

# Proximity ligation assays with peptide conjugate 'burrs' for the sensitive detection of spores

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## ABSTRACT

The proximity ligation assay (PLA) has previously been used for the sensitive and specific detection of single proteins. In order to adapt PLA methods for the detection of cell surfaces, we have generated multivalent peptide–oligonucleotide–phycoerythrin conjugates ('burrs') that can bind adjacent to one another on a cell surface and be ligated together to form unique amplicons. Real-time PCR detection of burr ligation events specifically identified as few as 100 *Bacillus anthracis*, 10 *Bacillus subtilis* and 1 *Bacillus cereus* spore. Burrs should prove to be generally useful for detecting and mapping interactions and distances between cell surface proteins.

## INTRODUCTION

The sensitive and accurate detection of spores is of key importance for both clinical and biodefense applications. Because of their extraordinary sensitivities (1), PCR-based methods are widely utilized for the detection and identification of nucleic acid sequences associated with spores (2–4). However, the accurate identification of specific bacterial species often requires that multiple gene targets be detected in parallel (3); otherwise, the amplification of genes from closely related, non-target organisms can occur (5). In addition, the detection of protein as well as nucleic acid targets can help to guard against the detection of false positives. Alternative methods for spore detection have relied upon either ELISA (6) or the binding of fluorescently labeled antibodies or peptides to spore surfaces, followed by microscopy or FACS (7–9).

Since most protein detection methods are not as sensitive as PCR, it would be useful to couple methods for the identification of spore surfaces with PCR amplification. Although an immuno-PCR approach should be possible (10), such methods require that unbound antibody–DNA conjugates be separated from bound conjugates, and are inherently prone to generating false positive results owing to non-specific binding.

Therefore, we have adapted the proximity ligation assay (PLA) (11,12) to couple spore coat recognition and real-time PCR amplification. Proximity ligation is an innovative technique in which small DNA tags are co-localized on a protein surface and subsequently ligated together, creating a unique amplicon that can be sensitively detected using real-time PCR. PLA has previously been used to detect zeptomole amounts of proteins (11).

We reasoned that co-localization of DNA tags on a cell surface, rather than on a single protein molecule, might lead to the specific and sensitive detection of cells. In the present study, we have adapted peptides that bind specifically to either *Bacillus anthracis*, *Bacillus subtilis* or *Bacillus cereus* spores (9,13) to PLA. Peptides and DNA tags were conjugated to the fluorescent protein phycoerythrin (PE), creating multivalent 'burrs' that could detect spore surfaces. Following ligation, the amplicons associated with burrs could be used to specifically detect as few as 100 *B.anthraxis* and 10 *B.subtilis* spores, and down to 1 *B.cereus* spore.

## MATERIALS AND METHODS

### Bacterial strains and spores

The *Bacillus* strains used in this study and their sources were as follows: *B.subtilis* (ATCC 6051) and *B.cereus* (ATCC 14579) were obtained from the American Type Culture Collection. Spores were produced by growing the respective bacteria (50 µl) in 500 µl of Luria–Bertani (LB) broth for 3 days to an optical density at 600 nm (OD<sub>600</sub>) of 1.6–2.0. The culture was then diluted to an OD<sub>600</sub> of 0.4–0.5 in synthetic replacement sporulation media (SRSM) (14) and incubated at 37°C on a shaker at 250 r.p.m. for 2 days. The culture was centrifuged at 10 000 g for 10 min using the JLA 16.250 rotor, and the pellet resuspended and lysed in 2 ml of the detergent B-Per (Pierce Biotechnology, Rockford, IL) and lysozyme (5 mg/ml). The lysate was placed on a lab rotator for 30 min at room temperature and then sonicated twice using a sonic dismembrator (Fisher Scientific, Hampton, NH) with a Branson model 102D horn fitted with a microtip at an

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amplitude of 15% for two 5 min intervals. The lysate was placed on ice between the two sonications. The sonicated lysate was centrifuged at 18 000 *g* for 15 min and washed twice with 3 ml of phosphate-buffered saline (PBS). During the last wash, the pellet was divided into five aliquots. Bacterial spores were separated from cell debris by density gradient centrifugation with sodium diatrizoate (15). Optimal conditions for separation were determined by resuspending the pellets in 2 ml of 25, 30, 35, 40 or 45% sodium diatrizoate in ddH<sub>2</sub>O. The 2 ml solutions were layered over 20 ml of 50% sodium diatrizoate and centrifuged at 11 000 r.p.m. for 45 min at 4°C. The broken vegetative cell debris floating in the supernatant was removed and the spores were washed three times with 2 ml of ddH<sub>2</sub>O. Although all the five concentrations of sodium diatrizoate could be used to separate spores from the broken vegetative cells, optimal separation was observed for pellets that were resuspended in 35% sodium diatrizoate.

