measure copy numbers of the respective genes when LATE-PCR is performed without RT. These experiments serve as controls with which to validate that our methods are sensitive down to single molecules of genomic DNA in single cells. They also allow us to compare several methods of reverse transcription in order to conveniently and reliably detect very low numbers of RNA molecules in single cells.

In collaboration with Brian Reid's laboratory at the Fred Hutchinson Cancer Research Center we will use our optimized protocols to construction of duplex and multiplexed LATE-PCR assays for measurement of mRNAs levels in single normal and neoplastic esophageal cells. The following transcripts, COX-2, hsp27, p53, are of particular interest because they are known to change in response to stress stimuli like heat, low pH, and bile salts. In parallel with these studies we are also carrying out single-cell LATE-PCR assays to measure loss of heterozygosity (LOH) in the region of CDKN2A(p16) or TP53(p53) primers. These genetic changes are hallmarks of Barrett's esophagus, the precursor to esophageal adenocarcinoma.

The Logic of LATE-PCR, Figures 1-3

LATE-PCR is an advanced, efficient form of asymmetric PCR which utilizes an Excess Primer and a Limiting Primer having a higher melting temperature (Tm). Phase I amplification results in exponential amplification of a limited number of double-stranded amplicons. Phase II amplification uses one strand of the double-stranded amplicon to linearly generate single-stranded amplicon. The single-strands accumulate to up to 10-20 fold more abundant than the double-strands. Single-stranded amplicons can be detected after the extension-step of the reaction, or at end-point, using low-Tm probes that are either sequence specific or mis-match tolerant.



PrimeSafe

is a temperature dependent inhibitor of DNA polymerase that acts throughout PCR amplification to suppress all forms of mis-priming. PrimeSafe® also makes it easier to construct multiplexed assays.



PurAmp

is a single tube method for lysing cells, reverse transcribing RNA, and amplifying cDNA using a serial dilution. PurAmp reduces the risk of sample loss and/or contamination.

