*RNA*GEM™ Tissue — Rapid RNA extraction for RT-qPCR From Cells numbers 1-150,000

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Introduction

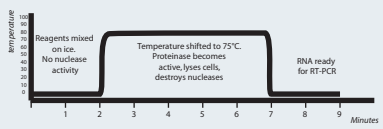
The use of cell lysates for direct RT-qPCR is requisite in many clinical and biomedical research applications. Cellular numbers can vary but often, single cells, need to be analyzed. Small samples are not readily prepared by solvent, column or precipitation based methods because of limited recovery and the potential for contamination. Furthermore, these samples are not suited to traditional quantitation methods (UV absorbance and fluorescent dyes) because of the limited sensitivity of the methods at the low levels of nucleic acids. In such cases the best method for quantitation is qPCR using genomic DNA as a template.

*RNA*GEM™, a rapid cell lysate preparation kit, delivers superior sensitivity for both gene expression profiling and sample mass normalization using qPCR. The kit makes use of a highly-active thermophilic protease which strips protein from nuclec acid templates whilst lysing cells and rapidly destroying RNAses.

The enzymes and reagents used in ZyGEM kits have been selected to be fully compatible with most downstream applications and so there is no need for stop solutions, solvent extraction or precipation steps. Hence, *RNA*GEM provides an approach that is far simpler than those common to chemical lysis or proteinase K methods. The result is an RNA extract that is immediately ready for use in sensitive RT-qPCR assays.

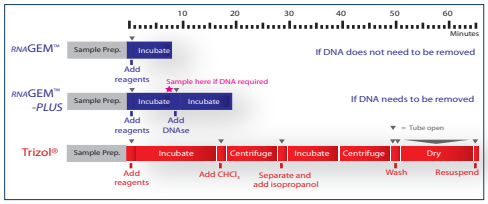
The *RNA*GEM single-step protocol releases RNA and DNA with excellent linearity across a wide range of cell numbers. The Method is automatable, closed-tube and does not require further purification of the RNA for accurate RT-qPCR analysis. Greater sensitivity is achieved - especially with low abundance transcripts Reduced handling, and efficient template preparation means that the *RNA*GEM kits generate mRNA profiles that are as close to the biological reality as possible.

Procedures



*RNA*GEM procedures are closed-tube and activated by a tempeature shift using a thermal cycler. Reagents are mixed on ice, and then a shift to 75°C activates the proteinase which then lyses the cells and destroys ribonucleases. Because all the *RNA*GEM buffers are compatible with RT and PCR enzymes, the lysate can be used directly without purfication.

Two *RNA*GEM Tissue kits are available from ZyGEM. Both use the same, simple procedure for extracting RNA but the *PLUS* kit includes RNAse-free DNAse I. At 37°C the *RNA*GEM proteinase is inactive allowing the DNAse to work. A temperature shift back to 75°C re-activates the proteinase which then destroys the DNAse. No EDTA needs to be added.



*RNA*GEM Tissue

1. Add:
   - Cell suspension or pellet
   - 10x Buffer SILVER
   - *RNA*GEM
   - Water
2. Vortex and incubate at 75°C
   < 50,000 cells - 5 min
   > 50,000 cells - 10 min
   A thermal cycler should be used for this step.

DNAse treatment (Steps are not needed for Intron spanning primers)

1. To the extract add:
   - 10x DNAse buffer
   - DNAse I
2. Incubate:
   37°C for 5 minutes
   75°C for 5 minutes
3. Add 10 x TE Buffer (provided) and store at -20°C or below.

Scalability and small sample sizes

The method provided with the kit is designed for cell numbers of less than 100,000 per extraction, although the procedure can be scaled for larger numbers. Typically an extraction would use the following volumes and incubation times.

Cells	Volume	*RNA*GEM	Time
50k -100k	50 - 100 µl	1 µl	10 min
5k - 50k	20 - 50 µl	1 µl	5 min
100 - 5k	5 - 20 µl	0.5 µl	5 min
<100	1 - 15 µl	0.2 µl	5 min

*RNA*GEM and RT-PCR

The SILVER buffer used by *RNA*GEM has been formulated to be compatible with polymerases, reverse-transcriptases and many other nucleic acid modifying enzymes, and so the extracts can be used without purification in PCR, RT-PCR, qPCR and RT-qPCR.

Figure 1 shows plots obtained when 5 µl of *RNA*GEM -extracts were added directly to an RT-qPCR. HeLa cell numbers from 10-50,000 were *RNA*GEM -treated, and plots generated from a high abundance mRNA (ACTB; ß-actin) and a low abundance mRNA (BRCA1; breast cancer early onset). The clean traces with gradients similar to the standards demonstrate the lack of inhibition.

