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Evaluation of a Solid Phase DNA Binding Matrix for Downstream PCR Analysis

D.E. Bader, G.R. Fisher and C.W. Stratilo
DRDC Suffield

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Abstract

A commercially available solid-phase DNA binding matrix (FTA® cards) was evaluated for its ability to capture and release DNA for downstream gene amplification and detection assays using polymerase chain reaction (PCR) analysis, as part of a project to determine the utility of FTA® cards for sampling, archiving, and transport of samples that may contain biowarfare (BW) or bioterrorist (BT) agents. PCR was performed using assays designed to amplify and detect two different *Bacillus anthracis* virulence genes, namely the lethal toxin gene (*lef*) found on plasmid pXO1, and the capsule B gene (*cap*B), located on plasmid pXO2. PCR assays were conducted using real-time, Taqman fluorescent probe detection on a Cepheid Smart Cycler® instrument. A baseline detection limit of about 300 gene copies was observed for each assay prior to DNA-FTA® card binding studies using *B. anthracis* Ames DNA. Direct PCR of DNA bound to FTA® discs resulted in a loss of sensitivity compared to DNA in solution, however, this method proved better than the heat-elution method since PCR signals were observed at earlier cycles. Furthermore, FTA® bound DNA generated a reproducible PCR signal for 12 of 12 replicate tests (100%), compared to 11 of 12 (92%) for a single heat elution treatment, and 5 of 12 (42%) replicates for a double heat elution treatment. Even after the FTA® bound DNA discs were heat-treated twice, the DNA was still detected directly from the discs in every replicate (12/12) and only 2.5 cycles later than untreated discs. This indicated that even after heat elution, considerable DNA was retained on the FTA® discs for subsequent PCR analysis. This study demonstrated that although there is a loss in PCR sensitivity for DNA captured on FTA® Whatman solid phase media, it still serves as a potentially useful media for the storage, archiving and transport of genetic material for downstream PCR analysis.

Résumé

Une matrice liante d'ADN en phase solide (cartes (FTA®) disponible dans le commerce a été évaluée pour sa capacité à capturer et à libérer l'ADN pour l'amplification de gênes et pour les biotests de détection, effectués en aval, utilisant l'analyse de réaction en chaîne de la polymérase (PCR). Cette évaluation faisait partie d'un projet visant à déterminer l'utilité des cartes FTA® pour l'échantillonnage, l'archivage et le transport des échantillons pouvant contenir des agents de guerre biologique ou des agents de terrorisme biologique. La méthode PCR a été effectuée en utilisant des biotests conçus pour amplifier et détecter deux gènes de différente virulence de *Bacillus anthracis*, dont le gène à toxines mortelles (*lef*) trouvé sur le plasmide pX01 et la capsule du gène B (*cap*B), située sur le plasmide pXO2. Les biotests PCR ont été effectués en utilisant une sonde de détection fluorescente Taqman sur un instrument Cepheid Smart Cycler®, en temps réel. On a observé une limite de détection de la ligne de base de 300 copies de gènes environ pour chaque biotest, préalablement aux études de liaison des cartes DNA-FTA® utilisant l'ADN Ames *B. anthracis*. La méthode PCR sur un ADN lié aux disques FTA® a résulté en une perte de sensitivité comparée à l'ADN en solution mais cette méthode s'est révélée supérieure à la méthode d'élution thermique puisque des signaux PCR ont été observés plus tôt dans les cycles. De plus, des ADN liés FTA® ont généré des signaux PCR reproductibles pour 12 des 12 tests effectués en parallèle (100%) comparé à 11 sur 12 (92%) pour un seul traitement d'élution thermique et 5 sur 12 (42%) des tests effectués en parallèle pour un traitement par élution thermique double. Même après avoir traités thermiquement deux fois les disques d'ADV liés FTA®, l'ADN a encore été détecté directement à partir des disques, dans chaque test effectué en parallèle (12 sur 12) et seulement 2,5 cycles plus tard que les disques non traités. Ceci indique que même après l'élution thermique, une grande partie d'ADN a été retenue sur les disques FTA® pour des analyses PCR ultérieures. Cette étude a démontré que bien qu'il existe une perte de sensitivité PCR pour l'ADN capturé sur les medias FTA® Whatman en phase solide, cela peut encore servir de média utile pour l'entreposage, l'archivage et le transport des matériaux génétiques pour les analyses PCR, en aval.

