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Short Communication

Application of gene specific mRNA level determinations in individual cells using flow cytometry-based PrimeFlow™ in immunotoxicology

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ABSTRACT

Determining changes in gene expression by measuring mRNA levels is an important capability in biological research. Real-Time Quantitative PCR (RT-qPCR) is the most ubiquitous technique for measuring changes in mRNA transcript levels, but heterogeneity of cell populations and low cell number are serious technical limitations. Recent advances in flow cytometric analytical techniques have enabled the quantification of mRNA levels in individual cells. Here, we present examples demonstrating the strength and challenges of concurrently measuring mRNA using PrimeFlow™ with other endpoints in immunotoxicological studies. Specifically, we demonstrate how measuring gene specific mRNA levels on a per cell basis was used to study: 1) markers of activation and differentiation; 2) cell signaling by measuring intracellular proteins in mature and developing cell types; and 3) a cell type that constitutes a minor population in peripheral blood. We also discuss cell type-specific modifications to the parent technique, which facilitated optimal performance in these cells. While the examples provided are focused on immunotoxicological questions and endpoints, this new strategy can be applied to a wide variety of toxicological research problems.

1. Introduction

Determining differences in gene expression by way of measuring changes in mRNA is central to mechanistic immunotoxicological investigations. The regulation of gene expression is involved in almost every aspect of immune responses, including: 1) cytokine production and cell surface receptors expression after activation (Chen et al., 2012; Phadnis-Moghe et al., 2015; Henriquez et al., 2017; Li et al., 2017); 2) clonal expansion (Sablitzky et al., 1985; van Stipdonk et al., 2001); and 3) terminal differentiation into mature effector and memory cell types (Rissoan et al., 1999). The most ubiquitous and reliable method of gene transcriptional analysis is Real-Time Quantitative PCR (RT-qPCR). RT-qPCR has become the gold standard for quantifying target gene specific mRNA levels in biological research (Giulietti et al., 2001).

Despite the prevalence and utility of RT-qPCR, this technique has several limitations including: 1) the need for large numbers of cells to obtain adequate amounts of mRNA for accurate quantification; 2) the inability to quantify gene expression in a specific subpopulation of cells within a heterogeneous cell preparation; and 3) the inability to measure protein and mRNA concurrently. In particular, studying transitional cell types undergoing development is a major challenge for immunotoxicological studies. Assessing the expression of the key transcription factors in distinct developmental stages is not feasible using RT-qPCR due to the heterogeneity of the cell population and the inability to concurrently measure mRNA and cell-stage specific markers. Furthermore, the concerns of inadequate cell number and mixed cell preparations are even a consideration when measuring mature immune cell populations. For example, plasmacytoid dendritic cells (pDC) and innate lymphoid cells (ILC) type 2 compose < 0.5% of peripheral blood mononuclear cells (PBMC) (Chang et al., 2011; Henriquez et al., 2017). Due to their rarity, obtaining an adequate number of cells to reliably quantify mRNA levels using traditional RT-qPCR is challenging and endpoints are often limited.

Recent advances in flow cytometric analysis have enabled the simultaneous analysis of protein and mRNA on a per cell basis. One of the first commercially available strategies is the Quantigene® PrimeFlow™ RNA assay by Affymetrix/Thermo-Fisher Scientific™ (Waltham, MA/Santa Clara, CA). This flow cytometry-based assay enables simultaneous quantification of gene specific mRNA levels and intracellular/membrane bound proteins and is already considered a valuable tool in

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immunological studies (Frank et al., 2015). Furthermore, the ability to identify cell populations using well defined surface proteins eliminates the need for cell purification and/or bulk lysis, thereby enabling analysis of gene specific mRNA levels in minor and transitional cell populations. In this way, significant insight into the mechanisms by which xenobiotics alter gene expression can be gained with minimal manipulation of the cells.

The overall objectives of this article are: 1) to raise awareness within the scientific community of this new and powerful technology; 2) to present examples on how this technology can be applied; and 3) to provide generalized guidance on key challenges and limitations of PrimeFlow™ technology that we have experienced. In the following sections, we illustrate how employing this new methodology enabled the study of toxicant-induced modulation of gene specific mRNA levels, which would not have been feasible using conventional RT-qPCR. Specifically, we show how PrimeFlow™ can be used to quantify: 1) gene specific mRNA level changes in a transitional cell population; 2) simultaneous gene specific mRNA levels with intracellular and cell surface proteins; and 3) expression of gene specific mRNA levels in a rare population of cells. We also highlight key parameters of the manufacture's protocol that are critical for assay success under “Materials and Methods” and include examples where modifications to the standard protocol facilitated optimum mRNA detection.

2. Materials and methods: critical conditions for PrimeFlow™ assay determinations

The most reliable results were obtained when PBMC were isolated from whole blood via Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient centrifugation. Cell number was also found to affect mRNA quantification such that 2×10^6 cells (human HSC, naïve B cell, or PBMC)/test produced optimum results.