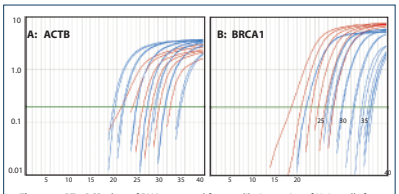


Figure 1: RT-qPCR plots of RNA extracted from a dilution series of HeLa cells from 10 - 50,000 cells. **A:** high copy number mRNA (ACTB). **B:** low copy number mRNA (BRCA1).
Red = standards; Blue = duplicate mRNA curves

Using the recommended method, the extraction efficiency of *RNA*GEM is constant over the range of 1 to approximately 100,000 cells (figure 2). Different ranges of linearity can be obtained by small modifications to the base procedure.

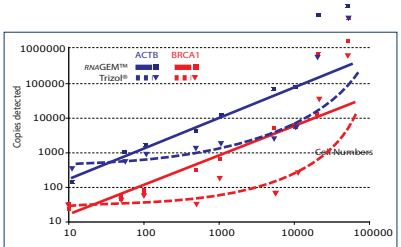


Figure 2: Log/Log plots of HeLa cell numbers versus mRNA copies detected. Both high copy number mRNAs (ACTB) and low copy number (BRCA1) are shown. Two extraction methods are used, *RNA*GEM and Trizol®

*RNA*GEM and DNA extraction for cell number estimation

*RNA*GEM is a whole nucleic acid extraction kit that gives linear yields of DNA between <10 cells and ~100,000 cells when using the recommended method (figure 3). If larger samples are needed, then the method is easily scaled (see www.zygem.com for details).

This linearity means that the DNA present in the lysate produced by *RNA*GEM can be used to determine the original cell number by qPCR.

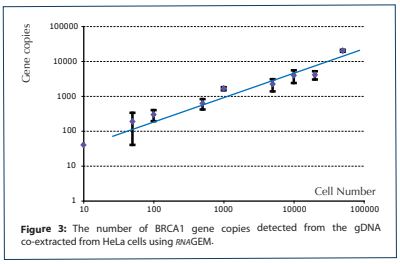


Figure 3: The number of BRCA1 gene copies detected from the gDNA co-extracted from HeLa cells using *RNA*GEM.

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

Sample normalisation

When calculating the copy number of an mRNA, or using an analytical method with a narrow tolerance for sample variation, it is important to normalise samples before the analysis is performed. With normalised samples, failure rates are reduced and comparative measures are more reliable.

The amount of RNA in a sample can be normalised using an RT-qPCR. Either a reference housekeeping mRNA, rRNA or a synthetic molecule can be used to provide an estimate of the total RNA concentration. The advantages and disadvantages of these methods are reviewed in Hugget et al. 2005. However, if RNA and DNA are simultaneously co-extracted with similar efficiencies, then gDNA copies can also provide a simple and direct estimate of cell numbers which in turn provides a normalisation factor for total RNA quantity.

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

In order to demonstrate *RNA*GEM's ability to produce RNA and DNA in consistent, linear proportions, 10-10000 HeLa cells were extracted using *RNA*GEM Tissue. The mRNA was quantified by RT-qPCR using qScript cDNA Supermix. DNA was quantified using a qPCR withPerfeCTA® SYBR® Green FastMix®, ROX™ (Quanta Biosciences) - figure 4.

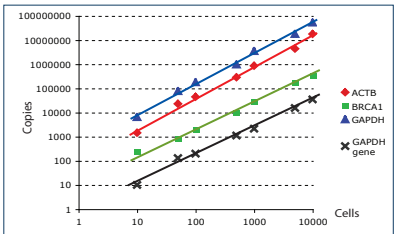


Figure 4: Copies of the human GAPDH gene detected by qPCR plotted alongside copies detected by RT-qPCR of three mRNAs. ACTB = 366 copies per cell; GAPDH = 2607 copies per cell; BRCA1 26 copies per cell.

Extraction linearity is seen for both low and high copy mRNA and also DNA. This ability means that it is possible to normalise *RNA*GEM extracts using a qPCR rather than an RT-qPCR - a simpler and less costly method.

Reference

Hugget et al. (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes and Immunity. ***6****:279-284*

Kit components

*RNA*GEM™ Tissue

- *RNA*GEM enzyme
- 10 x SILVER buffer
- 10 x TE storage buffer
- QuickStart Guide

*RNA*GEM™ Tissue *PLUS*

- *RNA*GEM enzyme
- 10 x SILVER buffer
- 10 x TE storage buffer
- RNAse-free DNAse 1
- 10 x DNAse Buffer
- QuickStart Guide

For more information visit: www.zygem.com
or email: info@zygem.com