Shayanfar et al. International Journal of Food Contamination (2015) 2:2
DOI 10.1186/s40550-015-0008-0

International Journal of Food Contamination
a SpringerOpen Journal

DATA ARTICLE Open Access

Fruit juice and puree characteristics influence enrichment requirements for real-time PCR detection of *Alicyclobacillus acidoterrestris*

Shima Shayanfar[1], Christina Harzman[2] and Suresh D Pillai[1*]

Abstract

Background: *Alicyclobacillus acidoterrestris* is a key spoilage causing bacterium commonly found in fruit juices and purees. Commercial real-time PCR based assays to detect this organism are available, but reportedly require 48 hours of enrichment for detection. The underlying hypothesis of this study was that fruit juice and puree characteristics influence the enrichment requirements of this organism, and that in some matrices, the organism can be detected within 24 hours even when present at low initial contamination. Thirteen different store-purchased fruit juice and purees were inoculated with 10 CFU/ml of *Alicyclobacillus acidoterrestris*. The inoculated samples were enriched for 24 and 48 h. Aliquots from the un-enriched, 24 hour, and 48 hour enriched samples were taken, total community DNA extracted, and the real-time PCR assay performed using commercially available kits.

Results: *A. acidoterrestris* was detected by real-time PCR within 24 h of enrichment in most matrices (except ketchup and orange concentrate) even from a low starting concentration (10 CFU/ml). Juice and puree samples with high soluble solids contents (i.e. Brix values) required longer incubation periods for lower *A. acidoterrestris* Ct values.

Conclusions: The soluble solids (Brix) content of fruit juice and purees influence the enrichment requirements for real-time PCR detection of *A. acidoterrestris*. Samples with high Brix values should be diluted to reduce the inhibitors of *A. acidoterrestris* proliferation during enrichment.

Keywords: Alicyclobacillus acidoterrestris; Fruit; Juice; Brix; Real-time PCR; Enrichment; Ct value

Background

Alicyclobacillus acidoterrestris is a thermophilic, acid tolerant, spoilage bacterium that withstands pasteurization temperatures typically employed in the juice industry. As the most commonly detected *Alicyclobacillus* species in spoiled fruit juices and purees, *A. acidoterrestris* converts lignin components to form guaiacol, contributing to the unpleasant 'hammy smell" in fruit juices undergoing spoilage (Eguchi et al. 2000; Silva and Gibbs 2001; Gocmen et al., 2005; Bahçeci et al., 2005; Chang and Kang, 2004; Groenewald et al. 2013).

Alicyclobacillus spp. spoilage is a global problem in the fruit juice industry (Cagnasso et al. 2010; Groenewald et al. 2009, McKnight et al. 2010, Walls & Chuyate 2000). Most instances of contamination have been reported in apple and orange juices (McKnight et al., 2010; Groenewald et al., 2009; Luo et al. 2004). In the US juice industry alone, *Alicyclobacillus* spp. related spoilage is estimated to be around 35% (Chang and Kang, 2004). Recent reports suggest that there are intriguing linkages between the genotype of *Alicyclobacillus spp.* and what is found in specific fruit juice matrices (Zeki Durak et al., 2010). Thermal processes to inactivate the organisms are ineffective, and therefore spoilage control relies primarily on efficient detection of the organism (Groenewald et al., 2013). The International Federation of Fruit Juice Producers (IFU) recommended method of conventional plating requires a minimum of 72 hours of enrichment prior to plating. Thus, conventional plating methods to detect this organism are generally very time consuming and ineffective as a quality

* Correspondence: s-pillai@tamu.edu
[1]National Center for Electron Beam Research, Department of Nutrition and Food Science, Texas A&M University, College Station, Room 418B, Kleberg Center, MS 2472, 77843-2472 Texas, USA
Full list of author information is available at the end of the article

control strategy (Luo et al., 2004; Lin et al., 2005). Considering the high product value of juices, storing fruit juices for an extended period of time until obtaining conventional plating results is not economically feasible (Lin et al., 2005). A variety of rapid detection methods for *Alicyclobacillus spp.* and other organisms have have been reported in the literature (Al-Qadiri et al., 2006; Luo et al. 2004, Jasson et al., 2009, Cagnasso et al., 2010, Connor et al., 2005, Chang and Kang 2004). Many of the published reports have, however, focused only on limited types of samples (Luo et al 2004; Connor et al., 2005). Real-time PCR-based kits which have been tested for their specificity and sensitivity are currently commercially available. These assays rely on at least a 48 h enrichment step.