*Bacillus anthracis Sterne* (BA) was purchased in the form of a vaccine from Colorado Serum (Denver, CO). The detergent-based spore suspension was centrifuged at 10 000 *g* for 45 min to pellet the BA spores. The spores were washed three times with 1 ml of 1× PBS and resuspended in 1 ml of ddH<sub>2</sub>O.

All spore preparations were titered using a haemocytometer (Hausser Scientific, PA).

### Peptides and primers

The spore-binding peptides used for PLA were synthesized by Biosynthesis Incorporated (Lewisville, TX). The sequences of the peptides were NH (*B. subtilis* specific), NHFLPKVGGGC-OH; A-TY (*B. anthracis* specific), ATYPLPIRGGGC-OH; and S (*B. cereus* specific), SLLPGLPGGC-OH. Fluorescently labeled peptides were synthesized by conjugation of the C-terminal cysteine with fluorescein maleimide, followed by reverse-phase HPLC purification.

DNA probes, splint oligonucleotide and primers were purchased from IDT (Coralville, IA) and were adapted from sequences in Ref. (11). The sequence of the DNA probes used were 3' oligonucleotide probe, 5'-P-GTCATCATTCGAATC-GTACTGCAATCGGGTATT-S-3' and 5' oligonucleotide probe, 5'-S-GTGACTTCGTGGAAGTATCTAGCGGTGTA-CGTGAGTGGGCATGTAGCAAGAGG-3', where '5'-P' indicates a phosphate and 'S' a thiol modification. The templating oligonucleotide (splint) sequence was 5'-AAGAAT-GATGACCCTCTTGCTAAAA-3'. The primers for PCR amplification were 5'-GTGACTTCGTGGAAGTATCTAG-CG-3' and 5'-AATACCCGATTGCAGTACGATTC-3'. For real-time PCR detection, we used the *TaqMan* assay and the probe 5'-FAM-TGTACGTGAGTGGGCATGTAGC-AAGAGG-BHQ-3', where FAM was 6-carboxyfluorescein and BHQ the Black Hole Quencher-1. All primers and probes were suspended in ddH<sub>2</sub>O to a final concentration of 1 mM each.

### PLA probe synthesis

R-PE was obtained from Prozyme (San Leandro, CA) and was purified from its ammonium sulfate buffer using a Microcon YM-100 filter (Millipore, MA) and resuspended in 250 µl of 1× PBS. The protein was activated using the heterobifunctional crosslinker sulfo-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC, Pierce, IL) as

described previously (16). In short, 0.1 mg of PE was incubated with 0.2 mg of Sulfo-SMCC for 1 h at room temperature. The activated PE was then desalted using a NAP-5 column and resuspended in 1 ml of 1× PBS. The final concentration of the activated PE was calculated using a Nanodrop ND-1000 (Nanodrop, Wilmington, DE).

Probe conjugation was mediated through the terminal cysteine residue on the peptide and either 3' or 5' terminal thiol modifications on the oligonucleotides. Before conjugation, the DNA probes were treated with 10 mM of DTT for 30 min at room temperature, desalted using a NAP-5 column, and resuspended in 1× PBS to the desired concentration. Conjugation was achieved by incubating 56 pmol of activated PE with a mixture of a spore specific peptide (400 pmol) and either the 5' or 3' DNA probe (400 pmol) in 20 µl of PBS overnight in the dark at 4°C. The PE conjugates were desalted using a Microcon YM-100 filter and resuspended in 100 µl of 1× PBS. Probe concentrations were measured using the Nanodrop ND-1000. The approximate stoichiometry of oligonucleotide-peptide-PE was determined for the ATY-conjugate probes by comparing the absorbance of the conjugates at 260, 280 and 566 nm. The stoichiometry was estimated to be 5:3:1.

### Fluorescence microscopy

Spore binding assays were prepared by combining ~10<sup>8</sup> spores with 40 nM fluorescein-labeled monovalent peptide-fluorescein or polyvalent peptide-PE conjugates in a 20 µl reaction. Samples were incubated for an hour at room temperature in 1× PBS and then washed three times with 100 µl of 1× PBS and 0.5% Tween-20. After each wash, the spores were centrifuged at 820 *g* at 4°C for 5 min. After the final centrifugation step, the spores were resuspended in 50 µl of PBS and fluorescence was detected using a Nikon Eclipse E800 microscope. Single band length excitation filters for FITC (501/16; 535/30) and Texas Red (568/24; 610/40) (Chroma, VT) were used to observe the monovalent peptide-fluorescein- and polyvalent peptide-PE-labeled spores, respectively. Spores incubated either without peptides or with unlabeled peptides served as controls for all microscopy experiments.

