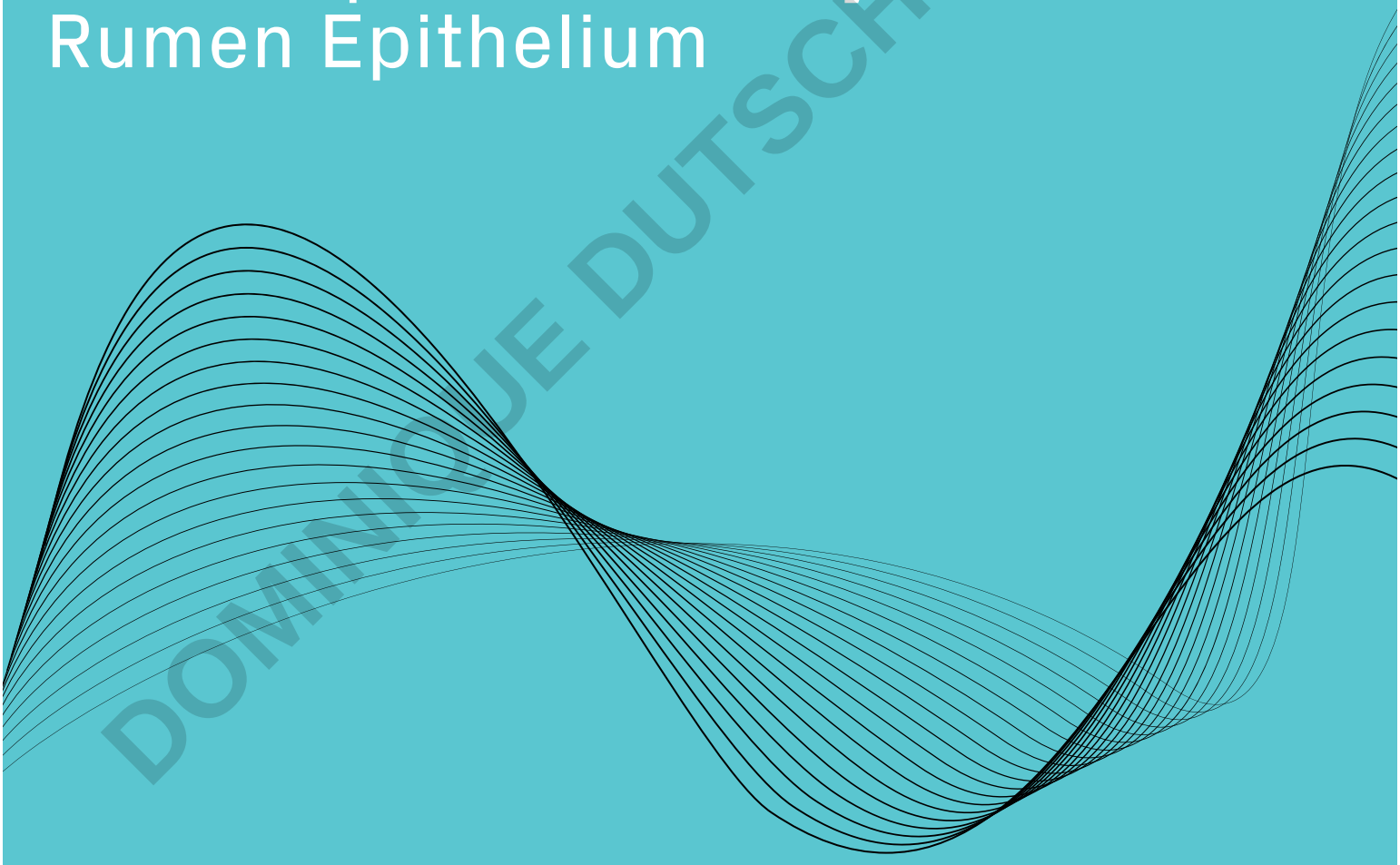
CONSUMABLES & INSTRUMENTS

APPLICATION NOTE

# Miniaturization of a qPCR Assay With Reduced Reagent Volumes to Achieve Cost Savings in Gene Expression Analyses in Rumen Epithelium

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## Introduction

The ruminal epithelium is characterized by high transport rates of short chain fatty acids (SCFA) and minerals like $Na^+$, $Cl^-$ and $Mg^{2+}$. However, to maintain stable physiological conditions in the rumen and to meet increased needs of nutrients (e.g. at onset of lactation), the transport capacity of the epithelium can even be significantly increased (ruminal adaptation).

To investigate the underlying mechanisms of adaptation, we analyse the expression of several carrier proteins under different feeding conditions using the technique of relative quantitative PCR, focussing on changes in gene-of-interest expression rather than on its absolute expression.

As quantitative PCR is a costly technique, we try to minimize expenses while at the same time keeping our results comparable to previous experiments and without losing sensitivity of the assay. We therefore tested a 40% reduction of the reaction volume per well using warp-free PCR plates with improved sealing properties efficiently preventing evaporation of smaller volumes.