The underlying hypothesis of this study was that fruit juice and puree characteristics will influence the enrichment requirements of this organism, and that in some matrices the organism can be detected within 24 hours even when present at very low initial contamination. Therefore, the objective of this study was to compare the detection of the commonly encountered spoilage-related species, *Alicyclobacillus acidoterrestris*, in different fruit matrices after specific enrichment time frames using a commercially available real-time PCR kit compared to the IFU method. We tested 13 different retail store purchased fruit juice and puree matrices (varying in soluble solids content) that were spiked in the laboratory with low levels (10 CFU/ml) of *A. acidoterresteris* spores. Previous studies have employed higher inoculation concentrations such as 10^4 CFU/ml and 10^5 CFU/ml (Yamazaki et al., 2008; Wang et al., 2013, (Funes-Huacca et al., Funes-huacca et al. 2004). We chose this low level since a previous study suggested that *A. acidoterrestris* contamination concentrations in US juices could be low (Pettipher et al., 1997).

Methods

Sample juice matrices

Thirteen different fruit juice and purees (that varied in pH and Brix values) were employed in this study (Table 1). The samples were purchased from a local retail store and used within 48 h for all testing purposes, all prior to their expiry dates. The samples were opened under aseptic conditions (in a biosafety cabinet) to avoid any potential cross-contamination. Samples were taken for pH and Brix value determination. Digital hand-held refractometers (Reichert 35H and 65H, Depew, NY, USA) calibrated with distilled water were used for obtaining the Brix values.

Screening for background *Alicyclobacillus* contamination

The samples were initially screened for background *Alicyclobacillus* contamination per the International Federation of Fruit Juice Producers method (IFU, 2007). The samples were heat-treated to 80°C for 10 min to eliminate background vegetative microbial populations and to enhance culturability of background *Alicyclobacillus* spp. spores. Portions (0.1 ml) of the samples were plated on K agar (HiMedia Laboratories, Mumbai, India) for enumerating background *Alicyclobacillus* spp. The plates were incubated for up to 5 days at 45°C per the IFU recommendations (IFU, 2007). The K agar plates were scored for growth/no-growth for *Alicyclobacillus* characteristic colonies. A commercial guaiacol detection kit (Kyokuto Pharmaceutical Co., Ltd., Japan) was used to confirm the presence of *Alicyclobacillus* in these colonies. The protocol provided by the kit manufacturer was followed to confirm the presence of guaiacol production by the colonies. The characteristic brown color change, as compared to the control blank samples, was used as an indicator for positive guaiacol formation.

Bacterial strain and growth conditions

An *Alicyclobacillus acidoterrestris* strain (ATCC 49025) was obtained from the American Type Culture Collection (Microbiologics®, USA), and cultured at 48°C in BAT broth (HiMedia Laboratories, Mumbai, India) for 5 days to obtain a cell titer of 10^7 CFU/ml. The culture was diluted in BAT broth to serve as the spiking inoculum.

Spiking of juice and puree samples

Aliquots (1 ml) of the juice and puree samples were aseptically pipetted (in triplicate) into separate 15 ml sterile conical tubes. Sterile BAT broth (9 ml) was added and the tubes were heated to 80°C for 10 min. To this, 0.1 ml of inoculum (containing 10 CFU of *A. acidoterrestris* cells) was added in triplicate. The inoculated samples were incubated in a shaking incubator at 45°C and 30 rpm for up to 48 h. Aliquots (1 ml and 0.1 ml) were taken at 24 hours and 48 hours from the inoculated samples. These samples were used for plating (0.1 ml) on K agar (Bevilacqua et al., 2008) and for DNA extraction (1 ml) for the real-time PCR assay. The "0 hour" sample was obtained from the heat-treated un-spiked sample.

