A novel enzymatic treatment to remove contaminating free DNA in bacteriophage-treated samples for use in routine testing

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Introduction

A common hurdle of PCR based assays for detection of microbial pathogens in food is often amplification of DNA from dead cells. In routine testing, the feasibility of confirming presumptive positive results is a key performance indicator for a laboratory or a method. With regard to molecular methods, such a capability might be thwarted by the presence of free bacterial DNA originating in food processes that aim at sterilizing food products (e.g. thermal, chemical or irradiation processes). Food processing itself may be an intrinsic source of free DNA contamination. This is the case when bacteriophage spraying is used as an intervention. For instance Listex™, which aims at preventing *Listeria*, may contaminate samples with DNA originating in the phage purification process from *Listeria* cell culture. To date, only tedious protocols involving hazardous chemicals with Ethidium Monoazide (EMA) or Propidium Monoazide (PMA) treatments are available to get rid of such free DNA.

The aim of this study was to evaluate an enzymatic approach to suppress free *Listeria* DNA present in the food sample due to Listex treatment without affecting the PCR outcome from living cells.

Methods

Presence of *Listeria* DNA in Listex

Serial dilutions of Listex were added to *Listeria* Special Broth (LSB) and incubated 24 hr at 30°C. Then 3 different enzymatic mixes were added to 100µL aliquots in the presence of a specific activating buffer. These enzymatic mixes (named free DNA removal solution or FDRS A, B & C) were designed to be specifically inactivated during the iQ-Check® DNA extraction protocol at high temperature (Figure 1). After a short incubation at 37°C, the DNA was extracted from these aliquots and amplified using the iQ-Check *Listeria* spp. assay according to the kit specifications. Cq values for *Listeria* targets and Internal Control (IC) obtained in the presence or absence of the enzyme mix were compared.

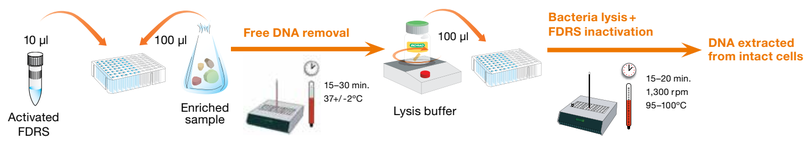
Listeria monocytogenes detection in cheese in the presence of Listex

L. monocytogenes (ATCC 35152) was spiked at 36 CFU/25g portions of cheese in 225ml of LSB. Inoculum was confirmed by plating on TCS medium. After 24 hr at 30°C, enrichment was serially diluted in a control broth supplemented with and without Listex. The 10-fold dilution series was then submitted to the free DNA removal solution: 100µl was incubated with reagent at 37± 2°C for 30 min. DNA extraction and real-time PCR analysis were performed with the iQ-Check *Listeria* spp. Cq values for *Listeria* and IC targets were determined. The sensitivity of the whole method was calculated and expressed as the limit of detection from 10 replicates of each dilution.

Free DNA cleaning from Listex — treated cheese inoculated with *L. monocytogenes*

The FRDS method was performed on Listex treated 25g cheese portions inoculated with 6.4 CFU of *L. monocytogenes*. After the enrichment step, two versions of the enzyme mix (B as shown above and D) were added to the samples and incubated at 37 ± 2°C for 15 min on a 10 fold serial dilution series of the samples. DNA extraction and PCR analysis were performed as above. *Listeria* and IC targets were determined and sensitivity was estimated accordingly.

Figure 1. Modified iQ-Check *Listeria* spp. assay involving the Free DNA Removal protocol



All Real-Time PCR assays were run on the CFX96 Touch™ Deep Well instrument.