### Real-time PCR amplification and optimization

All real-time PCR amplifications were performed with an MJ DNA Engine Opticon (MJ Research, MA). The reactions were initially optimized using a full-length DNA template (1 pM) that was analogous to the ligated PLA probes. A series of reactions were prepared using concentration gradients of MgCl<sub>2</sub> (4, 5 and 6 mM), dNTPs (50, 100 and 200 µM) and *TaqMan* probe concentrations of 75 and 100 nM. The buffer conditions for optimal amplification were 100 mM KCl, 5 mM MgCl<sub>2</sub>, 40 mM Tris-HCl, (pH 8.3), 0.4 U of T4 DNA ligase, 0.2 mM dNTPs, 500 nM primers (3' and 5' each), 75 nM *TaqMan* probe, 80 µM ATP and 0.5× Smart cycler additive [0.1 mg/ml non-acetylated BSA, 75 mM trehalose and 0.1% Tween-20 in 8.5 mM Tris buffer, (pH 8.0)] and 1.5 U of Platinum *Taq* polymerase (Invitrogen, CA). All reactions were conducted in a total volume of 50 µl. Real-time PCR was performed as follows: samples were heated to 50°C for 5 min and then cycled 50 times at 92°C for 1 min, 50°C for

1 min and 72°C for 1 min. The fluorescence intensity of the reaction was measured at the end of each cycle.

### PLA

PLA reactions minus enzymes were assembled at room temperature in 48.3  $\mu$ l of optimized PCR buffer. After the addition of Platinum *Taq* polymerase (0.3  $\mu$ l at 5 U/ $\mu$ l), ligation reactions were initiated by the addition of T4 DNA ligase (0.4  $\mu$ l at 1 U/ $\mu$ l). The reaction mixtures were incubated for an additional 5 min and then placed in the thermocycler. Experiments in which burrs and spores were preincubated for 1 h in PBS before the addition of enzymes showed no apparent effect on signal or detection.

All reactions were repeated a minimum of three times and were conducted with at least two independent preparations of PE-conjugated probes. The cycle differences reported in all figures represents the cycle difference (C[T]) between the background amplification reaction (no spores) and amplification in the presence of varying amounts of target spores.

PLA optimizations were carried out with reactions containing 100 (Figures 3–5) and 10 spores (data not shown). The optimal probe concentration was determined for reactions containing 10 pM splint and probe concentrations of 100, 50, 10, 1 and 0.1 pM. The optimal splint concentration was determined for reactions containing 1 pM probe and splint concentrations of 100, 50, 10, 1 and 0.5 pM. Spore detection assays were conducted using optimized conditions, 10 pM PLA probe and 10 pM splint. Reactions contained 10 000, 1000, 100, 10, 1 or 0 spores.

## RESULTS AND DISCUSSION

### The concept of peptide conjugate ‘burrs’ for spore recognition and PLA

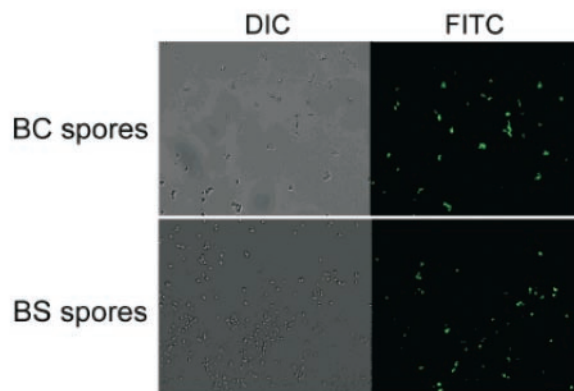
PLAs have previously been shown to be a sensitive and specific method for protein detection and analysis. The method relies on two independent affinity reagents that bear oligonucleotide tails binding in proximity to one another; the oligonucleotides can then be ligated together, yielding an amplicon that can be detected by PCR or other amplification methods. PLA was initially developed using DNA aptamers that either bound to individual subunits of a dimeric protein or to different epitopes on the same protein (11). The method has since been expanded to include antibody–DNA conjugates (11,12). We now further expand PLA to the use of peptide-based affinity reagents that can bind specifically not to proteins, but to the surfaces of spores.