DNA extraction and real-time PCR amplification

The commercial kits employed for DNA extraction and real-time PCR detection of *Alicyclobacillus* spp. and *A. acidoterrestris* in this study were the **food**proof® Short-Prep II Kit and the **food**proof® *Alicyclobacillus* Detection Kit (BIOTECON Diagnostics, Germany) respectively. The **food**proof® *Alicyclobacillus* Detection Kit uses 5' nuclease, Taqman (hydrolysis) probe chemistry to separately detect *Alicyclobacillus* genus (HEX channel) and *A. acidoterrestris* (FAM channel) as well as an internal amplification control (ROX channel) in one single assay. The kit manufacturer reports both *Alicyclobacillus* and

Table 1 - The effect of enrichment time on the Ct values of *Alicyclobacillus* and *A. acidoterrestris* in different juice and puree samples by real-time PCR

			Alicyclobacillus spp. (Ct values)			*A. acidoterrestris* (Ct values)		
Sample	**^{0}Brix**	**pH**	**Background**	**24 h**	**48 h**	**Background**	**24 h**	**48 h**
Orange concentrate	43	4.09	30.8 ± 3.1	33.9 ± 2.2[a]	34.3 ± 2.2[a]	ND	36.2 ± 2.4[a]	34.7 ± 2.7[a]
Apple concentrate	40	4.00	33.8[a] ± 3.9	47.7 ± 3.9[a]	40.2 ± 3.1[a]	32.3 ± 0.0	34.9 ± 1.5[a]	34.8 ± 1.7[a]
Ketchup	27	3.69	46.8 ± 5.5	46.3 ± 5.5[a]	39.3 ± 2.7[a]	ND	35.7 ± 4.4[a]	35.3 ± 0.5[a]
Tomato paste	26	3.69	39.0 ± 0.8	29.6 ± 11.6[a]	23.4 ± 13.7[a]	ND	25.6 ± 6.8[a]	18.9 ± 10.2[a]
Apple puree	17	4.08	ND*	34.0 ± 2.2[a]	21.4 ± 2.2[b]	ND	29.0 ± 4.8[a]	20.3 ± 9.9[a]
Peach puree	17	4.08	ND	UD	27.8 ± 2.7	ND	38.0 ± 1.6[a]	25.3 ± 2.1[a]
Apple juice (filtered)	12	3.8	27.1 ± 3.9	29.2 ± 2.2[a]	12.4 ± 2.7[b]	35.5 ± 0.9	26.6 ± 3.4[a]	10.4 ± 2.1[b]
Peach nectar	12	3.75	49.4 ± 5.5	19.8 ± 2.2[a]	14.3 ± 2.2[a]	35.4 ± 0.6	17.0 ± 2.10[a]	11.6 ± 0.2[a]
Apple juice (unfiltered)	11	3.73	37.0 ± 3.9	26.6 ± 2.2[a]	13.6 ± 2.2[b]	35.1 ± 0.7	23.9 ± 8.1[a]	11.0 ± 0.3[b]
Orange juice (with pulp)	11	4.03	38.2 ± 3.1	31.8 ± 2.4[a]	22.7 ± 2.2[b]	ND	26.7 ± 6.7[a]	20.6 ± 6.4[a]
Orange juice (filtered)	10	4.06	40.0 ± 5.5	22.5 ± 3.1[a]	19.3 ± 2.2[a]	35.2 ± 0.1	26.1 ± 8.3[a]	17.8 ± 7.7[b]
Tomato sauce	9	4.24	ND	31.7 ± 3.1[a]	16.3 ± 2.7[b]	37.0[a] ± 0.2	30.4 ± 3.8[a]	16.0 ± 3.0[b]
Tomato juice	6	4.15	37.6 ± 2.4	26.8 ± 1.7[a]	15.7 ± 1.7[b]	33.4 ± 1.7	26.1 ± 3.9[a]	14.1 ± 6.6[b]

Ct values with different superscript letters within each row indicate statistically ($p < 0.01$) significant differences as a function of enrichment time. Pairwise comparisons were performed using the Student's *t* test.
*ND = Not detected.

A. acidoterrestris amplification chemistries have a limit of detection of 7 genome equivalents, and 10^1-10^2 CFU/ml in combination with the DNA extraction kit. However, the manufacturer's verification documentation demonstrates detection at even 1 genome equivalent, though less than 100% (*data not included*). Though specificity testing was not a goal of this paper, the kit manufacturer's verification documentation states that 100% of 40 strains of non-target organisms including related genera (e.g., *Bacillus spp*., *Listeria spp.*) as well as strains commonly found in food were properly excluded (tested negative) (*data not included*).