For the miniaturized assay volumes we found identical threshold cycle (Ct) values as for the original larger volumes concluding that stability and sensitivity of the assay are maintained even when PCR reagents are reduced by 40%.

## Experiments

**Primer:** Four stock primers were tested (HSP70, Ubiquitin, GAPDH, G3PD).

**Template:** cDNA or dsDNA served as templates. The cDNA was prepared by reverse transcription of 100 ng mRNA (derived from primary ovine rumen epithelial cells, RNA integrity number= 10) using BioRad® iScript and dissolved in 200 µl nuclease free H20. As for the dsDNA, primer specific, purified PCR products were used at a concentration of 1 fg/µl. Per well, 5 µl of template were used and either 20 µl (setup 1) or 10 µl (setup 2) qPCR Mastermix were added.



FrameStar™ PCR plates for various PCR machines

| Cat. No. | Description | Quantity |
|---|---|---|
| **4Ti-0710** | 96well FrameStar PCR Plate Skirtless, BioRad iCycler®, Stratagene MX™ | 1 |
| **4Ti-0541** | Clear Seal Heat Sealing Sheets | 1 |
| **4Ti-0560** | Quantitative PCR Adhesive Seals | 1 |

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> "Reducing the SyBr Green consumption by 400/o means considerable cost savings to us."
>
> Dr. Carolin Deiner

**96 well PCR plate:** Warp-free 2component PCR plates were used (FrameStar product line from Azenta Life Sciences, cat.no 4ti-0710 suitable for BioRad iCycler and Stratagene MX). Rigidity results from the use of a robust polymer for the frame. The plate houses 96 wells moulded from a polymer identical to standard PCR tubes in which assays were originally established, thus providing ease of transfer of assays to higher throughput formats. The plates were heat sealed for 2-3 seconds using Clear Heat Seal (cat.no 4ti- 0541) for quantitative PCR applications.

**Experimental setup:** Triplets of each setup and primer pair were analysed on one and the same PCR plate for 35 cycles on a BioRad iCycler.

**Setup1: 25µl**
100% qPCR Mastermix and 100% Template

13.0µl BioRad SyBr Green Mastermix
0.5µl Primer sense
0.5µl Primer antisense
7.5µl Water

21.5µl→20µ per well
+ 5.0µl Template

**Setup2: 15µl**
60% qPCR Mastermix and 100% Template

7.8µl BioRad SyBr Green Mastermix
0.3µl Primer sense
0.3µl Primer antisense
2.5µl Water

10.9µl→10µ per well
+ 5.0µl Template

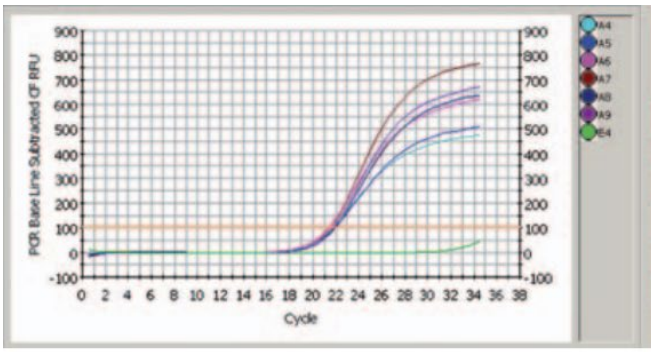
## Results

The mean Ct values of the genes analysed varied only minimally between the setups 1 and 2. In both setups, deviations from the mean triplet Ct values were similar and met the internal confidence value requirements (= maximum deviation of 0,5 from the mean Ct value). Ct values were 0,9 higher on average if template volumes were also reduced by 40% (data not shown).

The following Ct values resulted at a manually set threshold position of 100:

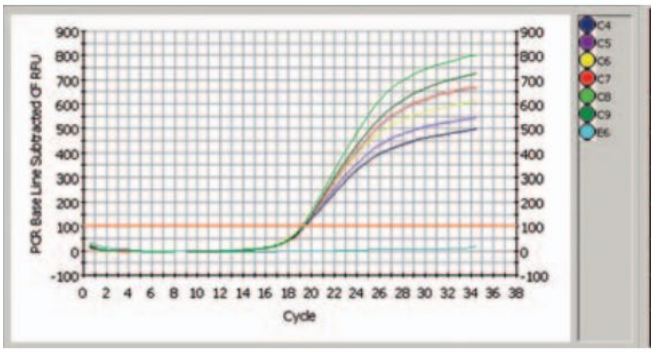
| | **Setup1: 25µ1** | | | | | **Setup2: 15 µl** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ct1 | Ct2 | Ct3 | **Mean** | SD | Ct1 | Ct2 | Ct3 | **Mean** | SD |
| **HSP 70** | 21,6 | 21,9 | 21,8 | **21,77** | ±0,15 | 21,8 | 21,9 | 21,4 | **21,7** | ±0,26 |
| **Ubiquitin** | 19,4 | 19,2 | 19,4 | **19,33** | ±0,12 | 19,4 | 19,3 | 19,1 | **19,27** | ±0,15 |
| **GAPDH** | 19,6 | 19,8 | 19,6 | **19,67** | ±0,12 | 19,5 | 19,6 | 19,6 | **19,57** | ±0,06 |
| **G3PD** | 23,3 | 23,6 | 23,5 | **23.47** | ±0,15 | 23,4 | 23,4 | 23,3 | **23,37** | ±0,06 |