Phage-displayed peptides have been selected that bind with high specificity to several different *Bacillus* spores [Table 1; (9,13)]. It was known that the peptides bound poorly as isolated, synthetic monomers (8,17), and our own preliminary

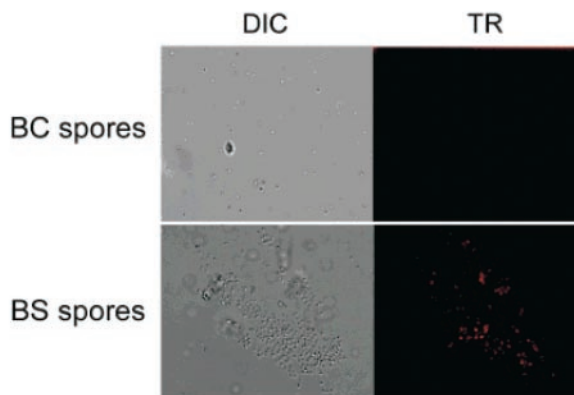
studies with fluorescent peptide derivatives indicated that there was a significant degree of cross-reactivity between different spores (Figure 1A). However, polyvalent presentation of the peptides either in the context of a fluorescently labeled phage or as PE conjugates was known to support specific recognition of spores, and we therefore decided to use PE as the basis for PLA affinity reagents. As Turnbough and co-workers previously observed, polyvalent peptide–PE conjugates proved to be highly specific for spores from *Bacillus* species (Figure 1B).

PLA affinity reagents were further developed by conjugating both peptides and oligonucleotides to PE, creating ‘burrs’ that had multiple opportunities to bind both to the spore surface and to position oligonucleotides for ligation reactions (Figure 2A). Peptides and oligonucleotides bearing thiol linkers were mixed with one another and then with PE activated with sulfo-SMCC. This joint immobilization procedure allows us to control the ratio of peptide–oligonucleotide. Starting with an equimolar ratio of peptide and oligonucleotide resulted in the conjugation of  $\sim$ 5 oligonucleotides and

#### A) Monovalent NH-peptide



#### B) Polyvalent NH-peptide



**Figure 1.** Specificity of monovalent and polyvalent probes. Fluorescent probes were constructed using the NH-peptide (BS-specific). BS and BC spores were incubated with either (A) monovalent NH-peptide–fluorescein conjugates or (B) polyvalent NH-peptide–PE conjugates. Specific binding was only observed when the polyvalent NH-peptide–PE probes were used. Spores were visualized using differential interference microscopy (DIC) and fluorescence microscopy with either fluorescein (FITC) or Texas Red filter sets (TR).

**Table 1.** Spore-specific peptides used for the design of PLA probes

Peptide name	Sequence	Spore specificity
NH S	NHFLPKVGGGC-OH SLLPGLPGGGC-OH	<i>Bacillus subtilis</i> <i>Bacillus cereus</i> , <i>Bacillus thuringiensis</i>
A-TY	ATYPLPIRGGGC	<i>Bacillus anthracis</i>

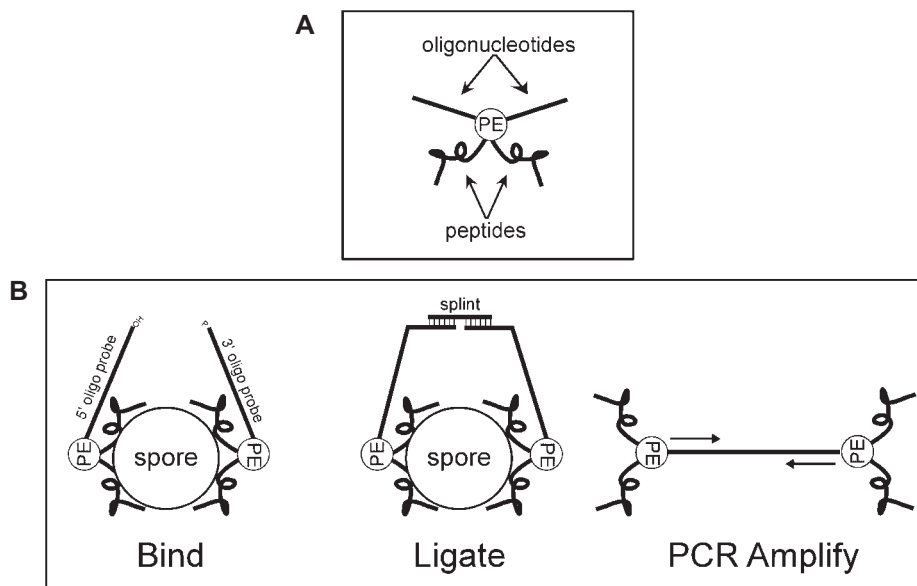


3 peptides per PE. When two burrs bind adjacent to one another on a spore surface, the pendant oligonucleotides can be aligned by an external template (splint) and ligated by T4 DNA ligase. The ligation event can be detected and quantified by real-time PCR (Figure 2B).

