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Highly multiplexed quantitation of gene expression on single cells

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Abstract

Highly multiplexed, single-cell technologies reveal important heterogeneity within cell populations. Recently, technologies to simultaneously measure expression of 96 (or more) genes from a single cell have been developed for immunologic monitoring. Here, we report a rigorous, optimized, quantitative methodology for using this technology. Specifically: we describe a unique primer/probe qualification method necessary for quantitative results; we show that primers do not compete in highly multiplexed amplifications; we define the limit of detection for this assay as a single mRNA transcript; and, we show that the technical reproducibility of the system is very high. We illustrate two disparate applications of the platform: a “bulk” approach that measures expression patterns from 100 cells at a time in high throughput to define gene signatures, and a single-cell approach to define the coordinate expression patterns of multiple genes and reveal unique subsets of cells.

Keywords

Single cell; Gene expression; T-cell activation; qPCR

1. Introduction

Cellular processes require the intricate coordination of expression of many gene products. Increasingly, our understanding of complex biologic systems depends on the ability to distinguish the genes critical in a cellular process from the myriad of other expressed transcripts. Determining the coordinate expression patterns for multiple genes uniquely defines cellular (and pathophysiological) states than cannot be achieved by measuring a single marker (Seder et al., 2008; Bedognetti et al., 2010). For these reasons, there is a need for methods that simultaneously measure many gene transcripts from a single cell.

Since its introduction over ten years ago, microarray technology has been used in a wide variety of settings (Shaffer et al., 2001; Caskey et al., 2011; Reddy et al., 2012) to identify gene signatures that describe cellular, disease, or vaccine processes. The power of

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microarrays lies in the high degree of multiplexing: more than 40,000 gene transcripts are analyzed from a single sample. However, typical methods require large numbers of cells (on the order of 10^6) or substantial nonlinear pre-amplification, limiting the sensitivity and utility of this technology. Importantly, information is lost about coordinate regulation of genes within a cell.

A system that combines the sensitivity and utility of single-cell qPCR with the multiplexed capabilities of micro-array analyses is therefore valuable. The Fluidigm Dynamic Array (or BioMark™ system) for single-cell gene expression was recently developed to address this need; the assay is performed on 96 samples simultaneously, and can measure 96 (or more) genes on each sample. It has been used recently in disparate biological settings (Flatz et al., 2011; Narsinh et al., 2011b; Citri et al., 2012); however, methodologic details for optimal and quantitative application of this technology have not been detailed.

Here, we describe methods for qualifying the TaqMan™ primers and probes used in the BioMark™ technology. We determined the optimal methodological parameters ensuring quantitative results, and defined quantitative aspects of assay performance to show, for example, that the limit of detection is a single mRNA transcript. We also investigated whether “endogenous controls” provide useful information in this setting.

We illustrate applications of this technology to immunologic assessments. We show that the measurement of small “bulk” (100-cell) samples, which we term “pooled-cell array,” provides remarkable sensitivity for identifying gene signatures. Finally, we demonstrate the unique power of this technology by applying it to single-cell samples. In particular, we identify genes that are modulated following T-cell activation, and illustrate the heterogeneity of gene expression patterns by activated T-cells.

2. Methods

2.1. Biological samples

PBMC were used from human and nonhuman primate specimens. Human specimens were obtained from fully anonymized donors and used under IRB (NIAID, NIH) exemption. NHP specimens were obtained from our cryo-repository of NHP specimens; all specimens were collected as part of IACUC (VRC, NIH)-approved studies.

2.2. Primer selection

Assay targets were selected based on their relevance to T-cell immunity, and included genes for T-cell homing, cytokines, cytolytic enzymes, apoptosis, signal transduction, and transcription factors. Primers were selected from the catalogue of TaqMan™ primers/probes qualified for qPCR (Life Technologies, Carlsbad, California). Where multiple primer/probe sets were available for the same transcript, the initial selection was based on: 1) crossing exon boundaries, to ensure that mRNA was amplified, 2) size of amplicon (140 bp or less), and 3) number of reference sequences. These criteria could not always be met, nor did they guarantee a primer/probe set would qualify.

2.3. RNA isolation for bulk and single-cell analyses

RNA was obtained from peripheral blood mononuclear cells (PBMC) or from cell subsets isolated by flow cytometric cell sorting. RNA was purified for bulk analyses using the QIAGEN RNeasy™ kit (Qiagen, Hildebrand, Germany). For single-cell experiments, cells were stained with a panel of fluorescent monoclonal antibodies (to identify various cell subsets, described below) and sorted directly into cell plates loaded with the reaction mix necessary for reverse transcription and cDNA synthesis.