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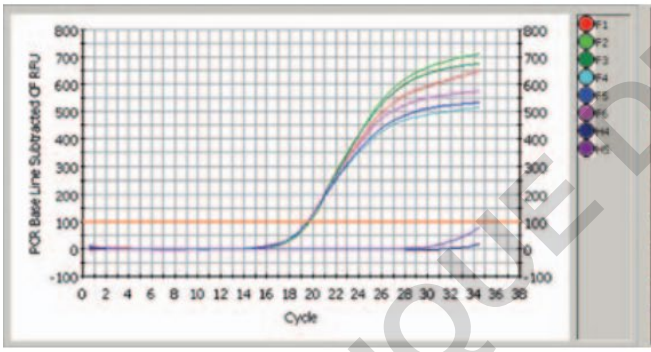

| | Threshold Cycle Ct | Identifier |
|---|---|---|
| A4 | 21.8 | hsp70 15-5µl templ |
| A5 | 21.9 | hsp70 15-5µl templ |
| A6 | 21.4 | hsp70 15-5µl templ |
| A7 | 21.6 | hsp70 25-5µl templ |
| A8 | 21.9 | hsp70 25-5µl templ |
| A9 | 21.8 | hsp70 25-5µl templ |
| E4 | N/A | neg hsp70 - 5µl |

**Figure 1:**
qPCR graphs of HSP70
A4 - A6: Setup 2
A7 - A9: Setup 1



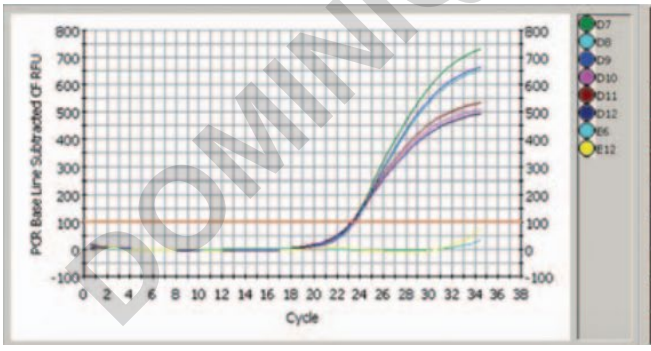

| | Threshold Cycle Ct | Identifier |
|---|---|---|
| C4 | 19.4 | ubi 15-5µl templ |
| C5 | 19.3 | ubi 15-5µl templ |
| C6 | 19.1 | ubi 15-5µl templ |
| C7 | 19.4 | ubi 25-5µl templ |
| C8 | 19.2 | ubi 25-5µl templ |
| C9 | 19.4 | ubi 25-5µl templ |
| E6 | N/A | neg ubi- 5µl |

**Figure 2:**
qPCR graphs of Ubiquitin
C4 - C6: Setup 2
C7 - C9: Setup 1




| | Threshold Cycle Ct | Identifier |
|---|---|---|
| F1 | 19.6 | GAPDH_25µl |
| F2 | 19.8 | GAPDH_25µl |
| F3 | 19.6 | GAPDH_25µl |
| F4 | 19.5 | GAPDH_15µl |
| F5 | 19.6 | GAPDH_15µl |
| F6 | 19.6 | GAPDH_15µl |
| H4 | N/A | negKo_GAPDH_15 |
| H5 | N/A | negKo_GAPDH_25 |

**Figure 3:**
qPCR graphs of GAPDH
F1 - F3: Setup 1
F4 - F6: Setup 2




| | Threshold Cycle Ct | Identifier |
|---|---|---|
| D7 | 23.3 | 82_25µl |
| D8 | 23.6 | 82_25µl |
| D9 | 23.5 | 82_25µl |
| D10 | 23.4 | 82_15µl |
| D11 | 23.3 | 82_15µl |
| D12 | 23.3 | 82_15µl |
| E6 | N/A | negKo_82_25µl |
| E12 | N/A | negKo_82_15µl |

**Figure 4:**
qPCR graphs of G3PD
D7 - D9: Setup 1
D 10 - D 12: Setup 2

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## Conclusion

Miniaturization of a qPCR assay is possible if original template amounts are maintained and evaporation is effectively prevented. Use of the warp-free FrameStar PCR plate and optimized sealing materials allows for a reduction of Sybr Green by 40% without significant changes in Ct values. The sensitivity of our method with the primer pairs established stays in the expected range even with smaller volumes, hence, previously gathered data in 25 µl-setups. This is an important advantage considering that there is already a huge database on diet dependent gene expression in sheep forestomachs. Reducing the SyBr Green consumption by 40% means considerable cost savings to us.

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**References**

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