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Technical aspects and recommendations for single-cell qPCR

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ABSTRACT

Single cells are basic physiological and biological units that can function individually as well as in groups in tissues and organs. It is central to identify, characterize and profile single cells at molecular level to be able to distinguish different kinds, to understand their functions and determine how they interact with each other. During the last decade several technologies for single-cell profiling have been developed and used in various applications, revealing many novel findings. Quantitative PCR (qPCR) is one of the most developed methods for single-cell profiling that can be used to interrogate several analytes, including DNA, RNA and protein. Single-cell qPCR has the potential to become routine methodology but the technique is still challenging, as it involves several experimental steps and few molecules are handled. Here, we discuss technical aspects and provide recommendation for single-cell qPCR analysis. The workflow includes experimental design, sample preparation, single-cell collection, direct lysis, reverse transcription, preamplification, qPCR and data analysis. Detailed reporting and sharing of experimental details and data will promote further development and make validation studies possible. Efforts aiming to standardize single-cell qPCR open up means to move single-cell analysis from specialized research settings to standard research laboratories.

1. Introduction

1.1. The promises of single-cell analysis

Organisms, organs and tissues are complex mixtures of cells and extracellular molecules that together carry out all types of biological functions. Cells can perform functions individually as well as interacting with the surrounding microenvironment. They show highly variable molecular profiles that respond to internal and external stimuli making every cell unique. The cell's profiles are also dynamic, adapting to the ever-changing microenvironment. Thanks to recent technological advances to handle and analyze single cells we now have the means to characterize all cell types, their different states and start to decipher their functions. Omni-omics, which is the measurement of multiple analytes in the same cell is feasible. By characterizing all the cell types that make up a tissue or organ we will learn about cell-to-cell interactions and how the cells act in concert to perform the complex biology of organisms.

1.2. Pros and cons of qPCR

Quantitative PCR (qPCR) is one of the most versatile and commonly applied methods in molecular biology and is available to researchers in most biological and medical laboratories. Quantitative PCR is also used in diagnostics to quantify biomarkers and to detect pathogens. Many molecular biologists know how to design and perform qPCR experiments, handle and evaluate data and set up new applications, such as single-cell analysis. Numerous detection technologies and instrumentations are available that can be applied at single-cell level (Kubista et al., 2006). Single-cell qPCR has been used in a wide range of applications, including insulin producing beta cells (Bengtsson et al., 2005), the influence of single-nucleotide polymorphisms on gene-expression phenotypes (Wills et al., 2013), astrocyte activation (Rusnakova et al., 2013; Ståhlberg et al., 2011), neuron activity (Liss et al., 2001), breast cancer stem cells (Akrap et al., 2016), colon cancer stem cells (Dalerba et al., 2011), cancer associated fibroblast activation (Busch

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et al., 2017), cell fate decision in stem cells (Guo et al., 2010; Narsinh et al., 2011) and cell cycle regulation (Dolatabadi et al., 2017). Compared to most other single-cell techniques the effort to move from bulk to single-cell analysis is rather straightforward when using qPCR. Regular assays and standard instrumentation may be applied. Single-cell collection, preamplification and some modified data analysis are the additional steps needed. An advantage of qPCR is that DNA, all types of RNA and many proteins can be analyzed even in combination, all generating Cycle of quantification (Cq) readouts (Darmanis et al., 2016; Genshaft et al., 2016; Ståhlberg et al., 2012; Tang et al., 2006). Another major advantage compared to other single-cell technologies is that qPCR data analysis is well established and can be performed with standard qPCR analysis software with no need for advanced bioinformatics and writing of scripts (Ståhlberg et al., 2013a). Quantitative PCR can also be used in combination with next generation sequencing (NGS) taking advantage of the strengths of both technologies (Kroneis et al., 2017). A limitation of qPCR is that only a limited number of target molecules, usually 1 to 96, can be assessed in a workflow. When the number of targets is larger other techniques such as NGS are preferred. Quantitative PCR and NGS are related technologies, since most NGS protocols include PCR (Gawad et al., 2016; Wen and Tang, 2016). Hence, qPCR and NGS workflows share many experimental features and limitations. Expression data measured with NGS and qPCR correlate and the methods should be exchangeable (Wu et al., 2014). In qPCR, each assay is optimized and the workflow is simpler, contributing to higher sensitivity and reproducibility compared to NGS (Kroneis et al., 2017). Another advantage of qPCR over NGS is standardized analysis workflow. Quantitative PCR data can be analyzed by most non-specialists, while NGS data analysis is still to be standardized and results may depend on the tools used, the assumptions made and the particular analysis workflow. User friendly analysis packages for non-experts are still few and have limited functionalities. For most applications, qPCR is also more cost-effective than NGS when considering all steps from sample handling to analyzed data, even if the reagent cost per gene and cell may be higher for qPCR. Quantitative PCR may also be substituted by digital PCR in single-cell analysis (Albayrak et al., 2016; Ottesen et al., 2006), but the cost per digital PCR is high and the throughput is low with current platforms, limiting its use.

2. Single-cell analysis using qPCR

2.1. The single-cell qPCR workflow

The single-cell field is still characterized by methodology development and biological proof-of-concept studies. Emerging data show that single-cell analysis can provide vital information about the cell that is not available when studying classical bulk samples. However, for single-cell profiling to become a mainstream methodology it needs to be transparent and to some degree standardized. To date, few reports and biological findings in the single-cell field have been verified in independent studies. A first step towards standardizing single-cell analysis is to report the entire experimental workflow in detail and make data publically available. This will make validation of results and confirmation of findings easier.

Quantitative PCR is a rather mature technology and MIQE (**M**inimum **I**nformation for publication of **Q**uantitative real-time PCR **E**xperiments) guidelines are available for its use on classical bulk samples (Bustin et al., 2009). Similar guidelines are also provided for digital PCR (Huggett et al., 2013). The goal of MIQE is to provide the basis for good experimental practice and encourage detailing protocols that allow for unbiased interpretation of qPCR data. Most aspects of the MIQE guidelines are relevant for single-cell analysis, but some experimental steps need adjustments and there are also some unique steps in single-cell analysis that must be considered (Table 1). Table 2 shows the additional and adjusted items that are specific to single-cell analysis.

