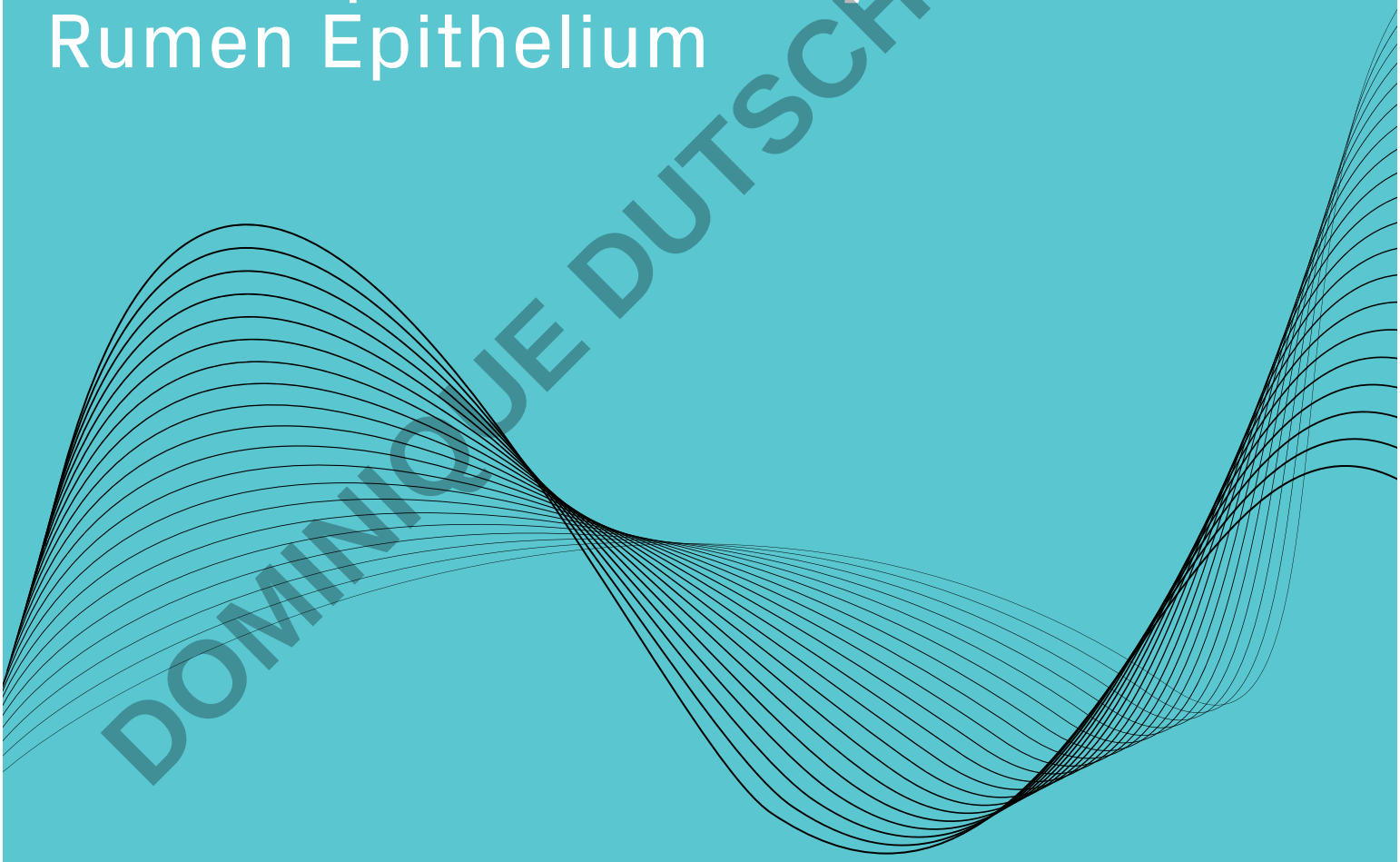
CONSUMABLES & INSTRUMENTS

APPLICATION NOTE

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

DOMINIQUE DUTSCHER SAS



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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