Executive summary

Introduction: A commercially available solid phase matrix (FTA® cards) has been developed for the purpose of collecting and preserving genetic material for down-stream analysis. According to the manufacturer, FTA® cards have been chemically treated to lyse cell membranes and organelles, entrap and protect released nucleic acid from nucleases, oxidation and UV damage, and rapidly inactivate organisms to prevent the growth of bacteria and other organisms including blood-borne pathogens. Such properties would clearly be highly desirable for sample processing, archiving and transport of samples suspected of containing biowarfare (BW) or bioterrorist (BT) agents for both laboratory and field-based applications. A first step in evaluating the suitability of FTA® cards for this purpose is to evaluate the efficiency of FTA® cards for trapping and releasing genetic material for downstream analysis. This study tested FTA® cards for the ability to capture and release DNA for gene amplification and detection using polymerase chain reaction (PCR). PCR was performed using assays designed to amplify and detect two different *Bacillus anthracis* virulence genes, namely the lethal toxin gene (*lef*) found on plasmid pXO1, and the capsule B gene (*cap*B), located on plasmid pXO2.

Results: A baseline detection limit of about 300 gene copies was observed for each assay prior to DNA-FTA® card binding studies using *B. anthracis* Ames DNA. Direct PCR of DNA bound to FTA® discs resulted in a loss of sensitivity compared to DNA in solution, however, this method proved better than the heat-elution method since PCR signals were observed at earlier cycles. Furthermore, FTA® bound DNA generated a reproducible PCR signal for 12 of 12 replicate tests (100%), compared to 11 of 12 (92%) for a single heat elution treatment, and 5 of 12 (42%) replicates for a double heat elution treatment. Even after the FTA® bound DNA discs were heat-treated twice, the DNA was still detected directly from the discs in every replicate (12/12) and only 2.5 cycles later than untreated discs. This indicated that even after heat elution, considerable DNA was retained on the FTA® discs for subsequent PCR analysis.

Significance: This study demonstrated that although there is a loss in PCR sensitivity for DNA captured on FTA® Whatman solid phase media, it still serves as a potentially useful media for the storage, archiving and transport of genetic material for downstream PCR analysis.

Future plans: Experiments are planned to evaluate FTA® cards for suitability as a live agent sample processing, archival and transport medium for downstream PCR analysis. The knowledge gained from this work will provide data on the suitability of FTA® cards as a fieldable BW agent transport medium in terms of safety and preservation of material for downstream processing and analysis.

Bader D.E., Fisher G.R. and Stratilo, C.W. 2005. Evaluation of a Solid Phase DNA Binding Matrix for Downstream PCR Analysis. DRDC Suffield TM 2005-226. Defence R&D Canada – Suffield.

Sommaire

Introduction: Une matrice liante d'ADN en phase solide (cartes (FTA®) disponible dans le commerce a été mise au point dans le but de collecter et préserver des matériaux génétiques pour les analyses en aval. Selon le manufacturier, les cartes FTA® ont été traitées chimiquement pour lyser les membranes cellulaires et organelles, encapsuler et protéger les acides nucléiques libérés des nucléases, de l'oxydation et des dommages causés par les UV et pour inactiver rapidement des organismes empêchant ainsi la croissance des bactéries et autres organismes dont les pathogènes à diffusion hématogène. En ce qui concerne les laboratoires et les applications sur le terrain, de telles propriétés seraient évidemment très désirables pour le traitement des échantillons, l'archivage et le transport des échantillons suspectés de contenir des agents de guerre biologique et de terrorisme biologique. Une des premières étapes visant à évaluer la pertinence des cartes FTA® pour ce but est d'évaluer l'efficacité des cartes FTA® à encapsuler et à libérer des matériaux génétiques pour les analyses en aval. Cette étude a testé les cartes FTA® pour leur capacité à capturer et à libérer l'ADN pour l'amplification et la détection de gènes en utilisant la méthode de réaction en chaîne de la polymérase (PCR). La méthode PCR a été effectuée en utilisant des biotests conçus pour amplifier et détecter deux gènes de virulence différente de *Bacillus anthracis* dont le gène à toxine létale (*lef*) trouvé sur le plasmide pXO1 et la capsule du gène B (*cap*B), située sur le plasmide pXO2.