Fig. 1 shows the workflow of single-cell qPCR analysis targeting nucleic acids. Experimental details of single-cell protein analysis have been detailed elsewhere (Darmanis et al., 2016; Genshaft et al., 2016; Ståhlberg et al., 2012). Single-cell DNA analyses using qPCR have been reported (Neves et al., 2014; Potter et al., 2013; Rygiel et al., 2015; Ståhlberg et al., 2012; Yang et al., 2014), but most single-cell studies so far has targeted RNA. The workflows for RNA and DNA analyses are similar, except that the RNA workflow requires a reverse transcription (RT) step, while the DNA workflow requires opening of the chromatin structure. Sample handling with cell dissociation and single-cell collection followed by direct lysis are specific to single-cell workflows. Single cells can be collected with several techniques, the most common being microaspiration, laser microdissection, and flow cytometry. Major focus is currently on throughput, where the introduction of droplet reaction containers has revolutionized the single-cell field (Ziegenhain et al., 2017). Attention is also on spatial information, linking cells molecular profiles to their localization in tissues and organs (Lee et al., 2014; Ståhl et al., 2016), and to specific compartments within the single cell (Sindelka et al., 2008). Preamplification is another common, but not an exclusive, single-cell step that is not discussed in the MIQE guidelines. Finally, data analysis is also in many ways unique for single-cell analysis. Carefully optimized protocols and adequate controls are important in all experimental work. In single-cell analysis the experimental setups are more challenging as the numbers of target molecules are very few. Successful single-cell workflows are characterized by efficient lysis and high reaction efficiencies as well as minimal material losses.

2.2. Experimental design

When designing single-cell experiments one of the first questions that arises is how many cells should be analyzed. In classical studies one sample per subject is usually collected, RNA is extracted and analyzed (Fig. 2). If a single gene target is analyzed its normalized expression in the studied groups is compared using univariate statistics to assess the significance of the measured difference. If multiple targets are analyzed multivariate methods are usually more powerful to classify the subjects. Technical replicates may be performed, where replicates upstream in the workflow, such as sampling replicates, reduce confounding variation more than downstream, such as qPCR replicates (Tichopad et al., 2009).

Table 1
MIQE checklist and additional single-cell items.

Item to check - MIQE	Single-cell level
Experimental design	Applicable
Sample preparation	Partly applicable
Nucleic acid extraction	Not applicable
Reverse transcription	Applicable
qPCR target information	Applicable
qPCR oligonucleotides	Applicable
qPCR protocol	Applicable
qPCR validation	Applicable
Data analysis	Partly applicable
Additional single-cell item to check	
Single-cell collection and direct lysis	
Preamplification target information	
Preamplification oligonucleotides	
Preamplification protocol	
Preamplification validation	

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Table 2
Single-cell qPCR items to check.

Item to check[a]	Item to check
Sample	**Preamplification oligonucleotides**
Description	Primer sequence and/or amplicon context sequence
Volume/mass of sample processed	Database identification number (RDML, etc)
Microdissection or macrodissection	Location and identity of and modification
Processing procedure	Purification method
If fixed, with what and how	**Preamplification protocol**
Tissue dissociation, with what and how	Complete reaction conditions
Enzymes, buffer, kit catalogue no. and manufacturer	Reaction volume
Evidence of dissociation optimization	Primer, Mg2+ and dNTP concentrations
Evidence of cell quality	Polymerase identity and concentrations
Biological and technical controls	Buffer/kit catalogue no. and manufacturer
Single-cell collection and direct lysis	Plates/tubes catalogue no. and manufacturer
Description of cell collection setup	Complete thermocycling parameters
Evidence of single-cell collection optimization	Reaction setup
Complete collection parameters	Manufacturer of PCR instrument
Biological and technical controls	Dilution of preamplified material
Manufacturer of single-cell collection instrument	**Preamplification validation**
Complete direct lysis conditions	Evidence of optimization
Lysis volume	Specificity evaluated by downstream qPCR
Buffer, kit catalogue no. and manufacturer	Calibration curves with slope
Storage of collected cells	r^2 of calibration curve
Preamplification target information	Preamplification efficiency with confidence interval
Sequence accession number	Linear dynamic range
Amplicon location	Confidence interval throughout the range
In silico specificity screen (BLAST, etc.)	Optimal number of preamplification cycles
Pseudogenes, retropseudogenes, or other homologes	Evidence of level of detection and quantification
Secquence alignment	**Data analysis**[b]
Secondary structure analysis of amplicon and GC content	Handling of missing data
Location of each primer by exon or intron (if applicable)	Description of preprocessing procedure
Where appropriate, which splice variants are targeted	Description of normalization method
	Cell inclusion/exclusion criteria

[a] For recommendations about experimental design, reverse transcription, qPCR target information, qPCR oligonucleotides, qPCR protocol and qPCR validation we refer to the MIQE guidelines (Bustin et al., 2009).
[b] Recommendations in addition to the MIQE guidelines.

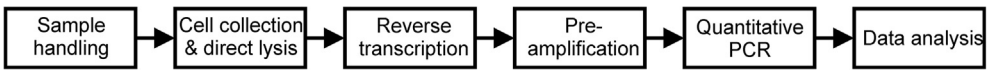

Fig. 1. Experimental steps in single-cell nucleic acids analysis using qPCR. Reverse transcription is performed when analyzing RNA. For protein analysis, reverse transcription is exchanged with an antibody to antigen binding step.

In single-cell analysis hundreds to several thousands of cells per sample are analyzed for the expression of multiple genes. Multivariate methods are used to analyze these data too, but this time cells are classified rather than the samples. The studied groups can then be compared based on the number of cells from each subject that are found in each of the different cell categories (Fig. 2). In single-cell profiling technical replicates are omitted, as analyzing more cells is statistically favorable than improving technical precision (Tichopad et al., 2009). Exception is when optimizing and validating protocols; technical replicates are then useful to assess reproducibility of the different experimental steps and compare processing noise to biological variability. For optimized protocols, cell-to-cell variability is normally much higher than the technical variability (Bengtsson et al., 2008; Kroneis et al., 2017; Ståhlberg and Kubista, 2014). The number of cells that needs to be analyzed in an experiment depends on several biological and technical factors. Comparing numbers of defined cell types under various conditions usually require fewer cells than when screening for novel subpopulations with unknown expression characteristics.

2.3. Sample preparation

Sample preparation and single-cell collection are arguably the most challenging steps in single-cell analysis. Quantitative PCR is flexible and most sample preparation protocols and single-cell strategies can be applied. Single-cell suspensions can be generated from tissues using enzymatic and/or mechanical methods. Numerous protocols, including different enzymes and physical disruption strategies have been described (Worthington Tissue Dissociation Guide). However, very little data are available about yield, reproducibility, viability and possible bias introduced by the various tissue dissociation procedures. Tissues contain many different cell types but also diverse extracellular material. Even within a seemingly homogenous tissue the cell composition and the surrounding matrix varies. This makes it very difficult to develop dissociation protocols that allow for full characterization of tissues. Therefore, detailed information about sample preparation, any optimization performed, generated cell numbers, and cell viability should be reported to facilitate for the community to validate and improve tissue specific dissociation protocols, *i.e.*, evidence of dissociation optimization and cell quality (Table 2). To determine if sample processing is unbiased the summed expression of the individual cells should be compared to that of a classical bulk sample (Ståhlberg et al., 2013b). Any deviation indicates there are problems such as apoptosis, induced stress, bias in the collection of cells, or that assays are amplifying also genomic DNA (Dzamba et al., 2016). Validation is always desired and can be sometimes be done with an independent technique, such as imaging and flow