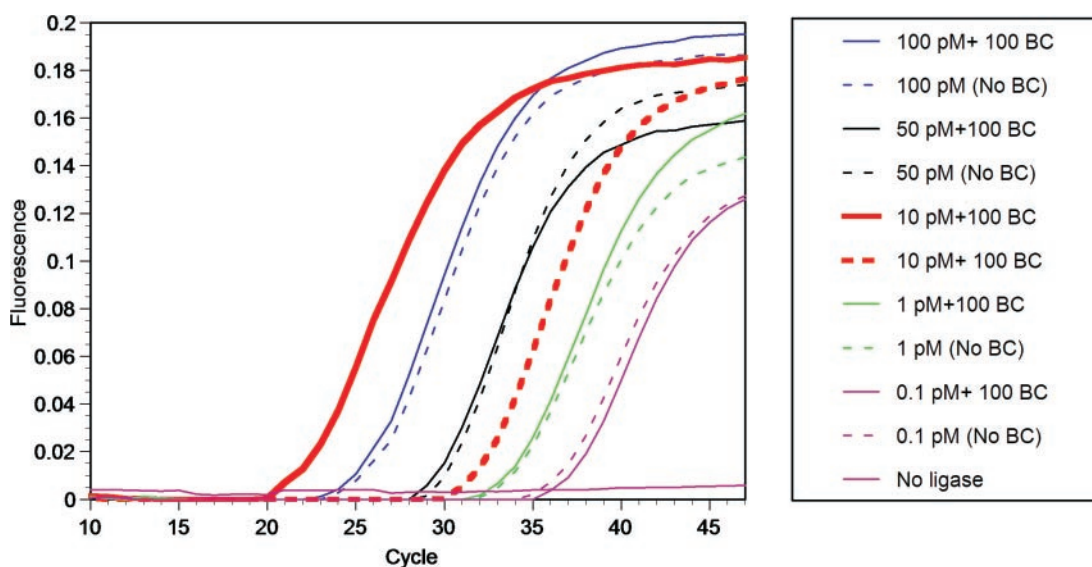
### Spore detection via burrs and PLA

Spore-specific burrs were mixed with *B. subtilis* (BS), *B. cereus* (BC) or *B. anthracis* (BA) and incubated for 5 min in optimized

PLA buffer before the addition of T4 DNA ligase and *Taq* polymerase. Since we wished to preferentially capture proximity events, ligation was carried out for a very short period of time (5 min), and then ligated sequences were amplified via real-time PCR. In addition, since the splint can potentially promote the ligation of the burrs even in the absence of spores we carried out negative controls without spores. After PCR, the spore-dependent signal is represented as the shift in the number of PCR cycles required for amplification to a given cycle threshold [C<sub>T</sub>] value (18).



**Figure 2.** Construction and ligation of burrs. (A) Burrs: oligonucleotides and peptides are separately conjugated to PE. There are two distinct oligonucleotide conjugates, one linked through its 5' end and one linked through its 3' end. (B) Burr ligation and amplification. When simultaneously bound to a spore target, burrs can be aligned by a splint oligonucleotide and ligated to generate a unique amplicon. The sequences of the 5' and 3' oligonucleotide probes as well as the splint oligonucleotide are given in Materials and Methods.



