RNAGEM™ Tissue

Antarctica - the source of RNAGEM™ (Camp, Ross Ice Shelf)

Sample normalisation with RNAGEM™ Tissue

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Introduction

When working with low cell counts or cell lysates, most rapid methods designed for estimating total RNA quantity are insufficiently sensitive or affected by material in unpurified lysates.

An alternative way to normalise samples is to rely on the cell numbers in the starting material. These can be determined with a cell counter or by using a microscope and haemocytometer. However, with low counts, small sample volumes or high-throughput systems, direct cell counting is impractical and unreliable.

One solution is to estimate cell numbers from DNA content. qPCR can precisely quantify DNA and this in turn can be used to estimate cell numbers. Ploidy and cell growth-phase need to be accounted for in calculating cell numbers from gene copy number, but in general, DNA quantification provides an accurate way to normalise samples.

For DNA to be used for for providing an estimate of cell counts, it must be present in the lysate and there must be a linear relationship between cell numbers and DNA yield over the expected working range.

RNAGEM™ and extraction of DNA for cell number estimation

RNAGEM is a whole nucleic acid extraction kit that gives linear yields between <10 cells to ~50,000 cells when using the recommended method (figure 1). If larger samples are needed, then the method is easily scaled (see www.zygem.com for details).

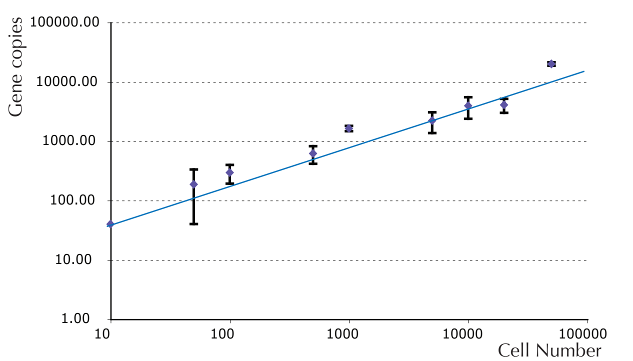

Figure 1: The number of detected gene copies detected from the gDNA co-extracted from HeLa cells using RNAGEM

This linearity means that the DNA present in the lysate produced by RNAGEM can be used to determine the starting cell number by qPCR.

Because different primers and PCR reagents have different amplification efficiencies, it is worthwhile calibrating the DNA yields from a range of counted cells. Also, be cognizant of the copy number of the gene, the presence of pseudogenes and the growth cell cycle all of which can affect the outcome.

Using a reference mRNA for normalisation

Other methods of RNA normalisation use RT-qPCR to quantify. Either a reference housekeeping mRNA, rRNA or a synthetic molecule is used. The advantages and disadvantates of a number of these methods are reviewed in Hugget et al. 2005.

However, if RNA and DNA are co-extracted with similar efficiencies, then gDNA copies can provide a simple and direct predictive estimate of the RNA yield without needing to rely on reference RNA molecules. An advantage of using endogenous gDNA rather than an internal RNA standard is that the DNA content of cells remains relatively constant for cells in the same growth phase and the cost and labour involved in performing a qPCR is less that that for an RT-qPCR.

A prerequisite for using this approach is that the efficiency of both DNA and RNA extraction remains constant over the range of cell numbers likely to be encountered. Furthermore, this should apply for both low and high copy mRNAs.

If DNA is to be used, then a calibration curve of DNA and RNA yield should be determined using a reference mRNA and RT-qPCR. From this calibration, a DNA:RNA yield ratio can be derived and used in subsequent experiments to estimate mRNA copies. If the copy number of the housekeeping gene in use is already known, then the total ng yield of RNA can also be estimated. In short, if an extraction method produces both DNA and RNA in constant proportions over a range of cell numbers, the need for carrying out an RT-qPCR on a reference mRNA can be side-stepped as long as cell growth conditions are kept consistent and a calibration standard is production.