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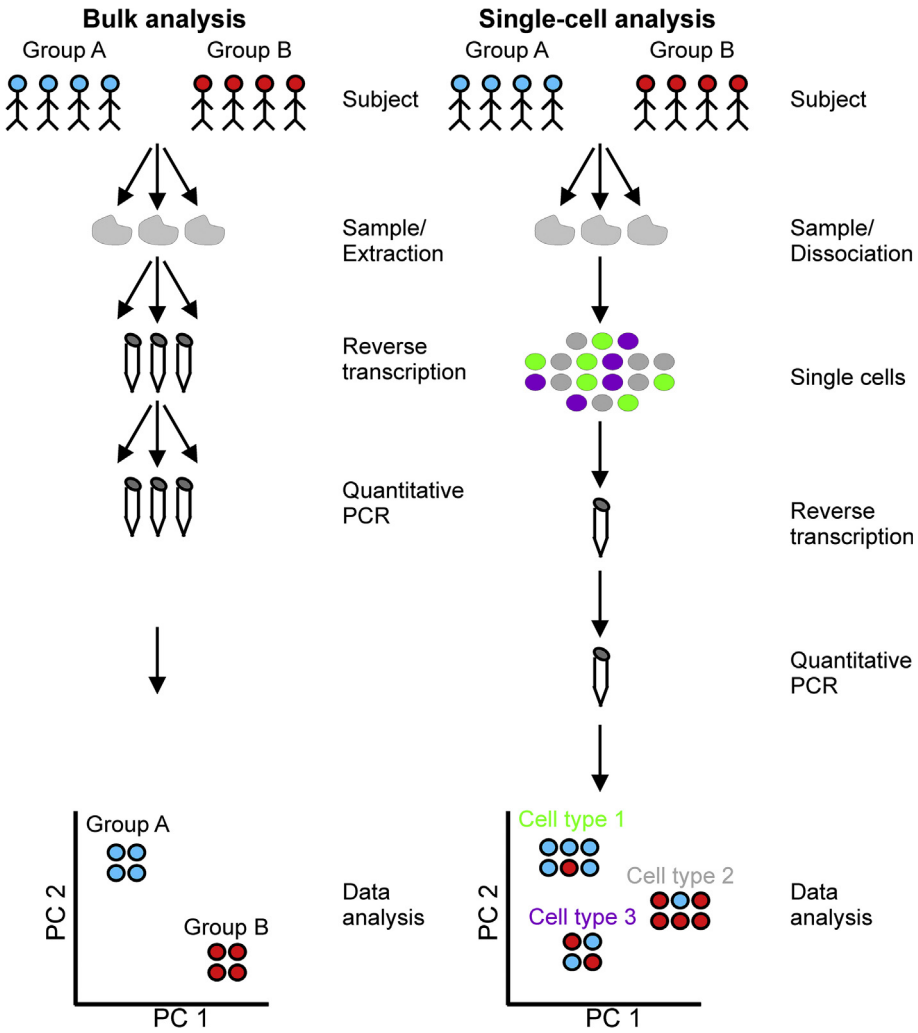

Fig. 2. Comparison between classical bulk and single-cell experimental design. In bulk analysis subjects are compared, while cell types/states are commonly compared in single-cell analysis.

cytometry. By staining cells for viability, apoptosis and cell type specific markers sample handling and dissociation protocols can be evaluated.

2.4. Single-cell collection and direct lysis

Setting up, establishing and validating a single-cell collection approach is an effort that requires careful consideration (Hodne and Weltzien, 2015). The total number of available cells and the throughput are important parameters to consider when choosing a platform for single-cell analysis. For example, fluorescence activated cell sorting (FACS) requires large numbers of cells to start with, while microaspiration techniques can essential recover all the viable cells, even if they are few. On the other hand, the total number of cells that can be collected with microaspiration is usually low. If the cells of interest are rare, it may be possible to enrich those based on markers, morphology or other cell specific features. Another possibility to consider is to link the collected single cells to their location in the tissue and take advantage of spatial heterogeneity. Laser microdissection preserves spatial information, but usually requires fixation, which is associated with analyte loss and degradation (Nichterwitz et al., 2016; Gründemann et al., 2011). It may also be possible to trace the origin of the cell based on staining or other cellular characteristics such as pigmentation that is linked to spatial information (Sidova et al., 2015). In most high throughput methods, such as those based on droplets, spatial information is lost. The possibility to perform functional validation of living cells is often overlooked when setting up single-cell experiments. This is possible if the cell collection method can release viable cells. Microaspiration techniques are often gentle and induce minimum stress.

Many single-cell collection techniques are performed under non-optimal culturing conditions for the cells. Information about successfully collected cells, selection criteria and timing of the experiment are therefore important parameters to consider when interpreting data and should be reported. Controls are also motivated if conditions are changed for sampling. For example, does the molecular profile change with time or is it affected by other confounding experimental parameters? Information about collection volume is important, as it may impact on downstream enzymatic reactions and there is risk of contamination and inhibition from the medium. The risk is particularly large when surrounding cells are necrotic or apoptotic, leaking cellular material into the extracellular medium. Negative controls containing no cell and positive controls containing a spike or other standardized material, in the same volume should be collected to estimate the influence of background. Even, when volume is low cells may be contaminated with cellular material from other cells that is attached to their

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membrane. This can be tested by spiking in intact viable cells from a different species. Contaminating molecules can then be detected by species-specific qPCR assays. For many collection techniques, the capture mechanism takes advantage of certain cell features, such as size, which can introduce sampling bias. For example, using FACS large cells may be missed as they may be mistaken for being two cells and therefore not collected. Microfluidic systems, like the C1 Single-Cell Auto Prep System (Pollen et al., 2014) and filtration based systems such as the VyCAP (de Wit et al., 2015) also select cells based on size. In fact, total unbiased single-cell collection methods may not exist.