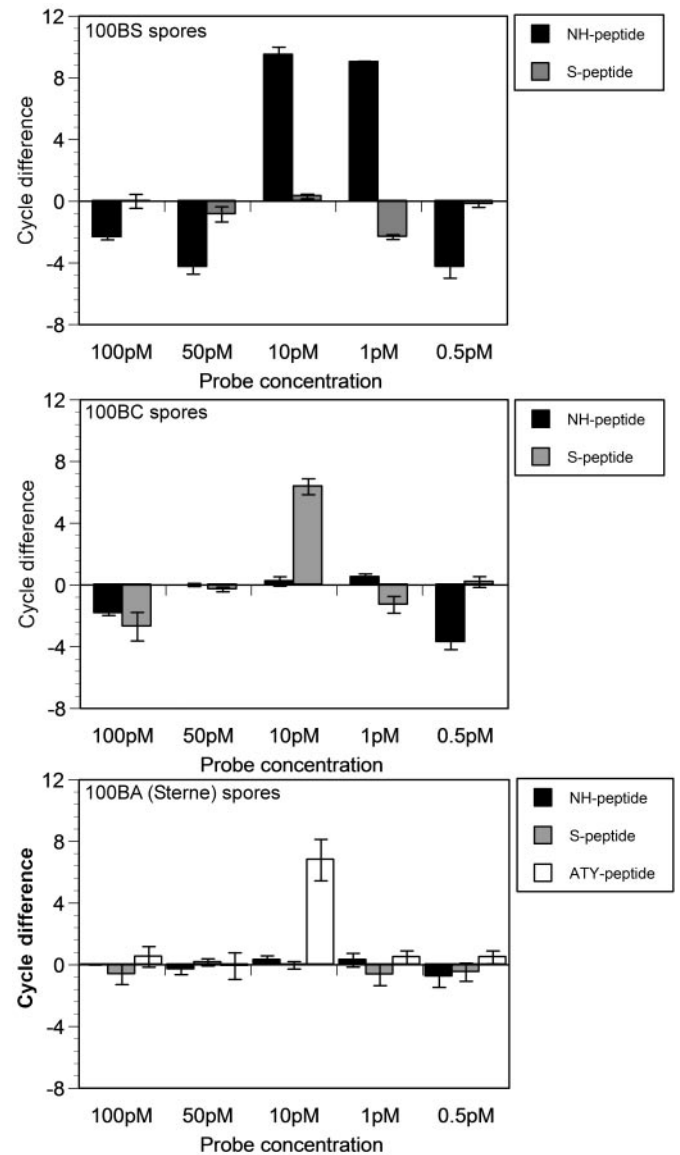
**Figure 3.** Optimization of PLA probe concentration for the detection of *B. cereus* spores. The real-time PCR data represent a single dataset in which the probe concentration was varied from 0.1 to 100 pM. PLA reactions conducted in the presence of 100 BC spores are indicated by a solid line and those conducted in the absence of spores by dashed lines. A positive, spore-dependent signal was only observed when reactions were conducted using 10 pM probe (bold red). Reactions were assembled as described in Materials and Methods.

Initially, we did not know how much burr would be necessary to achieve a significant shift in the cycle threshold. We, therefore, varied burr concentration from 0.5 to 100 pM while keeping the splint concentration (10 pM) and other variables constant. PLA reactions with only 100 BS and BC spores were conducted with burrs bearing either the NH- (BS-specific) or S-peptide (BC-specific), while reactions with 100 BA (Sterne) spores were conducted using probes bearing either the NH-, S- or the ATY-peptide (BA-specific). A single dataset generated with *B.cereus* spores is shown in Figure 3. A substantive real-time PCR signal was observed when the PLA reaction was conducted using a 10 pM concentration of burrs. Similar experiments were conducted with spores from all three bacterial species a minimum of three times. The averaged data from these experiments are shown in Figure 4. Again, spore-specific signals, indicated by a positive C[T] value, were reproducibly observed at some burr concentrations. PLA reactions in which the burrs and spores were pre-incubated for 1 h in PBS before the addition of enzymes gave similar results (data not shown).

The fact that only some burr concentrations should give large changes in C[T] values is not surprising; too many burrs in solution will yield a background of ligated templates that is not spore-dependent, whereas too few burrs will not generally bind adjacent to one another on a spore surface, will not ligate and again will not yield a spore-dependent signal. For 100 spores, 10 pM burr generally seemed to give a reliable signal. Gratifyingly, the BS-specific peptide never yielded a significant, positive C[T] value with BC and BA, and the BC-specific peptide did not give a positive C[T] value with BS or BA. Additional optimizations (Figure 6) revealed that the BA-specific peptide did not produce a signal in the presence of BS or BC spores. In some cases, a negative cycle difference ( $\sim 1-4$  cycles) was observed when reactions were conducted in the presence of spores. These negative C[T] differences may reflect the general inhibition of PCR reactions by spores or attendant organics in solution, and further emphasize the validity of the reproducible, positive C[T] values seen with cognate burr-spore pairs.

In addition to the affinity reagent concentration, the concentration of the splint oligonucleotide has been shown to be an important factor in the optimization of PLA detection (11,12). Therefore, we performed a series of assays in which we varied the splint concentration. Assays were conducted using a constant amount (10 pM) of burr and 100 BS, BC or BA (Sterne) spores. As shown in Figure 5, optimal spore detection was observed for reactions conducted with either 10 or 50 pM splint. The decrease in the observed cycle difference at the higher splint concentrations can be attributed to a decrease in the number of amplification cycles necessary to generate a signal in the absence of spores, indicating an increase in the number of spore-independent ligation events (data not shown). Most importantly, though, all reactions conducted with non-cognate spores again showed no positive signal.