Results

It was previously suggested that treating cheese with Listex may lead to *Listeria* PCR positive results. Therefore, our first goal was to demonstrate the presence of free *Listeria* DNA in the Listex solution, which may result in unconfirmed positive PCR result for treated samples. As shown in Table 1, Listex solution gave rise to positive *Listeria* DNA PCR, even on 10^7 fold dilutions (Cq = 39.52). Enzymatic pretreatment resulted in a significant Cq increase (Mean increase value: A: 5.9 Cq, B: 7Cq, C: 7.47 Cq), demonstrating that the Listex solution contained *Listeria* DNA that was reduced by an approximate 2 Log factor upon treatment with the enzyme mixes. Moreover, the IC Cqs were constant regardless of the protocol, thereby indicating that the enzymatic pretreatment did not affect the amplification of DNA present in the sample following the lysis/extraction step. Altogether these results show that Listex contains free *Listeria* DNA that can be removed by an enzymatic pretreatment of samples prior to iQ-Check *Listeria* PCR amplification.

Table 1. Efficiency of an enzymatic pretreatment to remove contaminating DNA from Listex solution

		Without treatment		Pretreated with enzymatic mix					
				A		B		C	
	PCR target	*Listeria* spp.	Internal Control	*Listeria* spp.	Internal Control	*Listeria* spp.	Internal Control	*Listeria* spp.	Internal Control
10 fold serial dilution of Listex	Pure	18.48	N/A*	25.44	N/A*	25.74	33.66	26.48	33.50
	10-1	20.64	33.64	27.42	31.32	27.59	31.11	28.36	31.31
	10-2	23.74	31.82	29.93	31.45	30.84	31.69	31.32	31.77
	10-3	27.12	30.86	32.87	32.14	33.57	32.20	34.29	32.04
	10-4	29.67	31.43	34.13	32.08	36.70	32.24	36.57	31.94
	10-5	32.76	31.94	38.04	32.20	39.98	32.03	40.22	32.00
	10-6	37.10	31.90	N/A	32.10	N/A	32.05	N/A	31.97
	10-7	39.52	32.28	N/A	32.21	N/A	32.08	N/A	32.02

* IC was not amplified due to competition with strong positive signal

We studied the interference of the FDRS with the detection of *Listeria* in a food sample. As shown in Table 2, enrichment of 25g portions of cheese that were initially spiked with 36 CFU of *Listeria* yielded a positive PCR with a Cq equal to ≈24. Serial dilutions of the enrichment in the absence of Listex indicated a dilution limit equal to 10^{-4}, as determined by the highest dilution at which 100% of the 10 samples were positive. Treating the samples with the activated Free DNA Removal Solution did not modify this dilution limit demonstrating that the pretreatment did not affect the detection of living cells.

In contrast, when Listex was added to the dilutions, all samples including the 10^7-fold dilution gave rise to a PCR positive signal due to the contaminating DNA. A Cq value of ≈37was observed on the enrichment in absence of living bacteria (0 CFU) corresponding to the DNA load brought by Listex. A constant Cq value of ≈37 was observed for dilutions 10^{-5} to 10^{-7}, demonstrating that below the 10^{-4} dilution limit of the *Listeria* target, contaminating free DNA was the main contributor to the positive signal. Implementation of the enzymatic pretreatment rescued the PCR signal specifically due to living cells, as demonstrated by the recovery of dilution limit comparable to the one obtained in absence of Listex contamination. Again, the IC signal was never affected, thus reinforcing the conclusion that the enzyme pretreatment was inactivated during the extraction step and therefore did not impact the PCR amplification of cell DNA. It is also important to note that a 5-6 Cq decrease was observed for the Listex solution in absence of *Listeria* inoculum, indicating that efficiency of the pretreatment did not vary with the presence of the cheese matrix.