RNA is generally not extracted from intact single cells. Rather, direct lysis is used with agents that make the molecules available, protect them from adsorbing to reaction container walls, and are compatible with downstream enzymatic reaction. This makes washing steps that would cause losses unnecessary (Svec et al., 2013). Cellular content may inhibit enzymatic reactions when concentrations are high, which may happen when lysis volume is small. For picoliter droplets this is often a problem, while for cells lysed in microliter volumes there is rarely inhibition. Direct lysis should disrupt the cell membrane and make the cytoplasmatic analytes accessible. Some detergents also lyse the nuclear membrane making its content available. Some cells, such Gram positive bacteria, require tougher conditions (Wang et al., 2015). Also mammalian cells show varying inertness to detergents and may require particular lysis conditions. Non-ionic detergents like NP-40 and Triton X-100 lyse the cell membrane but not the nuclear membrane of most cells, which can be used to purify RNA from the cell nuclei (Krishnaswami et al., 2016). Stronger chaotrophic agents, such as guanidine thiocyanate, lyse both membranes (Bengtsson et al., 2008). Many direct lysis buffers are supplemented with RNase inhibitors to minimize RNA degradation. For many single-cell workflows, however, there is no noticeable degradation of RNA by RNases. The reason is unclear; one speculation is that RNases, which are primarily extracellular, are washed away during the single-cell preparation with most protocols. When analyzing nuclear DNA, chromatin structure should be opened to make the histone bound DNA available. Proteinases and strong denaturing agents like guanidine thiocyanate can be used (Clark et al., 2017; Guo et al., 2015; Leung et al., 2016). Proteinases are inactivated by heat and/or inhibitors, but the very efficient proteinase K is rather thermostable and difficult to inactivate completely. If strong detergents are used they must be diluted not to inhibit downstream enzymatic reactions. In bulk analysis, nucleic acids are commonly purified by washing using a solid phase component like beads or membranes used in micro spin-column. In single-cell analysis purification is rarely applied to minimize analyte losses and for cost reasons (Svec et al., 2013).

2.5. Reverse transcription

For single-cell RNA analysis high RT yield and reproducibility are essential. Compared to most NGS approaches, qPCR have few constraints on the RT design. Essentially all conditions, including several NGS specific RT protocols are compatible with qPCR (Kroneis et al., 2017). The yield and reproducibility of RT depend on the reverse transcriptase, primers, additives and the temperature profile used (Ståhlberg et al., 2004a, 2004b; Ståhlberg and Bengtsson, 2010) and is target sequence dependent due to secondary and tertiary structure formation. Using synthetic RNA template the yield has been shown to vary between 0.4 and 100% depending on conditions (Ståhlberg et al., 2004b), emphasizing the importance of optimizing the RT reaction for sensitive measurements, such as single-cell profiling.

2.6. Preamplification

Analysis of many low abundant targets as is the case of single cells requires multiplexing or preamplification to avoid introducing sampling noise when the sample is divided into aliquots (Ståhlberg and Kubista, 2014). Multiplex qPCR can be applied to quantify several targets simultaneously by using different fluorophores without diluting the samples. The advantage of multiplexing is that it eliminates the need for preamplification that may introduce bias. Drawbacks with multiplexing are that only few targets can be analyzed and the multiplex reaction requires extensive optimization. Preamplification workflows are therefore usually preferred. For qPCR workflows target-specific preamplification is most common, where all primer pairs are mixed in a multiplex PCR and amplified a limited number of cycles (Andersson et al., 2015; Livak et al., 2013). The preamplification assays are usually the same as the assays used in the downstream singleplex qPCRs, although nested designs may be used (Genshaft et al., 2016; Wang et al., 2016). Global preamplification can also be used (Kroneis et al., 2017). Target-specific preamplification can be optimized for all the individual assays, while when using global preamplification assays for some targets will show poor performance and some targets may even be non-amplified. Advantages of global preamplification are convenience and flexibility as new targets can easily be added to an ongoing study.

Preamplification is in effect a highly multiplexed PCR run a limited number of cycles with reaction and thermocycling conditions similar to that of standard singleplex PCR. Successful preamplification is characterized by high yield and reproducibility as well as small variation of amplification efficiencies across the individual target assays. Target-specific preamplification is usually performed with 10–20 times lower primer concentrations than singleplex qPCR, with extended annealing time (3 min or more). The number of preamplification cycles is critical, as it depends on the initial number of target molecules, number of assays included, dilution factors and the singleplex qPCR reaction volume (Andersson et al., 2015; Livak et al., 2013). If too many preamplification cycles are performed the reaction will run short of some critical components introducing severe bias. If a highly abundant target, such as ribosomal RNA, is included in the preamplification it will not affect the general amplification of low abundant genes, since the preamplification primers of the ribosomal RNA will be consumed before the reaction runs out of dNTPs. Therefore, only abundantly expressed molecule may become confounded (Andersson et al., 2015). For most single-cell mRNA protocols 20 preamplification cycles will generate enough amplicons even for the BioMark 96x96 dynamic array, which employs reaction volumes of only 7.5 nL. In single-cell DNA preamplification, additional cycles may be used since most targets are present in few copies only. Twenty-two preamplification cycles is usually enough in most cases.

Like PCR preamplification is quantitative in its exponential phase. Therefore, preamplification can be monitored in real-time using a DNA intercalating dye, such as SYBR Green I the same way as qPCR, to reveal the maximum number of cycles that can be performed without problem (Fig. 3). The performance of the individual assays can be tested with downstream singleplex qPCR using dilution series to determine sensitivity, dynamic range, preamplification efficiency, and reproducibility (Andersson et al., 2015; Rusnakova et al., 2013). Bias may be introduced during preamplification if the reaction is not carefully optimized as even small deviations in efficiency can cause large quantitative errors. The standard MIQE recommendations for qPCR (Bustin et al., 2009) are applicable also for preamplification and are listed in Table 2.

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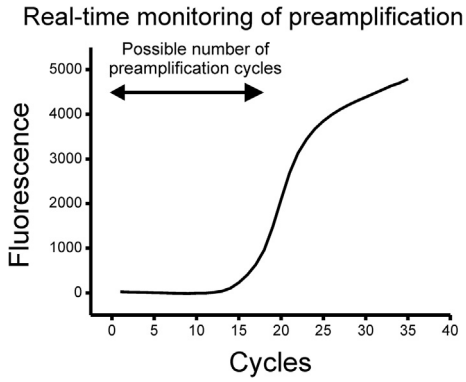

Fig. 3. Real-time monitoring of preamplification. To determine the optimal number of preamplification cycles fluorescent dyes, like SYBR Green I, can be included in the reaction to monitor the PCR product formation. Here, target-specific preamplification of 96 assays is shown using 100 copies of each target, more than usual observed in single cells (Andersson et al., 2015). The preamplification reaction should not exit its exponential growth phase to avoid technical biases, *i.e.*, around 18 cycles in this example.

2.7. Quantitative PCR

Our recommendations for singleplex qPCR performed after preamplification to quantify targets are in line with the MIQE guidelines (Bustin et al., 2009). For accurate quantification the assays should be exceedingly sensitive and specific, since often even single molecules should be detected. Our experience is that the sensitivity and specificity of many assays that perform well on traditional samples with more than some 100 target molecules are not good enough when only few target molecules are present. As a rule of thumb assays that generate non-specific primer-dimer products within 30–35 cycles on a conventional qPCR instrument are not suitable for single-cell analysis. Sensitive and robust PCR assays should also have efficiencies over 90%. Details about general qPCR challenges, including estimation of cycle of quantification and PCR efficiency, are discussed elsewhere (Kubista et al., 2006; Ruijter et al., 2013; Spiess et al., 2015, 2016; Ståhlberg and Bengtsson, 2010; Ståhlberg and Kubista, 2014; Svec et al., 2015; Tellinghuisen and Spiess, 2014).