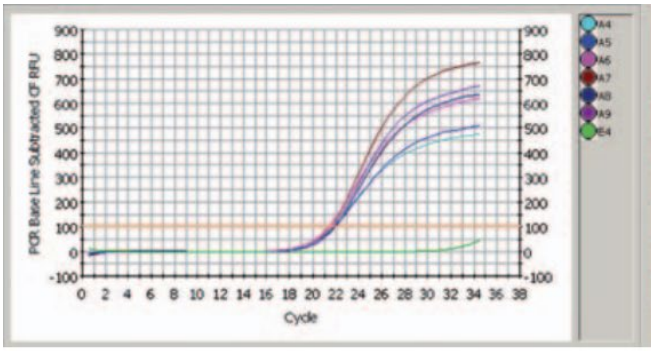
Results

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:

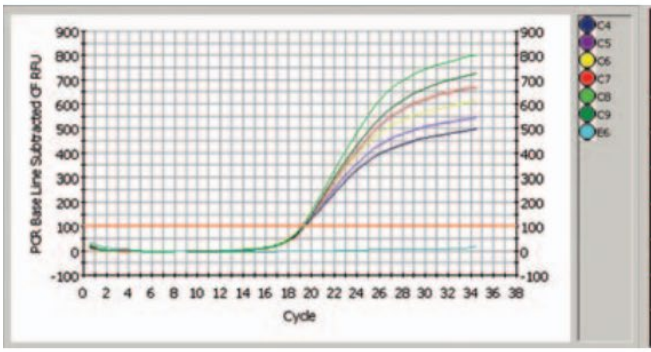
	Setup1: 25µ1					Setup2: 15 µl				
	Ct1	Ct2	Ct3	**Mean**	SD	Ct1	Ct2	Ct3	**Mean**	SD
HSP 70	21,6	21,9	21,8	**21,77**	±0,15	21,8	21,9	21,4	**21,7**	±0,26
Ubiquitin	19,4	19,2	19,4	**19,33**	±0,12	19,4	19,3	19,1	**19,27**	±0,15
GAPDH	19,6	19,8	19,6	**19,67**	±0,12	19,5	19,6	19,6	**19,57**	±0,06
G3PD	23,3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

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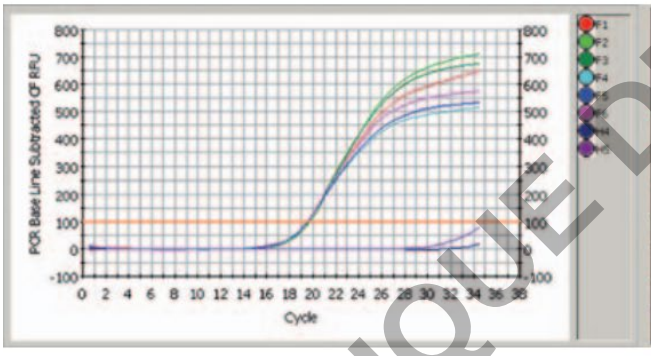
	Threshold Cycle Ct	Identifier
A4	21.8	hsp70 15-5µl templ
A5	21.9	hsp70 15-5µl templ
A6	21.4	hsp70 15-5µl templ
A7	21.6	hsp70 25-5µl templ
A8	21.9	hsp70 25-5µl templ
A9	21.8	hsp70 25-5µl templ
E4	N/A	neg hsp70 - 5µl

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



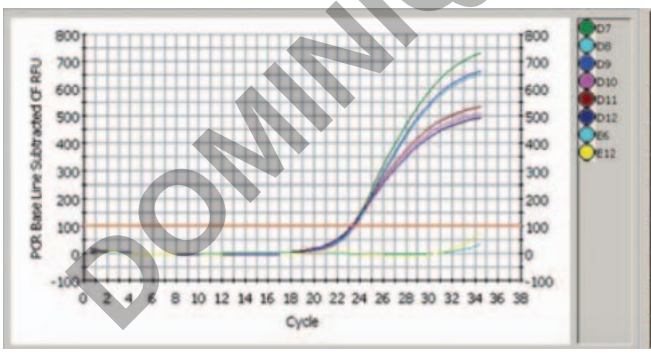
	Threshold Cycle Ct	Identifier
C4	19.4	ubi 15-5µl templ
C5	19.3	ubi 15-5µl templ
C6	19.1	ubi 15-5µl templ
C7	19.4	ubi 25-5µl templ
C8	19.2	ubi 25-5µl templ
C9	19.4	ubi 25-5µl templ
E6	N/A	neg ubi- 5µl

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

Axel Mohrle[1] . Isabel Dittmann[2], Zoran Nikolic[2], Carolin Deiner[2]

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