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Miniaturization applied to analysis of nucleic acids in heterogeneous tissues

Philip JR Day

Despite huge efforts in sample analysis, the measurement of marker nucleic acids within tissues remains largely nonquantitative. Gene analyses have benefited from sensitivity gains through *in vitro* gene amplification, including PCR. However, whilst these processes are intrinsically suited to highly reproducible, accurate and precise gene measurement, the term semiquantitative analysis is still commonly used, suggesting that other fundamental limitations preclude a generic quantitative basis to gene analysis. The most poorly defined aspect of gene analysis relates to the sample itself. The amount of cells and, particularly, cell subtype composition are rarely annotated before analysis; indeed, they are often extrapolated after analysis. To advance our understanding of pathogenesis, assay formats will benefit from resembling the dimensions of the cell, to assist in the analysis of cellular components of tissue complexes. This review is partly a perspective on how current miniaturization technologies, in association with molecular biology, microfluidics and surface chemistries, may evolve from the parts of a paradigm to enable the unambiguous quantitative analysis of complex biologic matter.

Expert Rev. Mol. Diagn. 6(1), 23–28 (2006)

CONTENTS

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KEYWORDS:
cells, DNA, microfluidics, miniaturisation, MRD, PCR, quantification, review, tissues, µTAS

Nucleic acid analysis & measurement in complex tissues

The determination of the amount of a marker nucleic acid species that is derived from a specific cell type, and relating the load of this gene to clinical outcome, remains a difficult proposition that is rarely achieved, but why? Seemingly, a relatively fundamental requirement for gene analysis is missing or often overlooked. This review offers suggestions for why the problem persists and argues in favor of miniaturized approaches to resolve the quantification of nucleic acids from any source of complex tissues.

Current nucleic acid analyses usually require sample selection, sample preparation, nucleic acid purification and, finally, nucleic acid analysis. Many tests are qualitative, highly robust and are readily interpreted, such as single nucleotide polymorphism analysis [1]. Quantitative analyses offer a greater challenge and are fast becoming an attractive possible means to measure and treat disease. This is perhaps best argued by observing caveats in assessing minimal residual disease (MRD) in leukemia, where therapy is directed following the monitoring of the dynamic change in the leukemic clonal population [2]. However, determination of the threshold of disease is rarely considered as a rationale for administering therapy, and both MRD and other disease threshold assessments, such as those that will relate to pharmacogenomics, are directly affected by technical constraints related to sensitivity of detection [3,4]. Furthermore, it is rarely considered that results are often directly influenced by how the sample is selected and handled. In this respect alone, the importance of implementing micro total analytic systems (µTAS) will be demonstrated, and the fundamental benefit to quantitative analysis from ill-defined complex tissues will hopefully become apparent [5,6]. µTAS is reviewed in [7].

Current measurement of genes in complex tissues

Tissues are typified by characteristic combinations of multiple differentiated cell types and a defined organization that lends to organ function, such as kidney and skin [8]. Therefore,

10.1586/14737159.6.1.23
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ISSN 1473-7159

because organs possess a 3D order, almost without exception, the sampling of tissue from an organ will be poorly replicated, as each sample contains a different complement of cell subtypes [9]. Even in the case where surgical removal of tissue is directed by diseased tissue morphology or binding of biomarkers, at the cellular level, there is a high likelihood that other unrelated cell types will also be harvested within the biopsy sample, as is the case with invasive cancers [10]. A recent example where this type of systematic sampling error is commonplace is the process of transcription profiling using gene arrays [11,12]. Some of the more rigorous protocols used for labeling ribonucleic acids have been developed for application on oligonucleotide microarrays [13,14], yet only a very low percentage of genes or transcripts can be analyzed with a high degree of statistical confidence [15,16]. Often, over 98% of transcripts cannot be clearly deciphered to demonstrate regulation, and replicates are poorly reproduced. For many researchers, these situations fall below expectation. However, the situation is markedly improved with the transcriptome analysis of cell lines. The problem of tissue heterogeneity is well known and studies using laser-microdissected (LMD) tissue sections aim to permit only highly selected cells to be collated and applied to gene microarrays [17]. Nucleic acid species form mixtures when released from the nuclei of cells and, if subjected to hybridization or PCR analysis, give results representing the average of each nucleic acid species (FIGURE 1). To circumvent this predicament, sensitive labeling strategies are being developed for LMD, and 200 or fewer cells can be used in current routine transcript analysis experimentation [18].