2.4. Reverse transcription and cDNA synthesis

Reverse transcription and cDNA synthesis were performed using the Invitrogen Cells Direct Kit™ (Life Technologies), per manufacturer's instructions. The optimal number of cDNA synthesis cycles was determined as described in the Results section.

2.5. BioMark™ data analysis

Forty cycles of PCR were performed, using the microfluidic chip developed by the manufacturer. The instructions provided for the assay were followed, with the exception that "failed" reactions (defined by the instrument's internal algorithms) were verified manually. In some cases, fluorescence data displayed characteristics of logarithmic amplification, and the expected "Ct" (threshold cycle) was achieved, so these reactions were designated "passed." For ease of data interpretation, "Et" (threshold expression) values are reported throughout the manuscript; these values are equal to 40-Ct, and thus increase with greater expression of a transcript; they are proportional to $\log_2$ RNA abundance.

2.6. Primer qualification

A primer qualification routine was developed to ensure three quantitative relationships held: (1) efficient amplification (*i.e.*, the signal doubles with each PCR cycle); (2) linearity (*i.e.*, the final signal is proportional to input RNA); and (3) multiplexing capability (*i.e.*, simultaneously quantifying multiple primers does not impact efficiency or linearity of any given set).

Qualification of primers is dependent on the number of mRNA transcripts present in the sample used for qualification. Therefore, the range of dilutions necessary for qualification differs dramatically between cell populations and primers. Thus, we use a mixture of bulk mRNA isolated from (*e.g.*) stimulated and resting T-cells for qualification. Furthermore, qualifications were performed with 12-point, two-fold dilution series (replicated eight times across a plate), segments of which were analyzed for linearity as follows.

Et values were plotted against the RNA quantity (expressed on a log scale; shown for a representative primer; Fig. 1). Every replicate, 12-dilution series was divided into seven sequential segments, each comprising six consecutive dilutions. Segments were evaluated in a step-wise, iterative manner, beginning with the lowest RNA amount (Fig. 1). For each segment, the linear least-squares correlation between the logarithm of the RNA amount and amplification cycles (Et; 40-Ct) was determined. The segment passed if the correlation was strong ($r^2 > 0.99$), and if the slope of this fit indicated a linear relationship between signal (2^{Et}) and RNA. If amplification was 100% efficient, the slope would equal 3.32 ($1/\log_{10}2$). The efficiency (E) was calculated from the formula: $E = 10^{1/\text{slope}} - 1$. We accepted slopes between 3.1 and 3.6 ($E = 90\text{--}110\%$). Preliminary experiments established that more primers qualified by this piecewise method (compared to using the whole series of dilutions), because of the wide variation in transcript levels for different genes.

2.7. Rhesus cross-reactivity

Peripheral blood mononuclear cells (PBMC) from Rhesus macaques were stimulated with 10 nM PMA and 1 $\mu\text{g/ml}$ ionomycin for 6 h, after which bulk RNA was isolated. Primer/probe sets designed for detection of human transcripts were qualified on Rhesus RNA, using the piecewise method described above. Similarly, primer/probe sets designed for Rhesus transcripts were tested on RNA samples isolated from human or NHP PBMC.

2.8. Discriminating leukocyte subsets by BioMark™

The ability to distinguish leukocyte subsets was tested as a means to establish the specificity of the technology, and to illustrate the power of the pooled-cell array approach. Human PBMC were stained with a panel of antibodies against cell surface markers that discriminated hematopoietic lineages. Cells were sorted by flow cytometry, based on expression of the following markers: T-cells (CD3+ CD20–), B-cells (CD3– CD20+), and monocytes (CD3– CD20– CD14+). For each cell population, a seven-point titration of number of cells sorted was performed, as follows (the number of replicates for each sort is indicated in parentheses): 100 cells (14), 50 cells (6), 25 cells (6), 12 cells (6), 6 cells (6), 3 cells (6), and 1 cell (15).

2.9. T-cell activation experiments

PBMCs were treated with Staphylococcal enterotoxin B (SEB) or medium (control) for time periods ranging from three to 12 h, in the presence of anti-human CD154 PE antibody (Chattopadhyay et al., 2005). Cells were then washed and stained with a panel of FITC- or APC-conjugated antibodies reactive to T-cell receptor (TcR) V families that are either known to bind SEB or known to be nonresponsive to SEB. Preliminary experiments based on intracellular cytokine staining demonstrated that cytokine and activation proteins were expressed primarily by the CD154+ SEB-responsive cells, and none by the CD154– SEB-nonresponsive cells. CD4+ T-cells were sorted based on TcR V and CD154 expression, into individual wells pre-loaded with primers, polymerase, and other reaction components for reverse transcription and BioMark™ analysis.

