

Frequently asked Questions on RNA amplification prior to QPCR

Why should I amplify when QPCR is already an amplification?

In small samples like biopsies, LCM, or sorted cell samples, the amount of RNA that can be isolated is often very limiting. Good QPCR experimental design can require large amounts of RNA due to the need for several types of controls and to allow for running triplicate reactions. Therefore, as the numbers of genes of interest increase, so does the sample amount requirement. In addition, many low abundance transcripts are naturally below the detection level of QPCR analysis. This limitation is even more critical in small and difficult samples. By amplifying the RNA prior to QPCR analysis, many low abundance transcripts are moved into the detectable range, resulting in higher levels of sensitivity and reproducibility. Thus, RNA amplification allows for better-designed, informative QPCR investigation of larger numbers of genes, with a wide range of abundance, in small homogeneous biological samples.

My sample is large, there is no reason for me to amplify, is there?

Yes, there is a reason. The fact that the biological sample is large – e.g. a whole organ – does not mean that the entire sample needs to be, or should be, used for QPCR.

First, one of the main goals of gene expression studies is to correlate an observed phenotype or pathology to expression patterns. This goal is best achieved by using as homogenous a biological sample as possible. Large numbers of different cell populations present in whole organs or even large sections of organs result in overlapping and low resolution expression profiles, masking the real biology. In these non-homogenous sources of RNA samples, it is often difficult, if not impossible, to correlate phenotype to expression profiles sensitively, accurately, or definitively. With new advances in LCM technologies, cell sorting, or other cell population classification approaches, most biological samples can undergo finer sub classification, yielding more homogenous populations of cells, which enables more valuable and accurate biological conclusions from gene expression analysis by QPCR.

Second, the differential expression of genes on an mRNA level is often the first indication of the underlying pathways involved in a response. For thorough investigation on the biology behind such indicators, one should look at e.g. protein expression, protein modification, in situ hybridization, or immunohistochemistry approaches. The use of RNA amplification allows the researcher to reserve the original biological material for downstream studies that may have larger sample requirements and use only a minor portion of the original sample for gene expression profiling. Additionally, the larger biological samples often consist of a mix of very different cell types and sub-types. RNA amplification enables deeper cell type segregation than by dissection or sorting - allowing separate investigation of different cell types in the same tissue sample.

I am only interested in a few genes, why would I amplify my RNA?

Today, your research may very well be focused on a small number of genes and you may currently not see a requirement for amplification. However, science is developing quickly with new technologies, discoveries, investigative and emerging analytical approaches. For example, new array data may become available in your area of interest or new publications may identify genes other than those you have investigated previously. When you generate an amplified cDNA sample, you are generating a more stable and plentiful copy of your original RNA sample. This cDNA may be archived for future QPCR applications increasing the number of genes you study at a given time, without the need to re-generate the amplified samples and the data and without depleting any more of the original RNA sample.

What is the impact of RNA amplification on my experimental design?

- RNA amplification significantly impacts experimental design. Now, you have more sample material for QPCR and therefore less demand for biological material. Consider the following study design issues with and without amplification:

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- Without amplification, you need to generate and collect a large number of samples that require pooling samples in order to have sufficient materials for a study.
- With amplification, you can:
 - Study each small sample individually reducing the inherent biological variability introduced by pooling.
 - Reduce the number of samples that need to be collected and generated (e.g. test animals).
 - Study subpopulations and specific cell types within the collected sample, therefore increasing your study sensitivity and obtaining more biologically significant data
- Without amplification, precious small clinical sample sets can be utilized for only a limited number of studies minimizing accessibility and the potential for collaborations.
- With amplification, you can:
 - Generate sufficient quantities of cDNA from your samples to complete your own studies and yet have enough materials to share with collaborators. You may also have sufficient original RNA sample left to embark on future collaborations, not considered today.
 - Gain access to other investigators extremely limited and rare patient materials, i.e. you are more likely to have access if you only require 50 nanograms of sample compared to 5 µg.
 - Organize consortiums around sample banks, pursue more sample sharing collaborations, and pool data and resources for difficult to procure samples.
- Without amplification, you have to design your study based on how limited your sample is, possibly foregoing sufficient controls, critical to the quality of experimental design.
- With amplification, you can:
 - Include appropriate number of both biological and technical replicates in all your studies.
 - Include sufficient internal controls in the same expression range as each of your genes of interest.
 - Determine the appropriate normalizing genes for your experimental system empirically and run as many normalizing genes as you chose.
 - Optimize your QPCR approach with your experimental samples not just a commercial control RNA, reducing variability even further.