When performing DNA extraction and real-time PCR, the kit manufacturer's recommended protocols were followed. Briefly, 1 ml from the 0 h (un-spiked), 24 h enriched (inoculated) and 48 h enriched (inoculated) samples were aseptically taken and used for DNA extraction. The un-spiked sample was included to identify possible natural contamination of the samples. For the real-time PCR assay, 5 μl aliquots of the extracted DNA samples were used as template according to the manufacturer's protocol. The real-time PCR amplifications were performed using duplicate technical replicates. The real-time PCR reaction mixture contained 20 μl of the premixed commercial PCR reagent (18.0 μl master mix, 1.0 μl enzyme solution and 1.0 μl internal amplification control) and 5 μl genomic DNA extract. For the negative and positive controls, 5 μl PCR-grade H_2O and 5 μl **food**proof® *Alicyclobacillus* Control Template respectively (provided in the kit) were added to the premixed commercial PCR reagent. Following the **food**proof® *Alicyclobacillus* Detection Kit protocol, the real-time PCR amplification parameters used in the Applied Biosystems 7900HT real-time PCR cycler were as follows: 37°C for 4 min (1 cycle) and 95°C for 5 min (1 cycle), and 95°C for 5 s followed by 60°C for 1 min (50 cycles). The enzyme solution of the **food**proof® *Alicyclobacillus* Detection Kit contains not only Taq polymerase, but also uracyl-N-glycosylase (UNG). The UNG is included to prevent false-positive reactions from previous PCR reactions as the dNTPs in the master mix contain dUTP rather than dTTP. Thus, should cross-contamination occur from previously made amplificates containing dUTP, they will be degraded by UNG during the initial 37°C incubation at the start of the PCR program. The inbuilt cycler software (SDS V.2.4, 2009, California, USA) was used for obtaining the real-time PCR cycle threshold (Ct) values.

Statistical analysis

The experiments were performed using three biological replicates for the fruit matrix samples with each of the real-time PCR amplifications performed in duplicate. The real-time PCR data as presented is the mean of six Ct values ± standard deviation. The correlation between Brix values and Ct values were determined using the Pearson product Moment Correlation using SigmaPlot (Systat Software, Inc.). To discern the effect of juice matrix, incubation time and the possible interaction between the juice matrix and time of incubation the Ct values were evaluated using ANOVA at a 1% level of significance using the commercially available Design-Expert™ (Ver 7.1.4) software. Significant differences between the

treatments if any, were determined using Student's *t*-test at a 1% level of significance ($P < 0.01$).

Results and Discussion

The 13 fruit juice and puree samples in the study had quite similar pH values which ranged from 3.69 to 4.24. However, they varied considerably in terms of their Brix values. Tomato juice had the lowest Brix value of 6.0, while the orange concentrate had the highest value with 43 (Table 1). The real-time PCR kit used provided separate amplification plots for the genus *Alicyclobacillus* and the species *A. acidoterrestris* as they were detected using different dyes. The Ct value in the real-time PCR assays was used as the benchmark for "detection". Ct values greater than 50 were scored (by the instrument software) as "not-detected". All samples, except for apple puree, peach puree, and tomato sauce showed the presence of background *A. acidoterrestris* contamination. Ten out of 13 samples (77%) showed background contamination of the genus *Alicyclobacillus,* while 7 out of the 13 samples (54%) showed the specific presence of *A. acidoterrestris* background contamination (Table 1). A number of reports have shown that *A. acidoterrestris* can be present in both fresh juices and juice concentrates (Pettipher et al. 1997; Luo et al., 2004; Groenewald et al. 2009; Groenewald et al. 2013; McKnight et al., 2010; Wang et al., 2013). The presence of background *Alicyclobacillus* spp. but not *A. acidoterrestris*, such as in orange concentrate, suggests that there may have been species other than *A. acidoterrestris* in this sample.

Based on the Ct values, it is evident that the background contamination levels of the target organisms varied in the samples (Table 1). Filtered apple juice had the highest background levels of *Alicyclobacillus* spp. contamination (Ct value =27.1) while ketchup had the lowest levels (Ct value = 46.8 ± 5.5). In terms of *A. acidoterrestris* background levels, apple concentrate had the highest levels (Ct value = 32.3) compared to tomato sauce which had the lowest levels (Ct value = 37.0 ± 0.2). There was only one sample (tomato sauce) where the assay detected the background presence of *A. acidoterrestris* but not the genus *Alicyclobacillus.* This result may be related to juice matrix components preventing effective amplification of the genus-specific sequences (compared to species-specific sequences) when these target sequences are present in low levels. This is further supported by lower Ct values for background levels of *A. acidoterrestris* compared to background levels of the genus *Alicyclobacillus* in the apple concentrate, peach nectar, apple juice, orange juice, and tomato juice samples (Table 1).