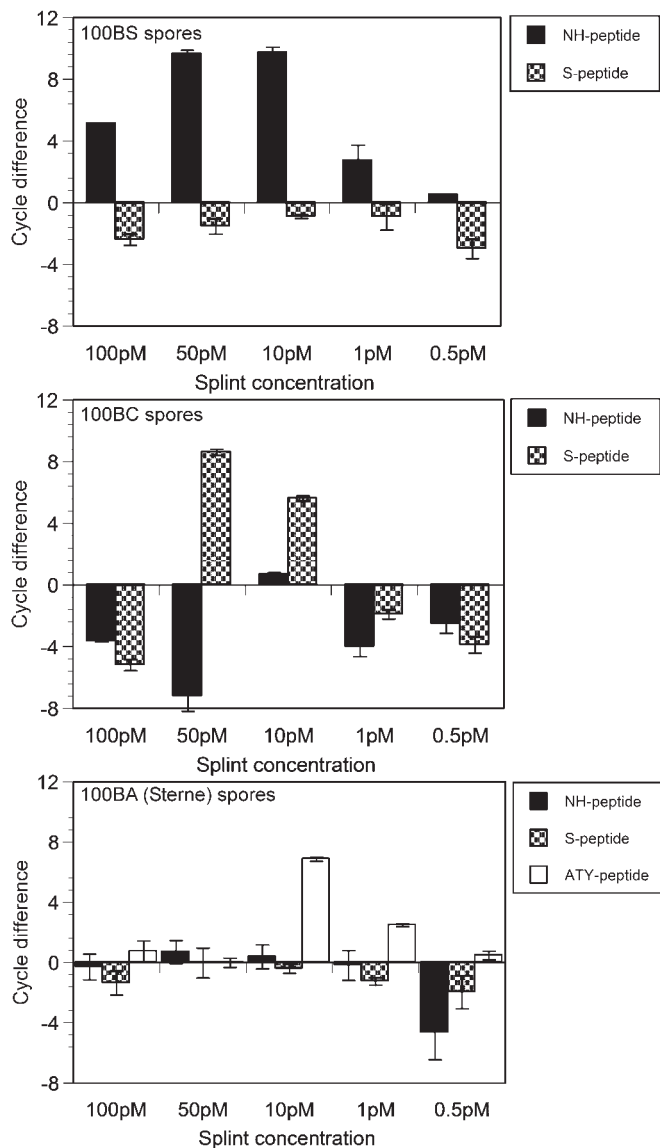
Finally, we carried out PLA reactions to examine the limits of detection with burrs. As shown in Figure 6, specific amplification is once again only observed for each burr with its cognate spore. The observed detection limits for optimized reaction conditions are as few as 10 BC or BS spores, and 100 BA (Sterne) spores. It should again be emphasized that



**Figure 4.** Optimization of PLA probe concentration for 100 *B.subtilis* and *B.cereus* spores. A splint concentration of 10 pM was used. The cycle difference represents the difference between the C[T] value of the background amplification reaction (no spores) and amplification in the presence of spores. Reactions containing BS spores and BC spores were carried out with burrs that presented either the NH- (BS-specific) or S-peptides (BC-specific). Reactions containing BA (Sterne) spores were carried out with burrs presenting either the NH-, S- or the ATY-peptides (BA-specific).

these are detection limits for the detection of the spore coat, not the spore genome, and thus that PLA with burrs is likely the single most sensitive method for the detection of spores themselves currently available.

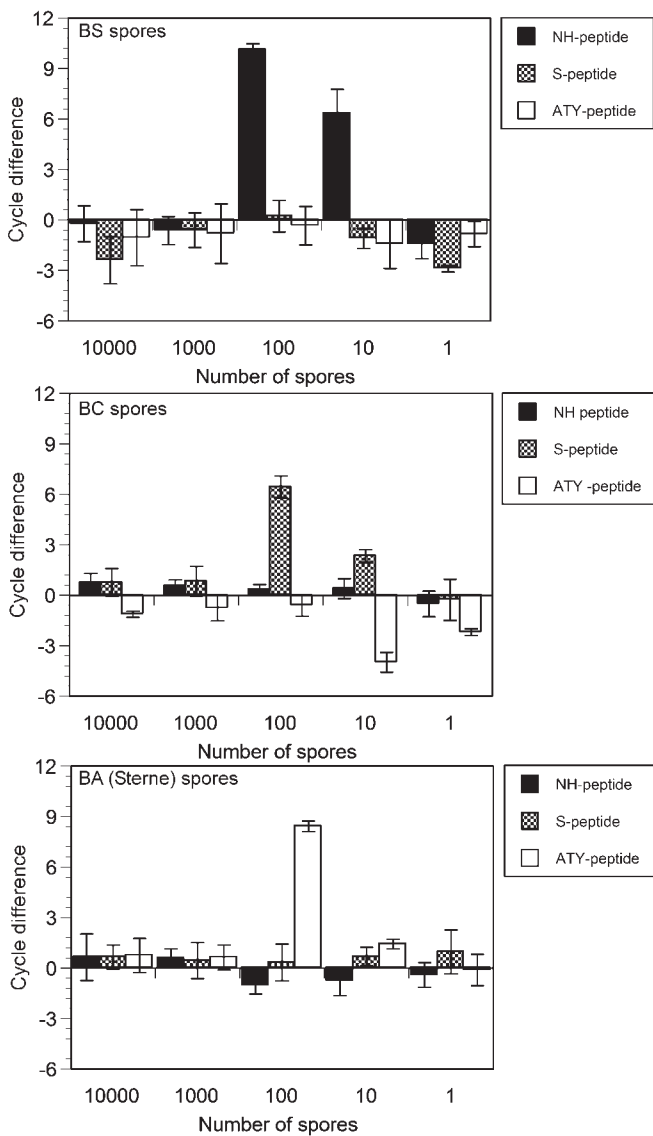
The loss of a positive signal at higher concentrations of spores is likely due to dilution of the burrs on the spore surface. At higher spore concentrations ( $10^3-10^4$  spores/ $50 \mu\text{l}$ ) the number of burrs binding to adjacent sites on the spore coat is decreased, leading to fewer or no ligation events. In keeping with this hypothesis, we reasoned that at lower spore concentrations there would be fewer spore-dependent ligation events but the same level of background ligation. If so, positive