2.10. Flow cytometry

Flow cytometric cell sorting was performed on a 20-parameter FACS Aria (BD), running FACSDiVa software (version 6.1.3, BD) to allow indexed sorting. B cells, T-cells, and monocytes were discriminated by staining PBMC with the following fluorescently-labeled, mouse anti-human monoclonal antibodies (conjugated in-house): anti-CD20 Cy5PE, -CD14 Alexa 700, -HLA-DR Alexa 700PE. In addition, the staining panel included the following commercial reagents: anti-CD3 Cy7APC (BD), -CD11c APC (BD), -CD123 PE (BD), and Aqua Blue LIVE/DEAD Fixable Dead Cell Stain (Invitrogen). T-cell activation experiments were performed with the following panel of antibodies (conjugated in-house, unless otherwise indicated): anti-CD3 Cy7APC (BD), -CD4 QD605, -CD8 QD705, -CD154 PE (BD), and -TcR V families (responsive V 12, 14, and 17 APC; nonresponsive V 1, 2, and 13.6 FITC). FlowJo v9.5 (Ashland, OR) was used to analyze the data.

2.11. Statistics

Statistics were performed using JMP version 10. Processing and automated curve fitting for qualification of primer/probes were performed with JMP scripts, which have been adapted to R (both are available by request).

3. Results

3.1. Linearity and sensitivity of multiplexed qPCR

3.1.1. Primer qualification—Although TaqMan™ primer/probe sets are nominally tested by the manufacturer for standard qPCR assays, reaction volumes and conditions in the BioMark™ system are significantly different. Therefore, it is important to qualify all primers for this system. Our initial tests showed that primers targeting highly abundant transcripts qualified with highly diluted bulk mRNA; the Et saturated with increasing concentrations of template RNA (e.g., CD7 and MAPK3, Fig. 2A). However, transcripts with low expression required initial template concentrations that were several logs greater

before the linear relationship with signal (necessary for primer qualification) was observed (*e.g.*, SFRP3 and XBP1, Fig. 2B). Because transcript abundance may not be known *a priori*, we developed a piecewise method for primer qualification (see Methods) that tested the efficiency of primer amplification across a wide range of concentrations. Primers qualified for the assay if at least one piecewise segment demonstrated 90–110% efficiency, *i.e.*, demonstrating exponential amplification and a linear dependence on input material over a three-log range.

When this method was applied to a primer set interrogating genes associated with T-cell immunity, 73% (572/788) of TaqMan™ primers qualified. Notably, we found that most transcripts of interest were present in samples stimulated with the mitogens PMA + ionomycin or SEB (93%), but 5% of the primers selected were unique to unstimulated samples. Hence, it is important to use a mixture of RNA from multiple sources when qualifying primers, to ensure that all primers being tested have template material for assessment. Summary data for all primers tested is included in Supplementary Table 2.

3.1.2. Primer competition—Early studies developing multiplexed, single-cell gene expression were limited by a loss of PCR fidelity, caused by primer competition. Therefore, we designed experiments (Fig. 3) that compared the expression of eight targets in samples reverse transcribed and pre-amplified with: a) single primers for each target, b) a set of primers for all eight targets, and c) sets of 24 or 96 primers that included the original eight targets (plus additional genes).

Experiments were performed with bulk RNA, using limiting amounts equivalent to a single cell or to 100 cells (to mimic conditions where template is limited vs. relatively abundant). Et values did not differ significantly across replicates of the four samples that were reverse-transcribed in the presence of one, eight, 24, or 96 primers (Fig. 3). These data demonstrate that the primers and probes can be multiplexed for this assay.