Résultats : On a observé une limite de détection de la ligne de base de 300 copies de gènes environ pour chaque biotest, préalablement aux études des cartes de liaison DNA-FTA® utilisant l'ADN Ames *B. anthracis*. La méthode PCR appliquée directement sur un ADN lié aux disques FTA® a résulté en une perte de sensitivité comparée à l'ADN en solution mais cette méthode s'est révélée supérieure à la méthode d'élution thermique puisque des signaux PCR ont été observés plus tôt dans les cycles. De plus, des ADN liés FTA® ont généré des signaux PCR reproductibles pour 12 des 12 tests effectués en parallèle (100%) comparé à 11 sur 12 (92%) pour un seul traitement d'élution thermique et 5 sur 12 (42%) des tests effectués en parallèle pour traitement par élution thermique double. Même après avoir traités thermiquement deux fois les disques d'ADV liés FTA®, l'ADN a encore été détecté directement à partir des disques dans chaque test effectué en parallèle (12 sur 12) et seulement 2,5 cycles plus tard que les disques non traités. Ceci indique que même après l'élution thermique, une grande partie d'ADN a été retenue sur les disques FTA® pour des analyses PCR ultérieures.

Portée des résultats : Cette étude a démontré que bien qu'il existe une perte de sensitivité PCR pour l'ADN capturé sur les medias FTA® Whatman en phase solide, la méthode peut encore servir de média utile pour l'entreposage, l'archivage et le transport des matériaux génétiques pour les analyses PCR en aval.

Plans futurs : Des expériences ont été prévues pour évaluer la pertinence des cartes FTA® pour le traitement d'échantillons d'agents vivants, comme medium d'archivage et de transport pour les analyses en aval. La connaissance acquise par ces travaux procurera des données sur la pertinence des cartes FTA® comme medium de transport d'agent de guerre biologique pouvant être mis en service en tant que matériel sécuritaire et de préservation pour le traitement et les analyses effectuées en aval.

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Introduction

In the event of a biological warfare (BW) attack or a bioterrorist (BT) attack, samples suspected of containing live agents need to be collected and identified so that the proper countermeasures can be taken. An important biological agent identification technology being evaluated and developed in our laboratory is a nucleic acid amplification technology called polymerase chain reaction or PCR [1-7]. PCR detects and amplifies specific nucleic acid sequences that can be used for identification purposes. PCR requires a source of genetic material (ribonucleic acid – RNA or deoxyribonucleic acid - DNA). Residual nucleic acid and/or genetic material contained within a biological warfare agent can act as source of material for PCR identification. It is important to protect this material from degradation until it can be analyzed. In the laboratory, material is maintained in a variety of forms such as dried, freeze-dried, frozen or refrigerated. These methods, while being resource intensive and logistically demanding to implement, are easier to accommodate in a laboratory setting than in a field setting. It would be advantageous to have a technology that is considerably less resource intensive and logistically demanding not only for field applications but also for laboratory applications as well.

Commercially available solid-phase matrices have been developed for the purpose of collecting and preserving genetic material for down-stream analysis. Solid phase matrices have been evaluated by PCR for various sample types such as blood samples [8] and wildlife samples [9]. A comparison study between IsoCode® STIX and FTA® Gene Guard Collection cards, for whole blood storage and processing for diagnosis of malaria by PCR, gave similar results for single-species malaria, but FTA® cards were more sensitive than IsoCode® STIX in mixed infections [8]. According to the manufacturer, FTA® cards have been chemically treated to lyse cell membranes and organelles, entrap and protect released nucleic acid from nucleases, oxidation and UV damage, and rapidly inactivate organisms to prevent the growth of bacteria and other organisms including blood-borne pathogens. This technology is an ideal technology to investigate for sample processing, archiving and transport of samples that may contain BW or BT agents for both laboratory and field-based applications.