Hypothetical study

Consider a study on a brain tumor in a mouse model system, which explores tumor development and RNA expression in 3 stages. From the earliest stage sample, approximately 50 ng of total RNA can be isolated. For later stages, about 100 ng of total RNA can be isolated. Experiments are done in triplicate, and QPCR is performed on 2 housekeeping controls and 20 target genes. Reactions are carried out as singleplex, using 10 ng of total RNA per reaction.

Without amplification:

- The study investigates 22 genes, requiring 14-15 animals for the earliest stage and 7-8 animals for the later stages. In total 28-30 animals are required.
- QPCR is performed and data is analyzed.
- Quite interesting data is obtained from the study. The involvement of some proteins and their localization is hypothesized.
- New animals now have to be generated for the protein localization experiment. Protein localization experiments are performed.
- The protein localization proves the hypothesis. However, there is still the open question of which additional genes may be involved. It is decided to run microarray experiments to identify new candidate genes. This requires triplicates for each stage - a total of 9 animals. Since it was understood that QPCR and protein localization experiments would follow, in parallel another group of 24 animals is generated. Tissue samples are isolated and stored at -80 °C. RNA data and protein data could not be directly linked.
- A QPCR analysis is performed with new genes, as well as protein localization and immune precipitation experiments.

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With amplification:

- The study investigates 22 genes, requiring 3 animals for each stage. In total 9 animals will be used for the study.
- QPCR is performed and data is analyzed.
- Quite interesting data is obtained from the study. The involvement of some proteins and their localization is hypothesized.
- New animals now have to be generated for the protein localization experiment. Protein localization experiments were performed.
- The protein localization proves the hypothesis. However, there is still the open question of which additional genes may be involved. It is decided to run microarray experiments to identify new candidate genes. This requires triplicates for each stage - a total of 9 animals. Since it is understood that protein localization experiments would follow, tissue samples are isolated and stored at -80 °C.
- The leftover cDNA of the microarray samples is used for QPCR analysis is performed on some new genes. The remainder of the tissue is used for some protein localization and immune precipitation experiments and the data can be directly correlated to QPCR data. The QPCR can then be performed on the first set of mice as well, to determine if the data correlates. Since no additional RNA has to be isolated, this process takes less time.

The advantage of amplification prior to QPCR in this study:

- Reduces costs; 3-4 times fewer animals are required
- Saves time; the same amplified cDNA is used for both microarray analysis and QPCR confirmation
- Enables direct correlation of protein and RNA data
- Facilitates comparing RNA data between batches of animals, increasing the total number of replicates
- Generates sufficient material left for future QPCR and array experiments



WT-Ovation™ RNA Amplification System Specifications

Cat No.: 2210-24, 24 reactions

Input: 5 - 50 ng total RNA

Yield: 1.5 - 4 µg single stranded cDNA

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NuGEN Technologies, Inc.
Headquarters USA

821 Industrial Road, Unit A San Carlos, CA 94070 USA, Toll Free Tel: 888.654.6544 Toll Free Fax: 888.296.6544 www.nugeninc.com
custserv@nugeninc.com techserv@nugeninc.com

Canada

MJS BioLynx Inc.
P.O Bag 1150, 300 Laurier Blvd.
Brockville, ON K6V 5W1
Toll Free: 1-888-593-5969
Tel: (613) 498-2126
Fax: (613) 342-1341
sales@biolynx.ca or tech@biolynx.ca
www.biolynx.ca/contact-biolynx.html

Europe

NuGEN Technologies,
Inc. P.O. Box 149,
6680 AC Bommel
The Netherlands
Tel: +31(0)13 5780215
Fax: +31(0)13 5780216
europa@nugeninc.com
www.nugeninc.com

Asia

MediBIC.
Daido Seimei Kasumigaseki
Building 8F, 1-4-2
Kasumigaseki, Chiyoda-ku,
Tokyo 100-0013, JAPAN
Tel: +81-3-5510-2313
Fax: +81-3-5510-2312
info@medibic.com
www.medibic.com

Australia

Integrated Sciences Pty. Ltd.
2 McCabe Place
PO Box 731
Willoughby NSW 2068 Australia
Tel: 02 9417 7866 or
1 800 252 204 (Australia only)
Fax: 02 9417 5066
tech@integratedsci.com.au
www.integratedsci.com.au/contactus.asp

Israel

ZOTAL Biological
& Instrumentation
4 Habarzel Street
Tel Aviv 69710, Israel
Tel: +972.3.6492444
Fax: +972.3.6496664
sales@zotal.co.il
www.zotal.co.il

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