3.1.3. Amplification necessary for single copy detection—The BioMark technology requires sufficient cDNA to distribute material equally into all 96 microfluidic PCR reaction chambers, so that individual amplification with each of 96 primer/probe sets is possible. Thus, some amplification of cDNA using all 96 primer pairs is necessary prior to loading onto the microfluidic chip (“pre-amplification”). Theoretically, about 1/2000th of the total pre-amplified cDNA is assayed in each qPCR microchamber; thus, even distribution of a single original mRNA into every chamber should require at least 12 preamplification cycles (resulting in 4096 amplicons loaded on the chip). We sought to determine the optimal extent of this initial amplification round; because differences in efficiencies are exponentially compounded with each PCR cycle, we chose the minimum number of cDNA pre-amplification cycles that allowed for reliable detection of a single transcript. Note, this amplification step is distinct from the amplification performed on the chip for determination of Et values. In particular, all 96 genes are co-amplified during this stage; on the chip, only one gene is further amplified in each nanowell.

Serial dilutions of RNA were reverse transcribed, and amplified for 9, 12, 15, or 18 cycles. cDNA products were loaded into BioMark™ chips; each of six representative transcripts present at varying concentrations in T-cells was amplified in 12 different channels on the chip. If pre-amplification was sufficient, then all 12 assays for each gene should have identical values; whereas if some of the 12 replicates did not amplify, then there was insufficient preamplification to deliver material equally to all wells. Fig. 4 demonstrates that, whether the target had low (*e.g.*, FoxP3) or high (*e.g.*, CCR7) expression, 18 cycles of cDNA synthesis reliably provided enough template for detection – in that essentially all replicates of a pre-amplified product would be positive.

Data from this experiment can also be used to ensure that the assay is capable of single copy detection (i.e., to confirm the limit of detection), as well as to define the signal achieved at the single-copy level. Poisson sampling predicts that, when a single mRNA copy is present in the starting material, 37% of reactions will receive zero copies on average after cDNA synthesis and amplification, resulting in failed reactions (Rawer et al., 2003).

At high RNA concentrations (>100 ng RNA; approximately 1000 cells), all four pre-amplification conditions (i.e., 9–18 cycles) yielded 12 positive reactions, as shown in Fig. 4A. However, at intermediate concentrations (0.1 ng RNA; 10 cells), six or more reactions for CD40LG, IFN γ , and FoxP3 failed after nine cycles of pre-amplification. At the lowest concentrations (<10 pg RNA; 1 cell), 18 cycles of amplification were required to provide sufficient cDNA for positive signals (Fig. 4A). Thus, the BioMark™ was capable of single copy detection from RNA quantities typically found in single cells, provided 18 cycles of cDNA synthesis were performed. When we examined the distribution of Et associated with occasional amplification (i.e., at the limit of detection), we found that an Et of 13.7 (range 12.4 to 15.1) corresponded to a single copy. (Fig. 4B). This Et corresponds to a total of 44 cycles of amplification of the original material (18 cycles of pre-amplification plus 26.3 on the chip).

3.1.4. Reproducibility—We next investigated technical variation in these assays. When replicate dilution series of bulk RNA were compared (Fig. 5), the difference rarely exceeded 5%, and this only occurred at the lowest Et (where initial mRNA copy number was limited). Indeed, the CV for 99.5% of the replicate assays was below 10% (Fig. 5). Given that biological variability is usually greater than 10%, variability in final analyses is unlikely to be technology-dependent.

3.1.5. Rhesus cross-reactivity—Because of the importance of *Rhesus macaque* animal models of human disease (particularly HIV), we tested the cross-reactivity of primers designed for human sequences. Primers varied in their cross-reactivity (Fig. 6A, Supplemental Table 2), with some (e.g., CD14) exhibiting similar amplification of human and Rhesus transcripts, while others were barely detectable in *Rhesus* (e.g., CCR4) or exhibited non-linear or inefficient in amplification (e.g., LTB).

When cells were studied (in the place of bulk mRNA), similar results were observed (Fig. 6B). For example, IgD exhibited similar amplification patterns in *Rhesus* and humans, TLR4 showed greater signal in *Rhesus*, and CD32 demonstrated reduced expression in B cells (but not monocytes). Thus, the cross-reactivity of primers designed for human sequences in *Rhesus* is unpredictable; they must be qualified in the species of interest using cell samples or bulk mRNA that contain detectable levels of the target. Summary results from our qualification and cross-reactivity tests are presented in Table 1 and Supplemental Table 2.

3.2. Application of BioMark™ to leukocyte studies

3.2.1. Relevance of endogenous controls—We examined the expression of genes that are theoretically expressed uniformly across all cells (often termed “endogenous controls” or “housekeeping genes”), in order to determine whether normalization improved quantification. Fig. 7A shows the distributions of Et values for five housekeeping genes tested for 5997 single cells. Among the single cells, values varied dramatically (4–20 Et) for the same housekeeping gene. Most importantly, however, expression levels did not correlate even between housekeeping genes with the least variation (ACTB vs. TBP, Fig. 7B), as would be required for normalization controls. Thus, normalization by housekeeping genes in single-cell assays is useless, since mRNA levels vary dramatically from cell to cell (as was

also shown recently (Livak et al., 2013)) and the control genes are not consistent with each other (McDavid et al., 2013).