The objective of this study is to evaluate the efficiency of FTA® cards for trapping and releasing genetic material from potential BW/BT agents for PCR analysis. PCR was performed using assays designed to amplify and detect two different *Bacillus anthracis* virulence genes, namely the lethal toxin gene (*lef*) found on plasmid pXO1, and the capsule B gene (*cap*B), located on plasmid pXO2. PCR assays were conducted using real-time, Taqman fluorescent probe detection on a Cepheid Smart Cycler® instrument.

Follow-on studies will investigate live BW/BT agents trapped onto the cards to measure the effectiveness of the FTA® card technology to inactivate or neutralize the agents and whether agent-specific PCR methods can be used to identify the agents after being applied to the FTA® cards.

Materials and methods

Preparation of DNA

Purified DNA was obtained from *Bacillus anthracis* strains in the DRDC collection which included *B. anthracis* strain ACB and Ames. DNA was purified using the method of Brumlick [10] which was adapted from Schraft and Griffiths [11]. Purified DNA preparations were subjected to sterility assessments, prior to removal from BSL3 containment, according to DRDC Suffield Bacterial Sterility Check Guidelines version 1.0, 9 January 2004. Briefly, 12% (v/v) of each preparation was aseptically inoculated into sterile brain heart infusion broth (BD Biosciences, 2280 Argentia Road, Mississauga, ON, Canada) and cultured at 35 °C for 7 days. A second aliquot of 12 % (v/v) of the preparation was directly plated onto sheep blood agar (SBA) plates (commercially prepared for DRDC Suffield and obtained through Medicine Hat Diagnostic Laboratory, Medicine Hat, AB, Canada), up to a maximum of 200 µL per plate and incubated at 35 °C for 7 days as well. After 7 days of incubation in broth culture, aliquots were removed and plated onto fresh SBA plates and incubated for an additional 7 days at 35 °C. Only preparations showing no observable growth on SBA plates either plated directly (7 days) or plated after broth culture (7 days) were removed from the BSL3 suite for analysis in the BSL2 laboratories. Purified DNA was quantified spectrophotometrically using the NanoDrop® according to the manufacturer's instructions (NanoDrop Technologies, Wilmington, DE, USA). DNA was diluted to working concentrations using TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Tris base and EDTA were from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. Copy numbers were calculated from molecular weight data obtained from published sources (Table 1).

Table 1. B. anthracis molecular weight data

Item	Source	Size in base pairs	Mol wt[a] (gm/mol)	% of total	Reference	Genbank reference no.
genome	BA Ames strain	5,227,293	3.45×10^9	95 %	[12]	NC003997
pXO1	Sterne strain	181,654	1.2×10^8	3.3 %	[13]	NC001496
pXO2	Pasteur	96,231	6.35×10^7	1.7 %	[14]	NC002146
total		**5,505,178**	**3.63×10^9**	**100 %**		

[a] based on molecular weight of 660 g/mol per base pair

FTA® Materials

All FTA® Whatman materials used in this study were obtained from VWR International Inc., Mississauga, ON, CA (Table 2).

Table 2. FTA® Materials

Item	VWR Catalog Number	Whatman Catalog Number
Indicating FTA® Classic Card	14222-802	WB12-0206
FTA® purification reagent	14222-816	WB12-0204
Harris Micro Punch (1.2 mm) with mat	14222-824	WB10-0005
Multi-barrier pouches for classic FTA® cards	14222-834	WB10-0010
Dessicant packets (1 gm)	14222-840	WB10-0003

DNA spotting onto FTA® Cards

Ten µL and 100 µL volumes of total DNA were applied by pipet onto indicating FTA® classic cards, generating spots with diameters of approx 6 mm and 17 mm, respectively (Table 3). The indicator dye turned from pink to white upon application. The card was allowed to air dry for a minimum of one hour at room temperature. Extra FTA® cards were spotted with DNA of various concentrations. Each card was placed into a multi-barrier pouch with a 1 gram desiccant packet and stored at room temperature until needed.