Once nucleic acids are released from cells, they are free to intermix and their subsequent analysis using PCR or hybridization will reflect the number of copies of the analyte sequence present in the total amount of nucleic acids extracted. Analysis of test gene X is typically achieved by undergoing a comparison with a known reference gene of stable expression or copy number and, therefore, an accurate quantification of genes is achieved for homogeneous cell populations. In the case for heterogeneous cell populations, the average analysis of the test gene may give misleading results with respect to cell content within the sample, thus failing to designate the true biologic event.

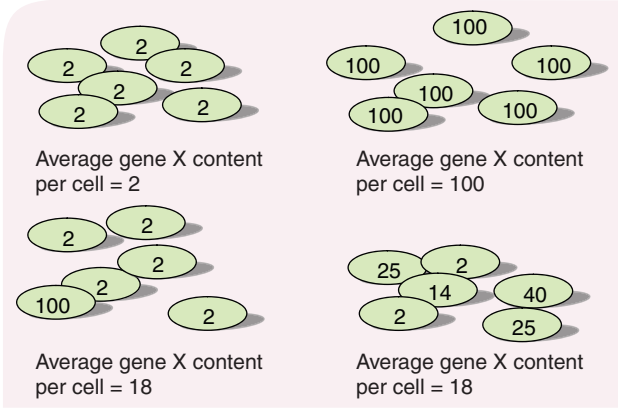

Figure 1. Importance of analyzing from defined cell populations when measuring nucleic acids. Once nucleic acids are released from cells, they are free to intermix, and their subsequent analysis using PCR or hybridization will reflect the number of copies of the analyte sequence present in the total amount of nucleic acids extracted. Analysis of test gene X is typically achieved by comparison against a known reference gene of stable expression or copy number, and thus, an accurate quantification of genes is achieved for homogeneous cell populations. In the case for heterogeneous cell populations, the average analysis of the test gene may produce misleading results with respect to cell content within the sample, and fail to designate the true biologic event.

The quandary concerning sample selection is precluding more rapid advancement in understanding pathogenesis processes. The situation related to sample collection is somewhat different with biologic fluids such as urine or blood. Fluids do not present the same paradox observed with sample collection from a solid tissue. Blood and solid tissues are both heterogeneous, possessing many differentiated cell types, but blood does not contain an architecture that holds the cell types apart in space in a manner that lends organs their function. Therefore, sampling blood benefits from the mixing of the circulating blood, and creates a medium where all samples are highly replicable and resemble the total population of cell types within blood. Single circulating cells are sampled at a given probability which relates to their Poisson distribution and, therefore, the sample volume has to be sufficiently large to detect these low copy-number cell types. This exact situation is prevalent in the case of analyzing dynamic changes in low copy-number leukemic cells in MRD treatment [19,20]. The situation in MRD further contrasts with that depicted in FIGURE 1, in that a unique fusion transcript is sought in MRD [21]. In this real-life scenario, the sensitivity of detection is directly affected by the number of other cells that do not express the fusion transcript (these impede and dilute the target sequences), and detection sensitivity between one and ten cancer cells per 100,000 normal leucocytes can be achieved [22]. The limit to detection sensitivity is used in MRD to orchestrate treatment and resembles a technical limit, rather than one based on more meaningful threshold disease progression.

Quantification of genes in complex tissues using miniaturized devices

Although microfluidic devices were originally structured in silicon or glass, polymers such as poly(dimethylsiloxane), poly(methylmethacrylate) and polycarbonate have increasingly been used as alternative substrate materials. Many recent microfluidic devices for PCR have been fabricated using SU-8, poly(ether ether ketone), poly(tetrafluoroethene), cyclic-olefin-copolymer or Zeonor® [23]. Several studies have investigated immobilizing DNA and proteins on the surfaces of microchannels made from these materials, for the promise of biologic activity [24,25].