Notably, unlike bulk qPCR, the precise number of cells deposited into a reaction is precisely determined by the fluorescence-activated cell sorter, obviating any need to control for input material. With single-cell deposition, we find signal in >98% of wells; the small CV's observed for replicate 100-cell depositions (Fig. 5) further support our finding that no normalization for input mRNA level is necessary.

3.2.2. Two modes of BioMark™ analysis—Our tests with bulk mRNA indicated that the platform has sufficient dynamic range for the assay using even 1000-fold more mRNA than what is found in a single cell. This led us to characterize the performance of the assay for “bulk” samples of 100 cells. This requires no changes to the reagents or amplification conditions used for single cells; 100-cell and single-cell samples can be analyzed on the same chip.

3.2.3. Pooled-cell array analysis—Analyzing the expression of 96 genes from 100 cells is akin to a scaled-down microarray experiment (hence the name “pooled-cell array”); on the BioMark™ chip, 96 such samples can be assayed simultaneously. To test the utility of the pooled-cell array application, we assayed samples with varying numbers of pure sorted T-cells, B-cells, or monocytes, and asked if a gene expression signature specific to each cell type could be identified. Unsupervised hierarchical clustering (Ward's minimum variance method) correctly discriminated the three cell lineages based on their gene expression profiles (Fig. 8). A subset-specific profile was recognized across all points in the cell titration, demonstrating that the assay can specifically discriminate cell populations, even at the single-cell level. However, the number of genes contributing to this profile decreased with lower numbers of cells. This is a consequence of the limited expression for some transcripts (*i.e.*, not all cells in a lineage express a given gene); thus, the signal for such genes only surpasses the limit of detection when higher numbers of cells were analyzed. This illustrates an important advantage of the pooled-cell array approach: rare transcripts are more likely to be captured, and the power to identify differences inherent to populations of cells is increased.

However, the pooled-cell array provides expression data for mixture of cells (like microarray), not single cells. Because of this, the co-expression of genes cannot be ascertained. This is illustrated in Fig. 9A, where expression of various genes was examined within 100-cell sorts of CD4+ T-cells from each of 50 individuals. Gene combinations, like CXCR5/CCL5, DPP4/TIA1, and IL16/TNFRSF25, appear to be uniformly co-expressed in these bulk samples. In contrast, when single CD4 + T-cells isolated from each of 10 individuals were tested, co-expression of these molecules was rare – and, in fact, the discordant expression of these genes thereby identifies unique subsets of CD4 + T-cells. Because the bulk cell sorts mix a variety of cell types into a single well, distinct expression of genes is lost and the frequency of co-expression does not reflect the biology of single cells.

Notably, when data was pooled from these single-cell samples and correlated to the signal from 100-cell samples of the same individuals (Fig. 9B), a strong correlation was observed. The line of fit has a slope of approximately 1.0. Discordance between 1 and 100-cell analysis was only evident at Et values below ~13 (corresponding to 1 mRNA molecule). We conclude that there is no loss of sensitivity or accuracy in single-cell analysis compared to 100-cell analyses.

3.2.4. Single-cell analysis reveals heterogeneity of expression—Highly multiplexed, single-cell qPCR technologies may provide a sensitive platform for monitoring T-cell activation or function. We stimulated PBMC with Staphylococcal enterotoxin B (SEB), which binds particular T-cell receptor (TcR) V chain variants; these variants are expressed by a subset of T-cells (approximately 5–20%, depending on the donor). As a consequence, T-cells bearing SEB-binding TcR become activated (SEB-responsive T-cells), while T-cells expressing other, non-SEB-binding TcR remain resting (SEB-nonresponsive T-cells). In a short-term stimulation assay, activated vs. non-activated CD4 T-cell populations can be identified by their expression of CD154 protein (Chattopadhyay et al., 2005).