A punchout disc was cored from the center of each sample spot using the 1.2 mm Harris micro-punch and mat, and placed into a 1.5 mL screw cap tube. The area of each disc was 1.13 mm^2. The disc was treated by adding 200 µL of FTA® purification reagent to the tube and incubating for five minutes at room temperature. The tube was gently mixed manually. The FTA® purification reagent was removed and discarded by pipet. This step was repeated two more times for a total of three treatments. Two hundred µL of TE buffer was then added to each tube and incubated for 5 minutes at room temperature. The TE buffer was removed with a pipet and discarded. This step was repeated one more time for a total of two washes. The disc was then dried by placing the tube into a heat block at 56 °C for 10 minutes. Negative control discs (no DNA applied) were processed in parallel using the above procedure from clean FTA® card punchouts.

Table 3. Dimensions of Spots and Discs

Volume of DNA spotted onto FTA® card	Diameter of DNA spot	Area of DNA spot	Area of punchout disc [b]	Ratio of Disc Area to Spot Area
10 µL	6 mm	28.26 mm^2	1.13 mm^2	1/25
100 µL	17 mm	226.86 mm^2	1.13 mm^2	1/200

PCR Analysis

Disc PCR

PCR was conducted directly on the punchout disc by placing the disc manually into a 25 µL Smart Cycler® tube using a fine tip pipet tip and then adding 25 µL of 1x PCR reaction mixture.

Eluate PCR

For PCR of eluted material, 50 µL of TE buffer was added to each tube containing the dried disc. The tube was placed in a 90 °C water bath for 10 minutes, then placed on ice during PCR setup. Five µL of eluate was added to 20 µL of a 1.25x PCR reaction mixture to make a final 1x concentration of PCR reaction components in a total reaction volume of 25 µL.

PCR Reaction Mix

The PCR reaction mix was prepared using SmartMix™ HM beads (Cepheid, Sunnyvale, CA, USA). The final reaction component concentrations were 4.2 mM HEPES buffer (pH 7.2), 200 µM dNTPs, 4 mM $MgCl_2$, 3U Hot start Taq Polymerase, 0.25 µM forward primer, 0.25 µM reverse primer, 0.1 µM probe (*lef*-FAM and *cap*B-TET).

Example of a mastermix set up for "Disk PCR" (ten 25 µL rxns @ 1x strength)

SmartMix™ HM beads [c]	5 beads
forward primer (5 µM)	12.5 µL
reverse primer (5 µM)	12.5 µL
probe (2 µM)	12.5 µL
nuclease-free water	212.5 µL

[b] based on the use of a 1.2 mm Harris punch
[c] one bead per 50 µL PCR reaction

Example of a mastermix set up for "Eluate PCR" (ten 20 µL rxns @ 1.25x strength)

SmartMix™ HM beads [d]	5 beads
forward primer (5 µM)	12.5 µL
reverse primer (5 µM)	12.5 µL
probe (2 µM)	12.5 µL
nuclease-free water	162.5 µL

PCR Primers/Probes

The PCR primers and probes used in this assay target the lethal toxin (*lef*) gene on the pXO1 plasmid and the capsule gene (*cap*B) on the pXO2 plasmid of *B. anthracis*.