Single-cell qPCR experiments are sensitive to contamination, since most targets are very rare. Negative controls can be introduced at each critical experimental step (Fig. 1) to test for contamination. A practical approach is to include cell-free controls at the cell collection step. If these come out negative it shows the cell medium, direct lysis buffer and reagents are not contaminated. Background in RT-qPCR analysis of RNA can also be due to amplification of residual genomic DNA. Even when assays are designed to span introns DNA contamination can give rise to background, as many genes have pseudogenes and pseudogenes often lack introns. In human, over 14,000 pseudogenes exist and the number of pseudogenes can be large for a given target (Karro et al., 2007; Pei et al., 2012). Also, for many targets assays spanning introns cannot be designed or the intron is too short to make amplification of genomic DNA negligible. To eliminate DNA background samples can be treated with DNase, preferably heat-labile double-strand specific nuclease to avoid introducing washing steps. Another strategy is to determine the degree of genomic DNA background using the ValidPrime assay and correct for it (Laurell et al., 2012).

Other potential sources of contamination are reagents and reaction mixes used. There is rarely any information about DNA and RNA contamination of reagents from the manufacturers, but we consistently observe amplification products using sensitive human assays, especially for highly abundant targets such as Alu repeats and mitochondrial DNA. Most reagents can be cleaned-up from double-stranded DNA using double-strand specific DNase (Andini et al., 2017; Krüttli et al., 2015).

2.8. Data analysis

Single-cell gene expression data analysis workflow is in many aspects the same as for classical samples, but there are some important differences. Profiling single cells the amount of missing data is considerable because of the temporal changes in RNA levels that occur due to transcriptional bursting (Chubb et al., 2006; Raj et al., 2006). Consequently, at any given time, for low expressed genes, there will be no target molecules present at all in many of the cells (Dzamba et al., 2016). Those missing data are correctly referred to as off-scale data, as they have biological origin rather than being due to technical error and should be handled differently (de Ronde et al., 2017; McCall et al., 2014; Ståhlberg et al., 2013a). Off-scale data due to too low expression can usually be distinguished from missing data caused by technical errors by correlations to the levels of other targets. In practice, using optimized and validated protocols, technical failures that affect just one or few genes in a cell are rare and all missing data can be considered being off-scale. In comparative studies the number of missing data scales inversely with the average expression across single cells and the expression in bulk (Ståhlberg et al., 2013b; Dzamba et al., 2016). Highly expressed genes like *GAPDH* are usually expressed in almost all cells, while transcripts from low expressed genes are detected only in a minority of the cells.

In single-cell analysis negative controls are essential. As most genes have transcripts in only a fraction of the cells, even few contaminating target molecules can introduce severe bias. Effect is particularly serious if the contamination is introduced before the preamplification step, as it will be amplified. In single-cell workflows it is common that cells are lost or damaged. If a cell generates few or no molecules in readout it is probably a technical failure. However, there are quiescent cells expressing few genes only, and there may also be pre-apoptotic cells with few transcripts. In a new experiment, it is not possible to tell if a cell with low transcript levels is an unusual cell or a technical failure and both options should be considered in downstream analysis, where correlations of transcript levels, expression patterns, gene function and pathway analyses may reveal the nature of those cells. Whatever strategy used, we recommend that the total number of cells collected and the number of cells used in the analysis are reported together with the applied selection criteria. Many single-cell publications lack information about negative controls and inclusion and exclusion criteria for the individual cells in analysis, making it hard for readers to grasp the complexity of the study and it makes it also impossible to reproduce it.

Analyzing traditional samples, comparison of gene expression profiles requires data to be normalized. Several normalization strategies exist, with normalization to multiple reference genes being most widely used (Andersen et al. et al., 2004; Vandesompele et al., 2002). Reference gene normalization is, however, not applicable to single-cell data, as no RNA (nor protein) is present at constant level due to temporal variations. However, this is rarely a problem as expression data are conveniently reported per cell, which is a natural and intuitive mode of normalization (Bengtsson et al., 2005). Single-cell expression levels are usually reported as relative quantities per cells, but data are also related to absolute transcript numbers as the workflow has negligible losses when based on direct lysis (Ståhlberg and Bengtsson, 2010). However, some special analyses require that cells or transcripts are normalized to define subpopulations of cells and identify gene networks (Livak et al., 2013; Ståhlberg et al., 2013b). Therefore, reports should

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detail data preprocessing as well as the analyses performed. We also encourage making single-cell data publically available, as it will simplify validation studies and serve as a robust reference to the less easily controlled global RNA-Seq approaches. Handling single-cell DNA and protein data additional preprocessing steps may be required. For example, in single-cell protein analysis negative controls show a background signal that must be considered (Darmanis et al., 2016; Genshaft et al., 2016; Ståhlberg et al., 2012).

3. Concluding remarks

Single-cell analysis using qPCR opens up new avenues to address numerous biological questions that cannot be resolved with bulk analysis. The experimental workflow is robust, flexible and can be implemented by most research laboratories. The MIQE guidelines have facilitated the use of qPCR with its recommendations and guidelines including how to report data and results to scientific journals, and they are very much applicable also to single-cell analysis. However, some aspects of single-cell work are different to bulk analysis and require attention. They are:

- Most single-cell approaches require cell dissociation, which is challenging as it is often associated with harsh treatments to generate single-cell suspensions.
- Important parameters in single-cell collection are throughput, spatial information, cellular stress caused by the collection method and any enrichment performed based on cell morphology, size or staining.
- Single cells are typically direct lysed avoiding purification protocols. Good direct lysis buffer maximizes the analyte concentration available and is compatible with downstream experimental steps.
- For most single-cell applications, nucleic acids must be pre-amplified to enable accurate quantification. Preamplification can be target specific or global.
- Expression data are presented per cells rather than normalized to the expression of reference genes.
- The amount of missing data is high and primarily caused by low expression rather than having technical failures. Those missing data are referred to as off-scale data and must be imputed for most multivariate analyses.

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References

Akrap, N., Andersson, D., Bom, E., Gregersson, P., Ståhlberg, A., Landberg, G., 2016. Identification of distinct breast cancer stem cell populations based on single-cell analyses of functionally enriched stem and progenitor pools. Stem Cell Rep. 6 (1), 121–136. http://dx.doi.org/10.1016/j.stemcr.2015.12.006.