We compared SEB-responsive CD154⁺ to SEB-nonresponsive CD154[−] T-cells to study genes modulated with stimulation. This analysis revealed a set of genes strongly associated with T-cell activation. Among the 75 measured (Supplemental Table 1), differential expression was statistically significant (after Bonferroni's multiple comparisons adjustment, $p < 0.006$) for 24 genes. Of the differentially expressed genes, 12 were strongly up-regulated within stimulated (responsive, CD154⁺) cells (Table 2). These genes included those known to be associated with T-cell activation and function (*e.g.*, CD154, IFN γ , IL2, and TNF), as well as markers not commonly assayed in immune monitoring studies (*e.g.*, SLAMF1/F5, TBX21, and CD109). Five additional genes showed modest up-regulation (in part because they were more frequently observed in resting cells, Table 2); these included CD48, CD3d, ICOS, IL6ST, and CD69. Finally, four genes were down-regulated among activated cells: CCR7, CXCR4, IL16, and CD5 (Table 2). Down-regulation in a 6 hour assay is remarkable, in that it reflects not only shutting off transcription, but degradation of the existing mRNA.

Fig. 10 shows the expression of these markers on a cell-by-cell basis. Unsupervised hierarchical clustering (Ward method) faithfully segregates activated from resting cells, based on expression of the 24 genes identified in Table 2. (Only five out of 163 total cells were misclassified.) Various subsets of activated cells can be identified from Fig. 10B, including populations of cells expressing IL2/IFN γ /TNF mRNA simultaneously (which may be similar to polyfunctional cells identified by intracellular flow cytometry), IL2 and TNF (without IFN γ), and TNF alone. Expression of ICOS differs between these subsets, with IL2+ IFN γ + TNF + expressing ICOS less frequently. Thus, this analysis reveals a far greater heterogeneity of activated T-cells than previously appreciated, and discriminates subsets that may have unique relevance to T-cell biology or immunity.

4. Discussion

Over the past five years, highly multiplexed, single-cell gene expression has been used increasingly to interrogate complex biological systems. These studies demonstrate that significant heterogeneity underlies cell populations previously thought to be relatively uniform, including early embryonic stages (Guo et al., 2010), neuronal populations (Citri et al., 2012), pluripotent stem cells (Narsinh et al., 2011a), and tumors (Powell et al., 2012). Comparatively little work, however, has been performed in immunological settings, where great cellular heterogeneity is already known (Bendall et al., 2012). For example, individual immune cells (isolated from finely-defined cell subsets, *e.g.* central memory T-cells) can vary not only in their resting state, but also in their activation-induced gene expression profiles (Shaffer et al., 2001). Moreover, activation profiles may vary with host genetics (Wilson et al., 1997; Jansen et al., 2005), or depend on antigen structure (Cemerski et al., 2007) or persistence (Petrovas et al., 2006). To ensure that gene expression assays (like the Fluidigm BioMark™ platform we use here) can accurately capture differences between study groups, even when cell populations are biologically heterogeneous, it is particularly important to minimize assay-related (technical) variation or noise. To that end, we

developed a rigorous methodology for performing quantitative immunoassays on this platform.

Our work extends past studies and protocol papers in a number of important ways. First, we present a method for qualification of primers, which accounts not only for the efficiency of cDNA amplification (Citri et al., 2012), but also for the wide variation in initial template (mRNA) levels that is observed across genes associated with immunity. Second, we demonstrate at the single-cell level that primer competition is not an appreciable problem in this highly multiplexed system. Third, we define the number of initial cDNA synthesis (“pre-amplification”) cycles that distributes template equally into all 96 microfluidic channels of the BioMark™ chip; past studies employ anywhere from 15 to 22 cycles without providing data to substantiate the choice (Guo et al., 2010; Marro et al., 2011). Finally, we demonstrate how sensitive the technology is, and describe a number of ways it can be employed in immune monitoring.

These studies demonstrate the exquisite sensitivity of multiplexed qPCR as a tool for single-cell analysis, and highlight the need for careful optimization studies. Standard qPCR and microarray technologies are highly dependent on the quality of an initial RNA isolation step, and typically require substantially more starting material (microgram quantities of RNA). In contrast, for our experiments, flow cytometric sorting deposits single cells directly into reaction wells and the resulting material (picogram quantities of RNA) is directly reverse-transcribed and amplified before transfer into the analysis chip (the “pre-amplification” step). This standardizes initial template concentrations from cell to cell (*i.e.*, sample to sample) in a manner not possible in other qPCR-based systems, obviating the need for endogenous controls.