Lethal toxin gene (*lef*)

CWSLP2 (*lef* forward primer)	5' ggtacaagaagtatttgcgaaagc 3'
CWSRP2b (*lef* reverse primer)	5' atcttgacagcatccgttga 3'
CWSprobe2 (*lef* probe)	5' FAM-tgcatattatatcgagccacagcatcgtga 3' BHQ-1
amplicon size	167 bp

Capsule B gene (*cap*B)

CWSRP3 (*cap*B forward primer)	5' gaagcgaaatataagactgtaggg 3'
CWSLP3 (*cap*B reverse primer)	5' ttctaaatcagcagcctctttaac 3'
CWSP3 (*cap*B probe)	5' TET-ctgcgttgctcaccgatattaggaccttc 3' BHQ-1
amplicon size	153 bp

The probes and primers were designed at DRDC Suffield using PREMIER Biosoft Beacon Designer v4.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Integrated DNA Technologies Inc., Coralville, IA, USA.

Real-time PCR Cycling and Analysis

The PCR cycling and analysis was performed on the Smart Cycler® using SCII I-core modules (Cepheid) and ver1.2b software. The cycling conditions included:

(1) 95 °C for 5 min – 1 cycle
(2) 95 °C for 15s + 60 °C for 60s (with optics "on") – 45 cycles

The data was analyzed by the Smart Cycler® software using the analysis settings presented in Table 4 and dye set FTTC25.

[d] one bead per 50 µL PCR reaction

Table 4. Smart Cycler® Analysis Settings

Channel	1-FAM; 2-TET
Target	-
Usage	assay
Curve analysis	primary curve
Threshold setting	manual
Manual threshold fluorescence units	30.0
Auto thresh #SD's	N/A
Auto minimum cycle	5
Auto maximum cycle	10
Valid minimum cycle	3
Valid maximum cycle	60
Background subtraction	ON
Boxcar average cycles	0
Background minimum cycles	5
Background maximum cycles	40

Results and Discussion

PCR Cycling Performance for *cap*B and *lef* Taqman Assays using *B. anthracis* Ames DNA

PCR cycling values (Ct) were determined over a seven log concentration range of *B. anthracis* Ames DNA for the *cap*B and *lef* Taqman PCR assays (Table 5).

Table 5. PCR Cycling Data for capB and lef Taqman Assays Using DNA from Bacillus anthracis (Ames strain)

fg total DNA per PCR reaction	log [fg/PCR] (Y)	Ct[e] run #1 *cap*B	Ct run #2 *cap*B	avg Ct value ± std deviation (X)	Ct run #1 *lef*	Ct run #2 *lef*	avg Ct value ± std deviation
$1.87x10^8$	8.27	16.67	16.87	16.77 ± 0.14	17.25	16.94	17.10 ± 0.22
$1.87x10^7$	7.27	19.95	20.23	20.09 ± 0.20	20.19	20.25	20.22 ± 0.04
$1.87x10^6$	6.27	23.44	23.44	23.44 ± 0.00	23.24	23.26	23.25 ± 0.01
$1.87x10^5$	5.27	27.21	27.23	27.22 ± 0.01	27.3	27.14	27.22 ± 0.11
$1.87x10^4$	4.27	30.52	30.52	30.52 ± 0.00	31	30.5	30.75 ± 0.35
$1.87x10^3$	3.27	33.98	34.13	34.06 ± 0.11	34.57	34.41	34.49 ± 0.11
$1.87x10^2$	2.27	42.77	44.98	43.88 ± 1.56	36.4	39.79	38.10 ± 2.40

A standard curve was generated for each assay and found to be linear over a six log range (Annex A - Figure 1 and 2). Linear regression analysis generated line equations that were used to calculate quantities from Ct values obtained in subsequent analyses for samples containing Ames DNA. The number of target gene copies for each assay were calculated to be about 300 copies based on the lowest amount of total DNA that gave a linear dose response (1.87 pg). This calculation assumed a single molecule of plasmid per molecule of the genome and one copy of the gene target per plasmid molecule. This assumption was used in all additional calculations.

pXO1 target (*lef* gene)

i. total DNA detected per PCR reaction = 1.87 pg
ii. % of total weight for pXO1 = 3.3 % (see Table 1)
iii. total weight of plasmid DNA = 0.033 x 1.87 pg = 0.062 pg
iv. copies of pXO1 plasmid
= (0.062 pg x $6.02x10^{23}$ molecules/mol)/($1.2x10^8$ g/mol * $1.0x10^{12}$ pg/g)
= 311 molecules of target plasmid
= 311 *lef* gene copies

