Comparison by 2 complementary assays of the measurement of the absolute copy numbers of plasmid calibrants used for the determination of a PCR cut-off

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Introduction

The 9th of March 2012, a real-time PCR assay developed by TNO Triskelion bv for the detection of ruminant DNA was officially validated by EURL-AP. As for other abundant natural PCR targets, late amplification signals are sometimes observed even in samples free of ruminant material and the determination of a reliable cut-off is therefore needed to interpret correctly the PCR results qualitatively. For the use of PCR methods by a network of laboratories, the EURL-AP developed and validated a transfer protocol based on the use of plasmid solutions containing the target as calibrants to determine the cut-off of a PCR platform (combination thermocycler-mastermix). An improvement of the protocol is the use of the digital PCR (dPCR) technology to determine more accurately the calibrant copy number. However the TNO ruminant PCR assay could not be used in a routine dPCR assay because of the presence of contaminant ruminant DNA in the original dPCR loading reagent (GE 20X buffer). Two alternative strategies were tested to solve this problem.

Material and methods

Samples

Two solutions containing ~ 500 copies/µl and ~ 8 copies/µl of linearised plasmid

Equipment

The experiments were realised on a « BioMark™ HD System » dPCR platform (Fluidigm Corporation, South San Francisco, CA, USA)

Figure 1 – digital PCR apparatus

Calibrator's plasmid and PCR reagents

The ruminant assay amplicon was firstly cloned in a pCR® 2.1-TOPO® plasmid using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) and then transferred in a pUC18 plasmid.
The map of the final calibrator's plasmid and the location of the PCR primers and probe are presented in Figure 2.

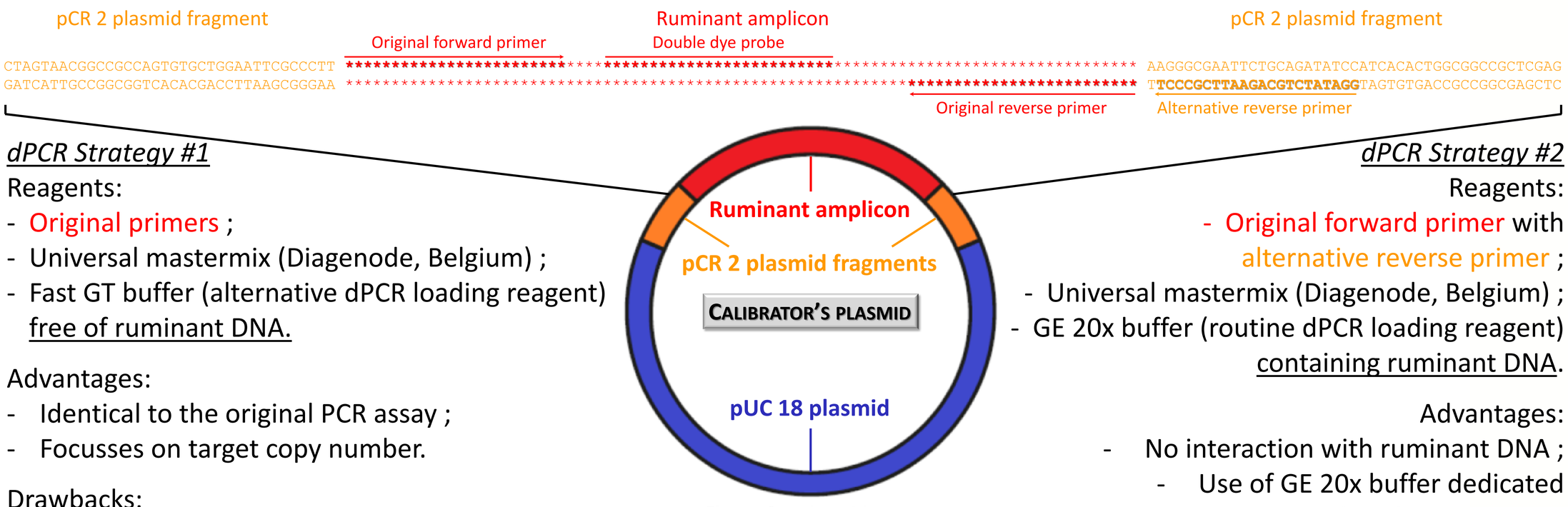

Figure 2 – plasmid map with primers and probe locations

Results

Sample (estimated copy number)	dPCR strategy	Nr of panels (x765 PCR reactions)	Measured copy number/µl	Average copy number/µl	Conclusion
500 copies/µl	#1	3	657 690 652	666 +/- 21	Final copy number determined on 20 panels with dPCR strategy #1: **637 +/- 27 copies / µl**
	#2	3	585 657 596	612 +/- 39	
8 copies/µl	#1	2	9 10	10 +/- 0,7	
	#2	2	15 9	12 +/- 4,2	

Conclusions

Whatever the strategy, the number of copies determined in the samples were comparable. Finally, the assay targeting the original amplicon (dPCR strategy #1) was chosen to continue the measurements. The calibrants prepared on basis of the exact copy numbers determined by digital PCR were successfully used in two collaborative studies conducted by the EURL-AP for the implementation of the PCR method for the detection of ruminant PAP in feed. In the future, digital PCR will be a key technology for the production of calibrants used as certified reference materials.

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