The **food**proof® *Alicyclobacillus* Detection Kit was designed to have a higher preference for the FAM-targeted *A. acidoterrestris* species amplification chemistry over the HEX-targeted *Alicyclobacillus* genus amplification chemistry. This preference is important at low DNA concentrations because if the species is found present, then naturally the genus is considered present as well. However, without this preference, the more specific *A. acidoterrestris* species information could be lost.

Table 1 also illustrates the effect enrichment time has on the ability of a commercial real-time PCR assay to detect *Alicyclobacillus* genus-specific sequences and *A. acidoterrestris* species-specific sequences when spiked at low initial concentrations (10 CFU/ml). When the samples were inoculated with a low inoculum of 10 CFU/ml, all samples (except for peach puree) showed detectable *Alicyclobacillus* spp. within 24 hours. By 48 hours of enrichment, all samples were "positive" for *Alicyclobacillus* spp. Within 24 h of enrichment, all 13 samples showed the presence of A. *acidoterrestris* (Table 1). The ability to detect *A. acidoterrestris* in all 13 samples after 24 hours of enrichment, even in peach puree, supports the finding of higher efficiency for the species-specific primer sequences compared to the genus-specific sequences. The variability in Ct values between 24 h and 48 h enrichments appears to be due to differences in the growth rate of *Alicyclobacillus spp.* within the different sample matrices. Previous studies have shown juice matrices to differentially influence *A. acidoterrestris* growth likely due to pH, total soluble solids, water activity, etc. (Silva et al., 1999; Bahceci and Acar, Bahçeci and Acar 2007; Collins, 2008; Goto et al., 2007). In a recent study by Wang et al. (2014), real-time PCR detection was coupled with immuno-magnetic separation (IMS) to detect low levels (10 CFU/ml) of *Alicyclobacillus* spp as well. They, however, state that in complex matrices there was low correlation between *A. acidoterrestris* numbers and Ct values. They attributed this low correlation to interfering sample debris. The influence of sample matrix effects can be deduced from the *A. acidoterrestris* Ct values of "filtered" samples and "unfiltered samples" (Table 1). In filtered orange juice there appears to be inhibition of *A. acidoterrestris* growth; hence, there is no significant difference ($p > 0.05$) in the Ct values between the 24 h and 48 h enriched samples. However, in orange juice "with pulp" samples, there was a significant difference ($P < 0.05$) between the Ct values reported after 24 h and 48 h enrichment, suggesting pulp may hinder the growth rate of *A. acidoterrestris* in such samples.

The analysis of variance indicates that both incubation time and the fruit sample matrix have a statistically significant ($p < 0.01$) effect on the Ct values for *A. acidoterrestris* detection (Table 2). Sample texture also appears to have a significant effect on Ct values. This is evident in the statistically significant differences in the Ct values for *A. acidoterrestris* detection after 24 h and 48 h enrichment in the tomato-based samples, i.e., tomato sauce, tomato paste and ketchup samples. Similar differences were observed in the apple-based samples, i.e., apple juice and apple puree

Table 2 Analysis of variance (ANOVA) to determine the interaction of sample type, enrichment time, and real-time PCR detection (Ct value) of *A. acidoterrestris*

Source	Sum of squares	df	Mean square	F Value	p-value
Model	9302.70	23	404.47	10.09	<0.0001
Sample type	6280.43	12	523.37	13.06	<0.0001
Enrichment time	2866.86	1	2866.86	71.53	< 0.0001
Sample type *X* enrichment time	1148.67	10	114.87	2.87	0.0035

samples (Table 1). The Pearson Product Moment Correlation analysis showed the Ct value for *A. acidoterrestris* detection tended to increase with increasing Brix values (correlation coefficient: 0.608; P value 0.02); suggesting that the higher soluble solids content suppressed the growth (i.e., higher Ct values) of the organism in the enrichment medium. These results are supported by previous reports which suggest that *A. acidoterrestris* growth is negatively influenced at Brix values above 18-20 (Collins, 2008; Sprittstoesser et al., 1994). In this study using commercially available kits, we found that these effects began at Brix values of greater than 27.