[e] Ct values represent the cycle number at which the fluorescence rises above the manual threshold fluorescence units setpoint which was set at 30 (default setting).

pXO2 target (*cap*B gene)

i. total DNA detected per PCR reaction = 1.87 pg
ii. % of total weight for pXO1 = 1.7 % (see Table 1)
iii. total weight of plasmid DNA = 0.017 x 1.87 pg = 0.032 pg
iv. copies of pXO1 plasmid
= (0.032 pg x 6.02×10^{23} molecules/mol)/(6.35×10^{7} g/mol * 1.0×10^{12} pg/g)
= 303 molecules of target plasmid
= 303 *cap*B gene copies

Ten-fold lower amounts were detected for the *lef* and *cap*B assays but at much later cycles (43.88 ± 1.56 and 38.10 ± 2.40, respectively). The detection limit is therefore between 30 and 300 copies for each assay using purified Ames DNA in solution. While the absolute detection limit may not be known for certainty, the relative sensitivity can be assessed when the same material is used for comparative analysis.

Disc Sensitivity Using the *cap*B Taqman Assay

A series of discs containing 9.3×10^{5} to 93 fg of total Ames DNA per disc was prepared and analyzed by direct PCR in nine replicate experiments (Table 6).

Table 6. Disc sensitivity for the capB Taqman assay

Total DNA (fg/disc)	Ct value (n=9)	Total number of PCR positive reactions
9.3×10^{5}	29.51 ± 0.43	9 of 9
9.3×10^{4}	33.27 ± 0.58	9 of 9
9.3×10^{3}	39.83 ± 2.17	7 of 9
9.3×10^{2}	43.83 ± 1.19	2 of 9
9.3×10^{1}	0.00	9 of 9

The amount of DNA that generated reproducible Ct values for all nine replicates was found to be 9.3×10^{5} fg/PCR (Ct = 29.51) and 9.3×10^{4} fg/PCR (Ct = 33.27). The Ct values one would expect to obtain for equivalent amounts of template in solution were determined from the standard curve (Figure 1) and found to be 24.65 and 28.13 respectively, or about 5 cycles earlier on average (4.85 and 5.14 respectively). This is equivalent to about a 32-fold difference in template copy number based on a doubling of material every PCR cycle (2^{5}). Alternatively, the amount of template in solution that would generate Ct values equivalent to those observed in disc PCR from the standard curve were 3.7×10^{4} fg/PCR and 3.07×10^{3} fg/PCR which represents 4% and 3.3% of the amount of DNA that was spotted onto the discs respectively. Thus there is a loss of sensitivity when target DNA is bound to the FTA® card compared to equivalent amounts of unbound DNA in solution.

PCR of Heat-Eluted Ames DNA versus Disc-Bound Ames DNA Using the *cap*B Taqman Assay

Heat elution of FTA® bound Ames DNA was investigated as an alternative method to direct disc PCR. A disc containing 93 pg of *B. anthracis* Ames DNA was heat-treated in 10 µL of TE buffer. The entire buffer volume was removed by pipet (eluate #1) and analyzed later by PCR (5 of 10 µL). The heat-treated disk was then subjected to a second round of heat treatment in 10 µL of fresh TE buffer. The entire buffer was removed for PCR (eluate #2) by pipet and then the heat-treated disc was PCR amplified (treated disc). A second disc containing the same amount of Ames DNA as the original heat-treated disc (93 pg) was PCR amplified directly without heat-treatment (untreated disc). Each sample was prepared in triplicate (3x) in 4 separate trials for a total of twelve replicates (Table 7).