Albayrak, C., Jordi, C.A., Zechner, C., Lin, J., Bichsel, C.A., Khammash, M., Tay, S., 2016. Digital quantification of proteins and mRNA in single mammalian cells. Mol. Cell 61 (6), 914–924. http://dx.doi.org/10.1016/j.molcel.2016.02.030.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64 (15), 5245–5250. http://dx.doi.org/10.1158/0008-5472.CAN-04-0496.

Andersson, D., Akrap, N., Svec, D., Godfrey, T.E., Kubista, M., Landberg, G., Ståhlberg, A., 2015. Properties of targeted preamplification in DNA and cDNA quantification. Expert Rev. Mol. Diagn 15 (8), 1085–1100. http://dx.doi.org/10.1586/14737159.2015.1057124.

Andini, N., Wang, B., Athamanolap, P., Hardick, J., Masek, B.J., Thair, S., et al., 2017. Microbial typing by machine learned DNA melt signatures. Sci. Rep. 7 (42097) http://dx.doi.org/10.1038/srep42097.

Bengtsson, M., Ståhlberg, A., Rorsman, P., Kubista, M., 2005. Gene expression profiling in single cells from the pancreatic islets of Langerhans. Genome Res. 15 (10), 1388–1392. http://dx.doi.org/10.1101/gr.3820805.

Bengtsson, M., Hemberg, M., Rorsman, P., Ståhlberg, A., 2008. Quantification of mRNA in single cells and modelling of RT-qPCR induced noise. BMC Mol. Biol. 9 (63) http://dx.doi.org/10.1186/1471-2199-9-63.

Busch, S., Andersson, D., Bom, E., Walsh, C., Ståhlberg, A., Landberg, G., 2017. Cellular organization and molecular differentiation model of breast cancer-associated fibroblasts. Mol. Cancer 16 (1), 73. http://dx.doi.org/10.1186/s12943-017-0642-7.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., et al., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55 (4), 611–622. http://dx.doi.org/10.1373/clinchem.2008.112797.

Chubb, J.R., Trcek, T., Shenoy, S.M., Singer, R.H., 2006. Transcriptional pulsing of a developmental gene. Curr. Biol. 16 (10), 1018–1025. http://dx.doi.org/10.1016/j.cub.2006.03.092.

Clark, S.J., Smallwood, S.A., Lee, H.J., Krueger, F., Reik, W., Kelsey, G., 2017. Genome-wide base-resolution mapping of DNA methylation in single cells using single-cell bisulfite sequencing (scBS-seq). Nat. Protoc. 12 (3), 534–547. http://dx.doi.org/10.1038/nprot.2016.187.

Dalerba, P., Kalisky, T., Sahoo, D., Rajendran, P.S., Rothenberg, M.E., Leyrat, A.A., et al., 2011. Single-cell dissection of transcriptional heterogeneity in human colon tumors. Nat. Biotechnol. 29 (12), 1120–1127. http://dx.doi.org/10.1038/nbt.2038.

Darmanis, S., Gallant, C.J., Marinescu, V.D., Niklasson, M., Segerman, A., Flamourakis, G., et al., 2016. Simultaneous multiplexed measurement of RNA and proteins in single cells. Cell Rep. 14 (2), 380–389. http://dx.doi.org/10.1016/j.celrep.2015.12.021.

de Ronde, M.W.J., Ruijter, J.M., Lanfear, D., Bayes-Genis, A., Kok, M.G.M., Creemers, E.E., et al., 2017. Practical data handling pipeline improves performance of qPCR-based circulating miRNA measurements. RNA 23 (5), 811–821. http://dx.doi.org/10.1261/rna.059063.116.

de Wit, S., van Alum, G., Lenferimk, A.T., Tibbe, A.G., Hiltermann, T.J., Groen, H.J., et al., 2015. The detection of EpCAM(+) and EpCAM(-) circulating tumor cells. Sci. Rep. 5 (12270) http://dx.doi.org/10.1038/srep12270.

Dolatabadi, S., Candia, J., Akrap, N., Vannas, C., Tesan Tomic, T., Losert, W., et al., 2017. Cell cycle and cell size dependent gene expression reveals distinct subpopulations at single-cell level. Front. Genet. 8 (1) http://dx.doi.org/10.3389/fgene.2017.00001.

Dzamba, D., Valihrach, L., Kubista, M., Anderova, M., 2016. The correlation between expression profiles measured in single cells and in traditional bulk samples. Sci. Rep. 6 (37022) http://dx.doi.org/10.1038/srep37022.

Gawad, C., Koh, W., Quake, S.R., 2016. Single-cell genome sequencing: current state of the science. Nat. Rev. Genet. 17 (3), 175–188. http://dx.doi.org/10.1038/nrg.2015.16.

Genshaft, A.S., Li, S., Gallant, C.J., Darmanis, S., Prakadan, S.M., Ziegler, C.G., et al., 2016. Multiplexed, targeted profiling of single-cell proteomes and transcriptomes in a single reaction. Genome Biol. 17 (1), 188. http://dx.doi.org/10.1186/s13059-016-1045-6.

Gründemann, J., Schlaudraff, F., Liss, B., 2011. UV-laser microdissection and mRNA expression analysis of individual neurons from postmortem Parkinson's disease brains. Methods Mol. Biol. 755, 363–374. http://dx.doi.org/10.1007/978-1-61779-163-5_30.

Guo, G., Huss, M., Tong, G.Q., Wang, C., Li Sun, L., Clarke, N.D., Robson, P., 2010. Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. Dev. Cell 18 (4), 675–685. http://dx.doi.org/10.1016/j.devcel.2010.02.012.

Guo, H., Zhu, P., Guo, F., Li, X., Wu, X., Fan, X., 2015. Profiling DNA methylome landscapes of mammalian cells with single-cell reduced-representation bisulfite sequencing. Nat. Protoc. 10 (5), 645–659. http://dx.doi.org/10.1038/nprot.2015.039.

Hodne, K., Weltzien, F.A., 2015. Single-cell isolation and gene analysis: pitfalls and possibilities. Int. J. Mol. Sci. 16 (11), 26832–26849. http://dx.doi.org/10.3390/ijms161125996.

Huggett, J.F., Foy, C.A., Benes, V., Emslie, K., Garson, J.A., Haynes, R., et al., 2013. The digital MIQE guidelines: minimum information for publication of quantitative digital PCR experiments. Clin. Chem. 59 (6), 892–902. http://dx.doi.org/10.1373/clinchem.2013.206375.

Karro, J.E., Yan, Y., Zheng, D., Zhang, Z., Carriero, N., Cayting, P., et al., 2007. Pseudogene.org: a comprehensive database and comparison platform for pseudogene annotation. Nucleic Acids Res. 35 (Suppl. l_1), D55–D60. http://dx.doi.org/10.1093/nar/gkl851.