To demonstrate RNAGEM's ability to produce RNA and DNA in consistent, linear proportions, 10-1000 HeLa cells were treated with RNAGEM Tissue. The mRNA was quantified by RT-qPCR using qScript ™ One-Step SYBR® Green qRT-PCR Kit, ROX™ (Quanta Biosciences). DNA was quantified using qPCR with Platinum® SYBR® Green qPCR Supermix-UDG with ROX (Invitrogen).

The gene used for the DNA quantification was the human BRCA1 gene (breast cancer early onset - Chrm 17: 3x copies in HeLa). The mRNA was ACTB (beta-actin). The results are shown in figure 2.

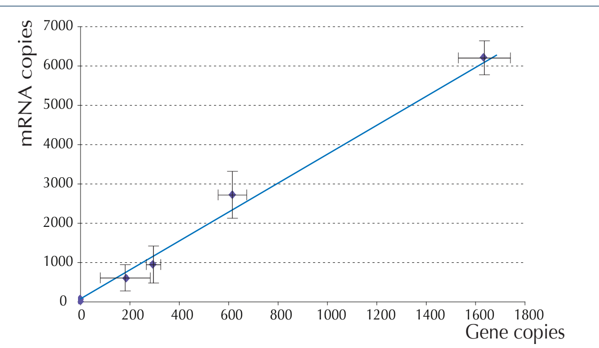

Figure 2: Graph of gene copies detected by qPCR plotted against mRNA copies detected by RT-qPCR. The linear release of DNA and RNA means that RNA yield can be estimated from a standard DNA qPCR.

In another experiment, we assessed this linearity on small sample sizes when co-extracting gDNA and a low and high copy number mRNA species.

The number of copies of the BRCA1 genes were determined by qPCR and number of mRNA copies for ACTB and BRCA1 by RT-qPCR (figure 3).

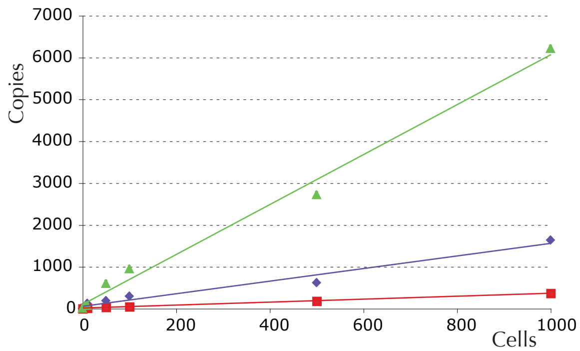

Figure 3: Graph of gene and mRNA copies detected plotted against cell number. Both the mRNA and the DNA yield is linear for this range of cell numbers. Relative mRNA copy number can be determined from the gradient of the plots. ◆ = BRCA 1 DNA (y = 5.97x + 92.20), ■ = BRCA1 mRNA (y = 0.36x + 8.42) and ▲ = ACTB mRNA (y = 1.50x + 49.2)

Calculating the ratio

The gradient and y-axis intercept of the lines can be determined from the linear regression of the plots. As the y intercept is expected to be at zero for all cases, this parameter can be ignored and so the DNA:RNA ratio is calculated from the ratio of gradients.

In this example the gradients are:

BRCA1 gene (reference DNA)	1.50
BRCA1 mRNA	0.36
ACTB mRNA	5.97

It is important to be aware that these figures are not the true, relative copy numbers of mRNA molecules in the cell. Although DNA yield is a good approximation of cell number, the measurement of mRNA copy number is affected by an number of factors that relate to expression and RT efiiciency. However, as a method for relative normalisation of cells and for providing an estmate of RNA yields in high-througput experiments, *RNA*GEM and DNA quantification offers a reliable and simple solution.

If you wish to use this method for your current workflow, then procedural consistency adhered to.

For help contact support@zygem.com

Reference

Hugget et al. (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes and Immunity, 1- 6.