Table 2. Free DNA cleaning from *L. monocytogenes* culture contaminated with Listex

		Without Listex				In the presence of Listex			
		Not activated FDRS B		Activated FDRS B		Not activated FDRS B		Activated FDRS B	
		% of positive results	Mean of *Listeria* spp. Cq values (10 replicates)	% of positive results	Mean of *Listeria* spp. Cq values (10 replicates)	% of positive results	Mean of *Listeria* spp. Cq values (10 replicates)	% of positive results	Mean of *Listeria* spp. Cq values (10 replicates)
Unspiked	0	0%	N/A	0%	N/A	100%	37.10	20%	42.02
Spike level before enrichment	36 CFU/23gr	100%	23.46	100%	23.15	100%	24.20	100%	23.66
10-fold dilutions of enrichement	10^{-1}	100%	26.46	100%	26.32	100%	27.14	100%	26.66
	10^{-2}	100%	29.82	100%	29.40	100%	30.21	100%	29.81
	10^{-3}	100%	32.83	100%	32.30	100%	33.47	100%	33.16
	10^{-4}	100%	36.20	100%	36.10	100%	35.74	100%	36.34
	10^{-5}	30%	39.04	70%	39.53	100%	36.96	60%	39.84
	10^{-6}	0%	N/A	20%	38.69	100%	37.13	20%	40.98
	10^{-7}	0%	N/A	0%	N/A	100%	37.36	20%	42.14

Finally, we tested FDRS directly on Listex treated cheese. In order to optimize the treatment, we compared two FDRS formulations (Table 3). For all dilutions, the mean Cq value for Listeria was ≈32 on cheese treated Listex samples subjected to inactive enzymatic mix B. Unspiked samples or 10^7-fold dilutions gave rise to PCR positive signal due to the high content of free DNA from Listex (equivalent to 10^5 bacterial genomes per gram of cheese). Pretreatment with activated enzymatic mix B allowed to get rid of the Listex free DNA without affecting the detection of the DNA from living bacteria. Mix B activated at 37 ± 2°C for 15 min was sufficient to recover a Cq similar to the one observed in untreated samples. In contrast, the enzymatic mix D failed to efficiently remove free DNA with 50% of the uncontaminated samples being still positive due to the presence of free DNA from Listex.

Table 3. Comparison of enzymatic mixes on Listex-treated cheese inoculated with *L. monocytogenes*

		Without Listex		Listex treated samples					
		No pretreatment		Not activated FDRS B		Activated FDRS B		Activated FDRS D	
		Positive result	Mean of *Listeria* spp. Cq values (10 replicates)	Positive result	Mean of *Listeria* spp. Cq values (10 replicates)	Positive result	Mean of *Listeria* spp. Cq values (10 replicates)	Positive result	Mean of *Listeria* spp. Cq values (10 replicates)
Unspiked	0 CFU	0%	N/A	100%	32.57	0%	N/A	50%	37.35
Spike level before enrichment	6.4 CFU/25g	100%	28.27	100%	28.72	100%	29.61	100%	37.59
10-fold dilutions of enrichement	10^{-1}	100%	31.92	100%	32.32	100%	32.91	100%	37.67
	10^{-2}	100%	34.65	100%	32.33	100%	36.01	100%	37.62
	10^{-3}	80%	37.10	100%	32.37	70%	39.27	100%	37.28
	10^{-4}	20%	38.67	100%	32.38	60%	40.95	100%	37.80
	10^{-5}	10%	39.40	100%	32.21	40%	41.50	100%	38.39
	10^{-6}	0%	N/A	100%	32.23	40%	41.98	100%	38.03
	10^{-7}	0%	N/A	100%	32.37	20%	42.24	100%	37.57

Conclusions

It was observed that treating cheese with Listex led to a DNA contamination of the samples. The contamination gave rise to a PCR signal in the 35-37 range detected by iQ-Check *Listeria* spp. When pretreated with the Free DNA Removal Solution, the signal of the dead cells was readily suppressed whereas the signal of the living cells was not affected. Furthermore, the Internal Control signal was not impacted, confirming that the lysis step with the Bio-Rad proprietary buffer efficiently inactivated the enzymatic mix. We optimized an enzymatic mix yielding optimal results with a pre-incubation step at 37°C ± 2°C for 15 to 30 min prior to the extraction with the iQ-Check *Listeria* spp. PCR assay with neither additional extraction steps nor supplementary equipment. This reagent which is now available (Figure 2) can be adapted to different matrices/broth/pathogen combinations and is easily automatable.

Figure 2. iQ-Check Free DNA Removal Solution

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