Primer qualification is an important quality control step; however, it is complicated by the unique low-volume amplification of the platform, the wide variation in transcript abundance from cell to cell (Elowitz et al., 2002; Swain et al., 2002; Livak et al., 2013), and the highly multiplexed nature of the assay. The methods developed here account for these factors, resulting in a 73% qualification rate for TaqMan™ primers in the BioMark™ system. In principle, our piecewise approach defines the range of Ct values associated with efficient amplification; therefore, experimental data falling outside this range could be excluded as outliers. We chose not to employ this approach because flagging experimental data based on unique, target-specific Ct ranges presents a substantial informatics challenge. Also, primers that fail our piecewise qualification often pass when redesigned (against an alternate sequence). Rather than assign restricted Ct ranges to analyses using a poor primer, it may be better to test alternative sequences. Still, establishing a range of qualified Ct values may have great value in some settings; particularly where primer alternatives are limited.

Our primer qualification system was also applied to NHP, an important model for immunity and infectious disease (Hu, 2005), where 59% of primers designed for humans qualified on NHP mRNA and 93% of NHP-specific primers qualified on human mRNA. It remains unclear whether primer cross-reactivity is driven by sequence differences between NHP and human genes, or differences in expression. This may be addressed in settings where the sequence of primers is known; however, in the TaqMan system used here, such information is proprietary.

Still, these results demonstrate the need for a primer qualification step in experimental design.

In defining the quantitative aspects of this assay, we showed that it was sensitive to single mRNA copies. Furthermore, we are able to define the scale associated with the measurement. We estimate that (using the experimental conditions defined here) an Et value

of ~13 corresponds to a single copy of mRNA in the sample. Thus, an Et of 23 corresponds to ~1000 mRNA molecules. We show that the platform has an excellent technical reproducibility, with 99.5% of assays showing <10% variability. Finally, we demonstrate no loss in sensitivity or accuracy at the single-cell level compared to the 100-cell level.

The assay of 100 cells at a time (pooled-cell array) represents a potentially useful adaptation of this platform. Our experiments sorting varying numbers of monocytes, B-cells, and T-cells (from 100 to single cells) show that, while single-cell analysis can reveal signatures that discriminate lineages, more genes comprise each signature in the pooled-cell array system. Pooled-cell array is a powerful platform for investigating the expression of a limited set of genes in a large number of specimens quickly and easily – requiring only 100 cells from each specimen.

Our efforts to develop rigorous analytical methodologies have contributed to new data analysis tools as well, described elsewhere (Finak et al., submitted for publication; McDavid et al., 2013). These tools are designed to enhance sensitivity and specificity beyond common statistical tests, which are limited by the sparse data obtained with this technology and by the broad distributions of gene expression (Finak et al., submitted for publication). We devised a single statistical test that summarizes both the frequency of genes expression (*i.e.*, on/off) as well as the amount of expression (when present) from each sample (McDavid et al., 2013). Finally, we developed a method for identifying extreme outliers from datasets that are most likely experimental artifact (McDavid et al., 2013).

5. Conclusions

Quantitative, high throughput qPCR is a powerful technology for identifying gene signatures and unique cell subsets associated with immunological states. As we show here, rigorous optimization and qualification of the technology can provide a wealth of information at either the many-cell level (pooled-cell array) or the single-cell level with similar sensitivity and accuracy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Piecewise primer qualification method

To account for variation in transcript levels among cells and conditions, we developed a qualification strategy that tests a broad range of RNA concentrations, searching for segments (“pieces”) where amplification is efficient (signal doubles with every PCR cycle, slope 3.1–3.6) and linear ($R^2 \geq 0.99$). When at least one segment within a replicate dilution series (1–8, shown for single, representative primer) meets these criteria, the primer is deemed suitable for use.

Fig. 2. Transcript levels vary dramatically among genes

Data from replicate dilution series is shown. Signals from highly expressed and common genes, like CD7 and MAPK3, saturate at high RNA concentrations. However, SFRP3 and XBP1 only show efficient amplification when RNA concentration is high. This confirms the need for piecewise qualification strategies shown in Fig. 1.

Fig. 3. Primer competition

The primers listed were tested in the reverse transcription (RT)/pre-amplification step separately, or combined for multiplexed assays of 8, 24, or 96 primers simultaneously. Experiments were performed with high (200 pg) or low (2 pg) RNA amounts. E_t did not differ significantly when primers were assayed singly versus multiplexed.

Fig. 4. Number of pre-amplification cycles needed for single copy detection

A) Varying RNA amounts were reverse transcribed and cDNA was pre-amplified for 9, 12, 15, or 18 cycles, before introduction into the microfluidic chip. When 18 pre-amplification cycles were performed, consistent signal was obtained even with low levels of input RNA (<1 cell equivalent). B) Based on Poisson statistics, the Et associated with single copy detection can be determined; this is the point where only occasional amplification occurs. This limit of detection differed slightly by gene, with a median of 13.7 Et.