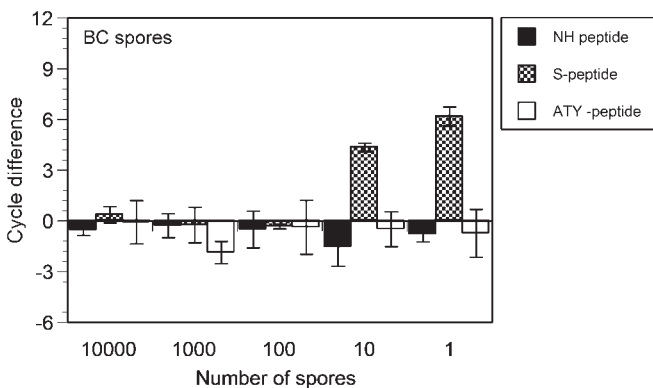
**Figure 5.** Splint optimization for 100 spores. A probe concentration of 1 pM was used. Reactions contained burrs as described in Figure 4. Cycle difference is as in Figure 4.

signals would be harder to acquire. Based on this, we attempted to rationally optimize the PLA detection method. Splint concentrations were lowered from 10 to 1 pM, in order to reduce the level of background ligation. As shown in Figure 7, this modification resulted in a further decrease in the detection limit to a single BC spore.

Although there appears to be a relatively narrow window in which specific spore-dependent amplification can be achieved, this window can be rationally manipulated and a variety of spore concentrations could potentially be detected by using several different burr-splint pairs in parallel. Each burr-splint pair would form a unique amplicon and would be present at a concentration that had previously been optimized for a given spore concentration. Thus, in a multiplex PCR, each burr-splint pair could detect a particular concentration range of a spore. Additionally, it may prove possible to



**Figure 6.** Specificity of spore detection assays. Reactions were carried out with 10 pM probe and 10 pM splint, and contained burrs bearing one of the three spore-specific peptides. Cycle difference is as in Figure 4.



**Figure 7.** Optimization of the limits of detection for BC spores. Reactions were carried out with 10 pM probe and 1 pM splint, and contained burrs bearing one of the three spore-specific peptides. Cycle difference is as in Figure 4.

improve detection by generating burrs that bear two different peptides for the same spore, or by synthesizing burrs with optimal oligonucleotide-peptide ratios.

The use of burrs is not merely an incredibly sensitive assay for cell surface epitopes, but should be an extremely powerful technique to probe the surfaces of cells. Although previous implementations of the PLA have indicated that multiple epitopes on the same protein or protein oligomer can be simultaneously detected, we have now extended this technique to multiple epitopes on the surfaces of cells. To the extent that type, number, or distribution of protein or other epitopes that can be identified by affinity reagents is diagnostic for a given cell or cell type, burr-based PLA may provide novel and interesting information about cell biology. For example, proteins that are ensconced within lipid rafts could be readily detected by burrs, even if the total concentration of proteins on the cell surface did not change. Similarly, burrs made from Annexin V could be used to identify when phosphatidylserine began to make an appearance on the cell surface, and thus could be used to monitor the earliest stages of apoptosis. As more applications for burr-based PLA are explored, it is even possible that oligonucleotides of differing lengths could be used as molecular rulers for probing the distances between target antigens on a cell surface.

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*Conflict of interest statement.* None declared.

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