The continued development of functionally related genomics is adding increased incentive to move away from the use of current units (moles of nucleic acid/unit weight of tissue) towards the quantitative measurement of numbers of molecules of marker nucleic acid per disease-associated cell type. The process,

starting from raw sample and proceeding through to analysis, needs to be substantially one of integration to produce a seamless protocol that is highly reproducible. Not too surprisingly, the scaling down of assay format to enable single cells to be maneuvered and treated is a mandatory requirement to achieve µTAS, and flow-assisted cell sorting is a universal example where manipulation of individual cells has been made possible using microchanneling [26]. The argument has already been voiced that, for the careful selection of cells, analysis should avoid the problems seen in FIGURE 1, but with microfluidics this problem can be dealt with differently. Already, some workers have used miniaturization to isolate cells for detailed analysis, but have not attempted to homogeneously analyze all of the target cells within a sample, thus losing valuable quantitative information [27]. FIGURE 2 demonstrates how, in the case of leukemia, preselection of cell numbers entering transcript analysis can be achieved, compared with the current method where blood volume (not cell numbers) is considered prior to MRD analysis.

FIGURE 2A shows the frequently used means to estimate MRD from an undisclosed number and mixture of cells. Both the total number of cells entering the analysis and the subpopulation of cancer cells are unknown but, owing to the fact that too many cells sequester the translocation PCR signal, it is best to use a preselected number of cells, which has been predetermined to allow fusion transcripts from a single MRD cell to be detected in a reduced background (FIGURE 2B).

The number of cells entering PCR needs to be tuned to the interpretation of the assay. Ultimately, the highest resolution possible is for single cells to be analyzed. To enable a full understanding of the state of disease it will be necessary for large numbers of single cells to be PCR analyzed to permit an effective statistical assessment of disease status. In effect, quantitative analysis for MRD and other diseases can be achieved by simply dividing a sample into smaller aliquots, as depicted in FIGURE 3.

FIGURE 3 shows how a sample can be split into smaller volumes to increase the precision for quantification of nucleic acids within the original test sample. The reaction volume can be adjusted to permit a specific amount of nucleic acid to enter, and this may resemble that of a single cell (or even many cells), provided that detection of the test sequence is not compromised by exceeding the detection sensitivity of the assay. Whole cells can also be manipulated similarly. Quantification is determined as the number of positive reactions present relative to total reactions conducted.

This concept has been used to develop microchannel voltage to generate a two-phase flowing stream of picoliter aqueous droplets and instrumentation with online optics and high-throughput PCR capabilities [28]. Emulsions have also been produced to create droplets for high-density reactions [29,30]. The hope is that this approach will yield MRD analysis from peripheral blood cells and render the requirement to undertake the painful procedure of bone marrow aspiration obsolete. Most uses of miniaturization for the µTAS quantification of nucleic acids are not fully integrated systems but relate usually

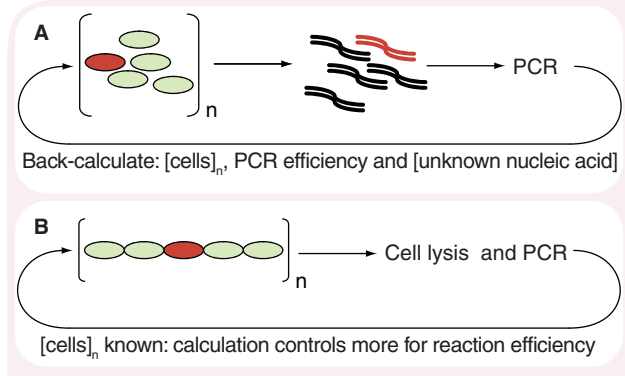

Figure 2. Minimal residual disease (MRD) analysis of nucleic acids from blood cell populations. (A) Frequently used methods to estimate MRD from an undisclosed number and mixture of cells. Both the total number of cells entering the analysis and the subpopulation of cancer cells are unknown. (B) However, since too many cells sequester the translocation PCR signal, it is best to use a preselected number of cells in a PCR that has been predetermined to allow fusion transcripts from a single MRD cell to be detected in a background of normal white blood cells.