Krishnaswami, S.R., Grindberg, R.V., Novotny, M., Venepally, P., Lacar, B., Bhutani, K., et al., 2016. Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. Nat. Protoc. 11 (3), 499–524. http://dx.doi.org/10.1038/

nprot.2016.015.
Kroneis, T., Jonasson, E., Andersson, D., Dolatabadi, S., Ståhlberg, A., 2017. Global preamplification simplifies targeted mRNA quantification. Sci. Rep. 7 (45219) http://dx.doi.org/10.1038/srep45219.
Krüttli, A., Bouwman, A., Akgül, G., Della Casa, P., Rühli, F., Warinner, C., 2015. Ancient DNA analysis reveals high frequency of European lactase persistence allele (T-13910) in medieval central europe. PLoS One 9 (1), e86251. http://dx.doi.org/10.1371/journal.pone.0086251.
Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonak, J., Lind, K., et al., 2006. The real-time polymerase chain reaction. Mol. Asp. Med. 27 (2–3), 95–125. http://dx.doi.org/10.1016/j.mam.2005.12.007.
Laurell, H., Iacovoni, J.S., Abot, A., Svec, D., Maoret, J.J., Arnal, J.F., Kubista, M., 2012. Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. Nucleic Acids Res. 40 (7), e51. http://dx.doi.org/10.1093/nar/gkr1259.
Lee, J.H., Daugharthy, E.R., Scheiman, J., Kalhor, R., Yang, J.L., Ferrante, T.C., et al., 2014. Highly multiplexed subcellular RNA sequencing in situ. Science 343 (6177), 1360–1363. http://dx.doi.org/10.1126/science.1250212.
Leung, M.L., Wang, Y., Kim, C., Gao, R., Jiang, J., Sei, E., Navin, N.E., 2016. Highly multiplexed targeted DNA sequencing from single nuclei. Nat. Protoc. 11 (2), 214–335. http://dx.doi.org/10.1038/nprot.2016.005.
Liss, B., Franz, O., Sewing, S., Bruns, R., Neuhoff, H., Roeper, J., 2001. Tuning pacemaker frequency of individual dopaminergic neurons by Kv4.3L and KChip3.1 transcription. EMBO J. 20 (20), 5715–5724. http://dx.doi.org/10.1093/emboj/20.20.5715.
Livak, K.J., Wills, Q.F., Tipping, A.J., Datta, K., Mittal, R., Goldson, A.J., et al., 2013. Methods for qPCR gene expression profiling applied to 1440 lymphoblastoid single cells. Methods 59 (1), 71–79. http://dx.doi.org/10.1016/j.ymeth.2012.10.004.
McCall, M.,N., McMurray, H.R., Land, H., Almudevar, A., 2014. On non-detects in qPCR data. Bioinformatics 30 (16), 2310–2316. http://dx.doi.org/10.1093/bioinformatics/btu239.
Narsinh, K.H., Sun, N., Sanchez-Freire, V., Lee, A.S., Almeida, P., Hu, S., et al., 2011. Single cell transcriptional profiling reveals heterogeneity of human induced pluripotent stem cells. J. Clin. Invest. 121 (3), 1217–1221. http://dx.doi.org/10.1172/JCI44635.
Neves, R.P., Raba, K., Schmidt, O., Honisch, E., Meier-Stiegen, F., Behrens, B., et al., 2014. Genomic high-resolution profiling of single CKpos/CD45neg flow-sorting purified circulating tumor cells from patients with metastatic breast cancer. Clin. Chem. 60 (10), 1290–1297. http://dx.doi.org/10.1373/clinchem.2014.222331.
Nichterwitz, S., Chen, G., Aguila Benitez, J., Yilmaz, M., Storvall, H., Cao, M., et al., 2016. Laser capture microscopy coupled with Smart-seq2 for precise spatial transcriptomic profiling. Nat. Commun. 7 (12139) http://dx.doi.org/10.1038/ncomms12139.
Ottesen, E.A., Hong, J.W., Quake, S.R., Leadbetter, J.R., 2006. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. Science 314 (5804), 1464–1467. http://dx.doi.org/10.1126/science.1131370.
Pei, B., Sisu, C., Frankish, A., Howald, C., Habegger, L., Mu, X.J., et al., 2012. The GENCODE pseudogene resource. Genome Biol. 13 (9), R51. http://dx.doi.org/10.1186/gb-2012-13-9-r51.
Pollen, A.A., Nowakowski, T.J., Shuga, J., Wang, X., Leyrat, A.A., Lui, J.H., et al., 2014. Low-coverage single-cell mRNA sequencing reveals cellular heterogeneity and activated signaling pathways in developing cerebral cortex. Nat. Biotechnol. 32 (10), 1053–1058. http://dx.doi.org/10.1038/nbt.2967.
Potter, N.E., Ermini, L., Papaemmanuil, E., Cazzaniga, G., Vijayaraghavan, G., Titley, I., et al., 2013. Single-cell mutational profiling and clonal phylogeny in cancer. Genome Res. 23 (12), 2115–2125. http://dx.doi.org/10.1101/gr.159913.113.
Raj, A., Peskin, C.S., Tranchina, D., Vargas, D.Y., Tyagi, S., 2006. Stochastic mRNA synthesis in mammalian cells. PLoS Biol. 4 (10), e309. http://dx.doi.org/10.1371/journal.pbio.0040309.
Ruijter, J.M., Pfaffl, M.W., Zhao, S., Spiess, A.N., Boggy, G., Blom, J., et al., 2013. Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. Methods 59 (1), 32–46. http://dx.doi.org/10.1016/j.ymeth.2012.08.011.
Rusnakova, V., Honsa, P., Dzamba, D., Ståhlberg, A., Kubista, M., Anderova, M., 2013. Heterogeneity of astrocytes: from development to injury - single cell gene expression. PLoS One 8 (8), e69734. http://dx.doi.org/10.1371/journal.pone.0069734.
Rygiel, K.A., Grady, J.P., Taylor, R.W., Tuppen, H.A., Turnbull, D.M., 2015. Triplex real-time PCR—an improved method to detect a wide spectrum of mitochondrial DNA deletions in single cells. Sci. Rep. 5 (9906) http://dx.doi.org/10.1038/srep09906.
Sidova, M., Sindelka, R., Castoldi, M., Benes, V., Kubista, M., 2015. Intracellular microRNA profiles form in the Xenopus laevis oocyte that may contribute to asymmetric cell division. Sci. Rep. 5 (11157) http://dx.doi.org/10.1038/srep11157.
Sindelka, R., Jonák, J., Hands, R., Bustin, S.A., Kubista, M., 2008. Intracellular expression profiles measured by real-time PCR tomography in the Xenopus laevis oocyte. Nucleic Acids Res. 36 (2), 387–392. http://dx.doi.org/10.1093/nar/gkm1024.