Fig. 5. Reproducibility

Replicate wells were compared across RNA dilution series. These wells differed by <5% (left panel), with the largest differences occurring near the limit of detection. The right panel graphs CVs across replicate dilutions of RNA. CV was generally less than 3%, well below biological variation.

Fig. 6. NHP cross-reactivity

A) Primer cross-reactivity is unpredictable. Some exhibit similar amplification across dilutions of bulk RNA isolated from human (black) and NHP (gray) PBMC (CD14, top), while others inefficiently amplify NHP transcripts (CCR4, middle and LTB, bottom). B) Cross-reactivity was also tested in sorted cell samples. In both species, expression was limited to the relevant cell types (B cells (blue) for IgD, monocytes (red) for TLR4, and both B cells and monocytes for CD32). IgD primers gave higher signals in humans than NHP, while TLR4 gave higher signals in NHP.

Fig. 7. Expression of endogenous control genes

A) At the single cell level, Et for six genes commonly used as endogenous controls differ dramatically, and can exhibit wide distributions. B) Expression levels between endogenous control genes do not correlate, even between genes with similar expression and narrow distributions (ActB and TBP), both at the single cell level (left) and the when higher cell numbers are examined (right, excluding undetectable signals).

Fig. 8. Analysis of cell signatures using single-cell and pooled-cell array approaches

Varying numbers of monocytes, T-cells, and B-cells were purified by flow cytometric sorting. Unsupervised hierarchical clustering identified genes that were uniquely detected in every cell type (blue heat map). These signatures were most easily recognized in samples with 25 cells.

Fig. 9. Gene expression compared between single-cell and 100-cell samples

A) 100-cell data suggests that genes are co-expressed; however, at the single-cell level the true, independent expression of these genes can be recognized (percent of cells expressing given marker combinations are shown at each quadrant). B) There is a strong correlation between single-cell and 100-cell gene expression data (normalized to single cells).

Fig. 10. Genes associated with T-cell activation

Unsupervised, hierarchical clustering faithfully distinguishes activated (red) from resting cells (black). Only six cells were misclassified (X). Clusters of cells differentially express TNF, IFNg, IL2 and ICOS, as described in the text.

Table 1

NHP cross-reactivity of human primers.

Primer specificity	RNA source		
	Human	Nonhuman primate	Both
Human	82% (249/346)	59% (161/273)	57% (155/270)
Nonhuman primate	83% (84/101)	93% (116/125)	83% (82/99)

Table 2

Genes significantly up- or down-regulated upon activation (after Bonferroni correction for multiple comparisons).

Gene	Percent		Et		Fold change	p-value
	Resting	Activated	Resting	Activated		
CD154	14.6	85.4	4.8	21.4	95303	0.0001
TNF	1.7	98.3	0.3	11.8	3067	0.0001
IL2Ra	10.1	89.9	1.6	11.2	746	0.0001
IL2	3.8	96.2	0.5	9.6	562	0.0001
IL10	2.9	97.1	0.3	7.4	137	0.0001
CD3d	32	68	10.2	16.6	81	0.0001
IL6ST	28.6	71.4	6.5	12.3	57	0.0001
FasLG	7.3	92.7	0.6	6.3	49	0.0001
IFNg	3.8	96.2	0.3	5.1	29	0.0001
TNFRSF9	8.4	91.6	0.6	5	22	0.0001
IL7R	36.3	63.7	13.4	17.9	22	0.0001
SLAMF1	7.5	92.5	0.5	4.5	17	0.0001
CD109	0	100	0	4	16	0.0001
ICOS	29.9	70.1	5.1	9.1	16	0.0001
SLAMF5	9.2	90.8	0.5	4.1	12	0.0001
CD48	36.5	63.5	11	14.6	12	0.0001
CD69	39	61	18.2	21.7	11	0.0001
IL2Rg	39.6	60.4	17.7	20.5	7	0.0001
TBX21	10.2	89.8	0.4	2.7	5	0.0005
CD5	73.3	26.7	9.2	2.6	-102	0.0004
IL16	79	21	11.9	2.4	-701	0.0001
CCR7	71.8	28.2	14.4	4.3	-1123	0.0001
CXCR4	71.8	28.2	15	4.5	-1444	0.0001