Table 3 illustrates the enrichment time requirements for the detection of *A. acidoterrestris* (by real-time PCR and growth on K agar) and the confirmation of guaiacol formation. For all 13 samples that were tested, real-time PCR was capable of detecting low initial contamination (10 CFU/ml) of *A. acidoterrestris* within 24 h of enrichment by real-time PCR. However, two samples (orange concentrate and ketchup) were negative even after 48 h of enrichment based on growth on K agar. All samples that showed positive growth on K agar after 24 h or 48 h enrichment were also positive for guaiacol formation (Table 3). There did not appear to be any pattern in culture-based detection or guaiacol formation as a function of the Brix values. Instead, having a minimum enrichment time of 48 hours was very important for the conventional IFU plating method to be effective. This study made use of the most commonly identified spoilage species *A. acidoterrestris,* which we exposed to a period of heat-stress for 10 minutes. Similar studies should be performed with other pertinent *Alicyclobacillus* species in samples that have been exposed to different processing conditions. Nevertheless, this study provides evidence that faster more accurate detection of the genus *Alicyclobacillus* as well as the species *A. acidoterrestris* is possible and should be further explored by fruit processing manufacturers to best optimize detection in their particular matrices.

Conclusions

Fruit juice and puree characteristics, specifically the soluble solids content (Brix values), significantly influence the enrichment and real-time PCR detection of the spoilage genus *Alicyclobacillus* as well as the species *A. acidoterrestris*. Samples with high Brix values impair

Table 3 Comparison of detection of *A. acidoterrestris* in different fruit matrices by real-time PCR and conventional plating using K agar and confirmation of guaiacol formation

Sample	^{0}Brix	Time requirement*	Growth on K Agar		Guaiacol Formation	
			24 h	48 h	24 h	48 h
Orange concentrate	43	24 h	-	-	-	-
Apple concentrate	40	24 h	+	+	+	+
Ketchup	27	24 h	-	-	-	-
Tomato paste	26	24 h	+	+	+	+
Apple puree	17	24 h	-	+	-	+
Peach puree	17	24 h	-	+	-	+
Apple juice (filtered)	12	24 h	-	+	-	+
Peach nectar	12	24 h	+	+	+	+
Apple juice (unfiltered)	11	24 h	+	+	+	+
Orange juice (with pulp)	11	24 h	-	+	-	+
Orange juice (no pulp)	10	24 h	-	+	-	+
Tomato sauce	9	24 h	+	+	+	+
Tomato juice	6	24 h	+	+	+	+

*enrichment time required for positive detection by real-time PCR.
-: not detectable.

microbial proliferation in the enrichment medium. The impaired microbial growth results in higher Ct values in real-time PCR assays. Thus, fruit juices and purees with high Brix values require longer enrichment periods or need to be diluted prior to being enriched for real-time PCR detection. This study also highlights the point that even with low starting concentrations namely 10 CFU/ml, the commercial real-time PCR kit used detected *A. acidoterrestris* within 24 h of enrichment. Thus, it may not be necessary to wait for 48 h of enrichment prior to real-time PCR analysis. The ability to detect the presence of this key spoilage organism in fruit juices and purees within 24 h will have significant economic value to the fruit juice industry.

Competing interests

There are no personal or financial competing interests in this study. SS and SDP are employees of the State of Texas. CH is an employee of BIOTECON Diagnostics which provided the molecular reagents for this study.

Authors' contributions

SS, SDP, and CH were involved in the experimental design of this study. SS and SDP performed the laboratory analyses. SS, SDP and CH were involved in the writing, editing, and proof-reading of the manuscript.

Acknowledgements

The authors wish to acknowledge Drs. C. Groenewald and K. Berghof-Jaeger of BIOTECON Diagnostics for their support and for kindly providing the real-time PCR reagents used in this study. This work was supported by funding to SDP from the Texas A&M AgriLife Research project H8708.

Author details

[1]National Center for Electron Beam Research, Department of Nutrition and Food Science, Texas A&M University, College Station, Room 418B, Kleberg Center, MS 2472, 77843-2472 Texas, USA. [2]BIOTECON Diagnostics GmbH, Hermannswerder 17, 14473 Potsdam, Germany.

Received: 17 October 2014 Accepted: 9 February 2015
Published online: 25 February 2015

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