The PrimeFlow™ assay was performed per the manufacturer's suggested protocol (available through the Thermo-Fisher™ website) and visualized in Fig. 1, except where indicated in the sections below. Overall, maintaining a consistent temperature of 40 °C to ensure successful amplification for the target mRNA was found to be the most critical condition within the protocol. To this end, placing a heating block in an incubator, both set to 40 °C, provided the best results. It is noteworthy that the *RPL13A* internal positive control can also be used as an indicator of cell viability, since standard methods of viability detection may not be compatible with PrimeFlow™.

The combination of PBMC isolation followed by treatment and analysis by PrimeFlow™ typically required > 11 h to complete from initiation to completion. To facilitate a more manageable workflow, we found that the best stopping point was after the hybridization step with the target probe (Fig. 1). Stopping the procedure at this stage and storing the cells overnight at 4 °C resulted in no loss of mRNA signal quality compared to continuous completion of the protocol.

2.1. Measuring changes in gene specific mRNA levels in transitional cell populations

The well-orchestrated development of hematopoietic stem cells into immunocompetent cells is regulated by the sequential expression of cell-stage specific transcription factors. Quantifying perturbations in expression of these transcription factors represents a major advancement toward elucidating the molecular mechanisms underlying the developmental immunotoxicity of xenobiotics. The ability of PrimeFlow™ to measure gene specific mRNA on a per cell basis enables quantification of cell stage specific gene expression in transitional cell populations during hematopoiesis. For instance, we have reported that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) treatment impairs the development of human hematopoietic stem/progenitor cells (HSPC) to lineage committed B cells (Li et al., 2017). To study the molecular mechanisms underlying the impaired B cell development, PrimeFlow™ was used to measure the changes in mRNA expression of lineage specific genes in the HSPC-derived heterogeneous cell population. We found that treatment with TCDD (1 nM) significantly suppressed the mRNA levels of Early B-cell Factor 1 (*EBF1*), a critical transcription factor that regulates B cell development (Fig. 2A–B).

To further characterize the *EBF1*-expressing cell population, we concurrently measured the intracellular protein level of CD79α, a cell marker demarcating early-B and pro-B cells. During the PrimeFlow™ staining procedure, we noticed that fixation after intracellular staining (Fixation II) was required to minimize the loss of intracellular protein detection during the subsequent RNA staining procedure (Fig. 2C–D). However, loss of mRNA amplification was observed after fixing with Fixation Buffer II (Fig. 2C–D). To obtain both optimal staining of intracellular proteins and retain RNA detection, the protocol was altered by either: 1) reducing the concentration of Fixation Buffer II by half and fixing for the recommended amount of time; or 2) fixing the cells with the recommended concentration of Fixation Buffer II for half the incubation time stated in the original protocol (Fig. 2E–F). The co-expression of *EBF1* mRNA and CD79α (Fig. 2E–F) suggests that the expression of *EBF1* is B cell lineage specific. The ability to quantify both

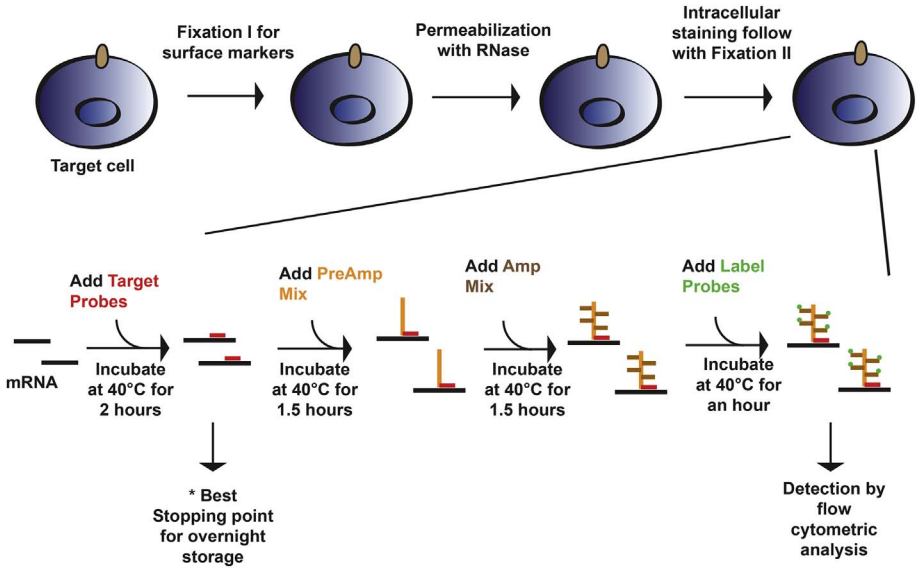

Fig. 1. Overview of the standard PrimeFlow™ assay protocol. Cells are first fixed with fixation buffer I, stained for surface markers, then permeabilized and treated with RNase inhibitors. Cells are then stained for intracellular markers and further fixed with fixation buffer II. To amplify the target mRNA, target probes are hybridized to the mRNA of interest. A preamplifier (PreAmp) oligonucleotide is then bound to the target probe/mRNA dimer followed by the binding of amplification (Amp) probes. Finally, fluorescent dye labeled probes are bound to the Amp probes and detected through flow cytometry.