to one of cell culture, sample extraction or PCR amplification [27,31–33]. There is agreement in the field to adopt a modularized approach to device format; however, few biologic problems have been fully addressed (as suggested by the µTAS concept). Lateral interests related to heterogeneous sample analysis have extended into optimization of conditions to provide faster PCR [34] and even single-molecule detection [35], real-time applications [36] and multiplexing [37,38]. Using integrated miniaturization to homogeneously traverse from raw samples to quantitative analyses is not currently a reality.

Whilst gene amplification for quantitative µTAS assessment of nucleic acids requires much effort to perfect, part integration of assays to attain qualitative analysis is making some good progress. Prokaryotic genomes have been detected [39,40] and reverse transcription and sequencing have been performed in picoliter reactors [29,41]. New efforts will extend into rolling circle amplification [42]. Particularly, aneuploidy and polymorphism detection are amenable to the purely qualitative analyses that tend to manifest with current µTAS nucleic acid analysis applications. On reflection, quantification of nucleic acids has never been universally achieved using macrofluidic approaches, but the attributes of µTAS renders nucleic acid quantification considerably more feasible .

Conclusion

The development of miniaturized devices for the analysis of genes in complex tissues is in its infancy; too often this challenge is inappropriately viewed too simplistically. Most miniaturization developments, while elegant, are typically interesting variations for conducting PCR. There is limited consideration of the requirements for a specific analytic test and how miniaturization can enhance current nucleic acid testing. Most studies are therefore limited to determining generic components that can be included in various µTAS

designs, although moves towards modularized µTAS are starting to manifest. Presently, for nucleic acid analysis, miniaturization has its main successes in repackaging PCR, but seems to sidestep the main advantage that scaling down to handle single cells could offer the high-resolution analysis of gene copy number within complex tissues.

Expert commentary

The miniaturization of nucleic acid analyses (and all other bioassays) to permit quantitative measurement from any tissue will induce a new era in quantitative functional genomics. The implications will profoundly impact the applied fields that encompass molecular biology, but this will be combined with increased automation and an enhanced ability to perform highly complex high-throughput procedures without the requirement for specialist personnel or facilities. The field of work has been pioneered by analytic chemists who have demonstrated proof-of-principle of technique, but improvement to specific applications is now required. A fascinating road is starting to be paved; however, first we need to assemble teams of highly multidisciplinary individuals to traverse traditional specialty boundaries and find ways to extract cells from tissues, nucleic acids from cells and signals from nucleic acid targets, while maintaining an ability to track a nucleic acid to its originator cell. This approach will permit detailed analysis that will derive the all-elusive quantitative databases that will enhance our understanding of bioprocesses.