Spiess, A.N., Deutschmann, C., Burdukiewicz, M., Himmelreich, R., Klat, K., Schierack, P., Rödiger, S., 2015. Impact of smoothing on parameter estimation in quantitative DNA amplification experiments. Clin. Chem. 61 (2), 379–388. http://dx.doi.org/10.1373/clinchem.2014.230656.
Spiess, A.N., Rödiger, S., Burdukiewicz, M., Volksdorf, T., Tellinghuisen, J., 2016. System-specific periodicity in quantitative real-time polymerase chain reaction data questions threshold-based quantitation. Sci. Rep. 6 (38951) http://dx.doi.org/10.1038/srep38951.
Ståhl, P.L., Salmén, F., Vickovic, S., Lundmark, A., Navarro, J.F., Magnusson, J., et al., 2016. Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. Science 353 (6294), 78–82. http://dx.doi.org/10.1126/science.aaf2403.
Ståhlberg, A., Håkansson, J., Xian, X., Semb, H., Kubista, M., 2004a. Properties of the reverse transcription reaction in mRNA quantification. Clin. Chem. 50 (3), 509–515. http://dx.doi.org/10.1373/clinchem.2003.026161.
Ståhlberg, A., Kubista, M., Pfaffl, M., 2004b. Comparison of reverse transcriptases in gene expression analysis. Clin. Chem. 50 (9), 1678–1680. http://dx.doi.org/10.1373/clinchem.2004.035469.
Ståhlberg, A., Bengtsson, M., 2010. Single cell gene expression profiling using reverse transcription quantitative real-time PCR. Methods 50 (4), 282–288. http://dx.doi.org/10.1016/j.ymeth.2010.01.002.
Ståhlberg, A., Andersson, D., Aurelius, J., Faiz, M., Pekna, M., Kubista, M., Pekny, M., 2011. Defining cell populations with single-cell gene expression profiling: correlations and identification of astrocyte subpopulations. Nucleic Acids Res. 39 (4), e25. http://dx.doi.org/10.1093/nar/gkq1182.
Ståhlberg, A., Thomsen, C., Ruff, D., Åman, P., 2012. Quantitative PCR analysis of DNA, RNAs and proteins in the same single-cell. Clin. Chem. 58 (12), 1682–1691. http://dx.doi.org/10.1373/clinchem.2012.191445.
Ståhlberg, A., Rusnakova, V., Forootan, A., Anderova, M., Kubista, M., 2013a. RT-qPCR work-flow for single-cell data analysis. Methods 59 (1), 80–88. http://dx.doi.org/10.1016/j.ymeth.2012.09.007.
Ståhlberg, A., Rusnakova, V., Kubista, M., 2013b. The added value of single-cell gene expression profiling. Brief. Funct. Genom. 12 (2), 81–89. http://dx.doi.org/10.1093/bfgp/elt001.
Ståhlberg, A., Kubista, M., 2014. The workflow of single-cell expression profiling using quantitative real-time PCR. Expert Rev. Mol. Diagn 14 (3), 323–331. http://dx.doi.org/10.1586/14737159.2014.901154.
Svec, D., Andersson, D., Pekny, M., Sjöback, R., Kubista, M., Ståhlberg, A., 2013. Direct cell lysis for single-cell gene expression profiling. Front. Oncol. 3 (274) http://dx.doi.org/10.3389/fonc.2013.00274.
Svec, D., Tichopad, A., Novosadova, V., Pfaffl, M.W., Kubista, M., 2015. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. Biomol. Detect. Quantif. 3, 9–16. http://dx.doi.org/10.1016/j.bdq.2015.01.005.
Tang, F., Hajkova, P., Barton, S.C., Lao, K., Surani, M.A., 2006. MicroRNA expression profiling of single whole embryonic stem cells. Nucleic Acids Res. 32 (2), e9. http://dx.doi.org/10.1093/nar/gnj009.
Tellinghuisen, J., Spiess, A.N., 2014. Comparing real-time quantitative polymerase chain reaction analysis methods for precision, linearity, and accuracy of estimating amplification efficiency. Anal. Biochem. 449, 76–82. http://dx.doi.org/10.1016/j.ab.2013.12.020.
Tichopad, A., Kitchen, R., Riedmaier, I., Becker, C., Ståhlberg, A., Kubista, M., et al., 2009. Design and optimization of reverse-transcription quantitative PCR experiments. Clin. Chem. 55 (10), 1816–1823. http://dx.doi.org/10.1373/clinchem.2009.126201.
Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (7) http://dx.doi.org/10.1186/gb-2002-3-7-research0034. RESEARCH0034.
Wang, J., Chen, L., Chen, Z., Zhang, W., 2015. RNA-seq based transcriptomic analysis of single bacterial cells. Integr. Biol. 7 (11), 1466–1476. http://dx.doi.org/10.1039/c5ib00191a.
Wang, L., Brooks, A.N., Fan, J., Wan, Y., Gambe, R., Li, S., et al., 2016. Transcriptomic characterization of SF3B1 mutation reveals its pleiotropic effects in chronic lymphocytic leukemia. Cancer Cell 30 (5), 750–763. http://dx.doi.org/10.1016/j.ccell.2016.10.005.
Wen, L., Tang, F., 2016. Single-cell sequencing in stem cell biology. Genome Biol. 17 (71) http://dx.doi.org/10.1186/s13059-016-0941-0.
Wills, Q.F., Livak, K.J., Tipping, A.J., Enver, T., Goldson, A.J., Sexton, D.W., Holmes, C., 2013. Single-cell gene expression analysis reveals genetic associations masked in whole-tissue experiments. Nat. Biotechnol. 31 (8), 748–752. http://dx.doi.org/10.1038/nbt.2642.
Worthington Tissue Dissociation Guide. http://www.worthington-biochem.com/tissuedissociation/default.html (accessed 17 may 08).
Wu, A.R., Neff, N.F., Kalisky, T., Dalerba, P., Treutlein, B., Rothenberg, M.E., et al., 2014. Quantitative assessment of single-cell RNA-sequencing methods. Nat. Methods 11 (1), 41–46. http://dx.doi.org/10.1038/nmeth.2694.
Yang, Y., Swennenhuis, J.F., Rho, H.S., Le Gac, S., Terstappen, L.W., 2014. Parallel single cancer cell whole genome amplification using button-valve assisted mixing in nanoliter chambers. PLoS One 9 (9), e107958. http://dx.doi.org/10.1371/journal.pone.0107958.
Ziegenhain, C., Vieth, B., Parekh, S., Reinius, B., Guillaumet-Adkins, A., Smets, M., et al., 2017. Comparative analysis of single-cell RNA sequencing methods. Mol. Cell 65 (4), 631–643. http://dx.doi.org/10.1016/j.molcel.2017.01.023 e4.

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