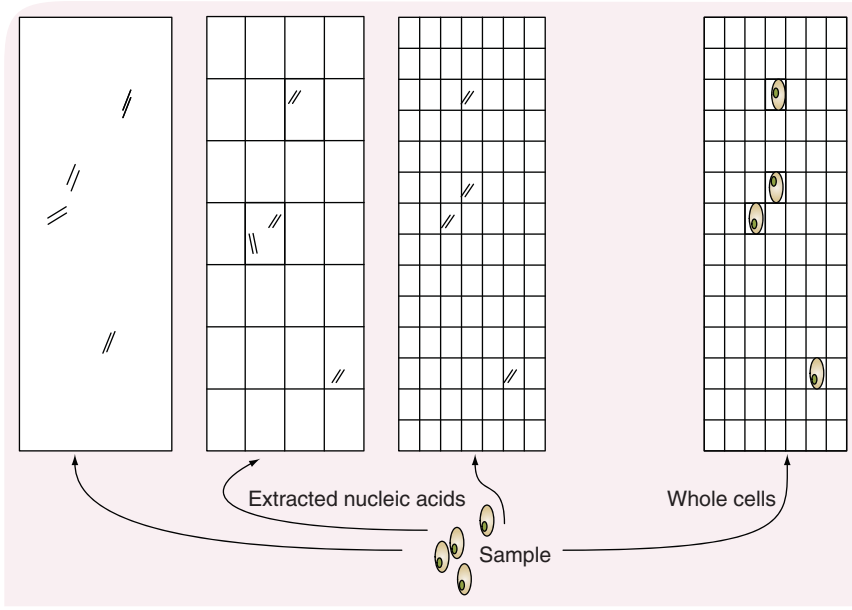

Figure 3. Division of a sample to increase sensitivity of analyte detection. Demonstrates how a sample can be split into smaller volumes to increase the precision for quantification of nucleic acids within the original test sample. The reaction volume can be adjusted to permit a specific amount of nucleic acid to enter; this may resemble that of a single cell or even many cells (provided that detection of the test sequence is not compromised by exceeding the detection sensitivity of the assay). Whole cells can also be manipulated. Quantification is determined as numbers of positive reactions present compared with total reactions conducted.

Five-year view

There exists enormous interest in the development of miniaturized devices for use in conjunction with nucleic acid analysis, and this outwardly unusual union will undoubtedly continue to touch the imagination and bring together engineers, chemists and bioscientists more intimately. Akin to the quantum change experienced by the molecular sciences following the annotation of the human genome, this is another example of technological innovation advancing nucleic acid and (hopefully also) medical applications. The annealing of the disciplines is expected to gain in momentum, particularly when further fuelled by the expansion of interests in the direction of systems biology (of the single cell) where miniaturization will help to isolate cells for detailed testing [43]. The realization that quantification of nucleic acids cannot be fully achieved without more careful selection of biologic matter needs to further manifest. The microfluidic device is an excellent packaging medium to select and present cells for subsequent treatment and analysis. Increasingly, analysis of the single cell is taking center stage, and reporting disease in terms of numbers of molecules of disease-associated molecules per disease-causing cell is set to prevail. However, for benefits to be appreciated, developments need to detract away from pure engineering, and the challenge undertaken should be more focused on the biologic question. To this end, the development of future devices must necessitate a fully integrated approach with associated dedicated miniaturized instrumentation. This fits fully with the concept of µTAS. An active move away from generic devices for application on relatively meaningless test model systems is to be encouraged. A prediction is that, despite the current goals that are highly technical and related to microdevice fabrication, surface treatments and analysis, the results generated from the associated instrumentation are the most valuable commodity, and these will be logged into a database to help increase understanding of pathogenesis and improve design of therapy regimen. Perhaps the frustration that will ensue will be the recognition that quantitative nucleic acid data can be generated, but there exists a total inability to interpret and fully apply quantitative data to patient welfare. However, the longer term challenge exceeding this 5-year perspective will be to take the quantitative raw data and help meet the expectation of pharmacogenomics in defining and treating individuals in terms of their specific risk group, for a given disease [44].

Key issues

- Miniaturization has an absolute prerequisite to deliver reproducible and meaningful quantification of nucleic acids. There is an innate requirement to analyze small, numerically defined cell populations.
- Microdevices need to be fabricated to achieve integrated micro total analytic systems (μTAS). The devices should be designed to meet the exact requirements of the specific bioassay, and therefore multidisciplinary teams of experts will be required.
- There needs to be a move away from reporting mole quantities of genes per gram weight of tissue, and active movement towards measuring disease-causing molecules per disease-related cell subpopulation.
- The future of quantification of nucleic acids requires rigorous standardization, which can be met by dimensionally shrinking the architecture associated with bioassays to enable the cell to be moved and treated as a unit entity.
- The resultant long-term value of measuring bioanalytes using μTAS (e.g., in the case of pharmacogenomics and systems biology) will be the production of databases that will become definitive recordings of how to judge the meaning of change in amount of analyte, definition of disease-related thresholds and therapy.

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