Table 7. PCR Analysis of Disc-bound Ames DNA vs Heat-Eluted Ames DNA using the capB Taqman Assay

	untreated disc[f]	Eluate #1[g]	Eluate #2	treated disc
Average Ct value	33.33 ± 0.70	35.93 ± 1.55	36.19 ± 0.74	35.66 ± 0.96
No. of PCR positive replicates	12	11	5	12
% positive	100%	92%	42%	100%

The untreated disc generated the lowest average Ct value and the lowest standard deviation of the four sample types tested giving an average Ct value of 33.33 ± 0.70 cycles (n = 12). Even after two heat treatments, PCR of the treated disk resulted in Ct values of only 2.33 additional cycles indicating that DNA template is still available for subsequent rounds of PCR even after heat elution is performed. Another interesting observation was that a Ct value was observed for every replicate when PCR was performed directly from the disc (untreated or treated), whereas only 11 of 12 replicates were positive for the first eluate, and only 5 of 12 replicates were positive for the second eluate. Thus direct PCR from the disc generated greater reproducibility than PCR of heat-eluted DNA.

PCR of Heat-Eluted Ames DNA versus Ames DNA in Solution

The first heat-elution treatment (eluate #1) resulted in an average Ct value of 35.93 ± 1.55 cycles using the *cap*B assay for a disc that was loaded with 93 pg of total DNA (Ames). This is equivalent to a maximum of 46.5 pg of total DNA available for amplification in the eluate

[f] Contained 93 pg of Ames DNA per disc per PCR reaction
[g] Five of 10 µL was used in the PCR reaction for both eluate #1 and eluate #2

assuming 100% elution from the disc and the fact that half of the eluate (5 of 10 µL) was used in the PCR reaction. In terms of copy number, 46.5 pg of total Ames DNA is approximately equivalent to about 7500 copies of the *cap*B target sequence. From the standard curve, a Ct value of 35.93 is equivalent to about 85 copies of target DNA which represents 1.1% of the available target DNA that was amplified (85 copies/7500 copies). Alternatively, a Ct value of 29.23 was calculated from the *cap*B standard curve for 46.5 pg of total DNA (Ames). This represents a difference of about 6.7 cycles or about a 100-fold difference between Ames DNA in solution versus heat-eluted Ames DNA.

A second experiment comparing Ct values between DNA in solution and heat-eluted DNA from FTA® cards using *B. anthracis* strain ACB DNA revealed a similar trend (Table 8). A difference of 6.64 cycles (100 fold) was observed for the *lef* target and 5.14 cycles (35 fold) was observed for the *cap*B target from 7.4 pg of total DNA per PCR reaction.

Table 8. PCR of Heat-Eluted DNA versus DNA in Solution Using B. anthracis (ACB strain) DNA

Total DNA conc. (pg/µL)	Volume spotted onto FTA® card (µL)	Total DNA on FTA® card (pg)	Total DNA on disc[h] (pg)	Total DNA per PCR[i] (pg)	CT values[j]					
					lef			*cap*B		
					unbound	eluted	Δ[k]	unbound	eluted	Δ
460	10	4600	184	7.4	31.32	37.96	6.64	31.57	36.7	5.13
46	10	460	18.4	0.74	34.06	0	NA	34.96	40.11	5.15
4.6	10	46	1.84	0.074	37.84	0	NA	38.82	0	NA

NA = not applicable

[h] based on a disk to spot ratio of 1:25
[i] based on using 2 µL of 50 µL of eluate per PCR reaction
[j] based on average of two trials
[k] Δ = Ct (eluted) – Ct (unbound)

Conclusion

FTA® Whatman solid phase media was evaluated as a DNA capture and recovery medium for PCR analysis. This study revealed a decrease in the PCR signal (increase in Ct values) for DNA recovered from FTA® cards either by direct PCR or after heat elution. Direct PCR of FTA® bound DNA provided better sensitivity and greater reproducibility than PCR of heat-eluted DNA. Observable PCR signals were observed from FTA® discs that had undergone multiple heat elution treatments indicating that residual template was still available for subsequent rounds of PCR. Although there is a loss in PCR sensitivity for DNA captured on FTA® Whatman solid phase media, it still serves as a potentially useful media for the storage, archiving and transport of genetic material for downstream PCR analysis.

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Annex A – Figures

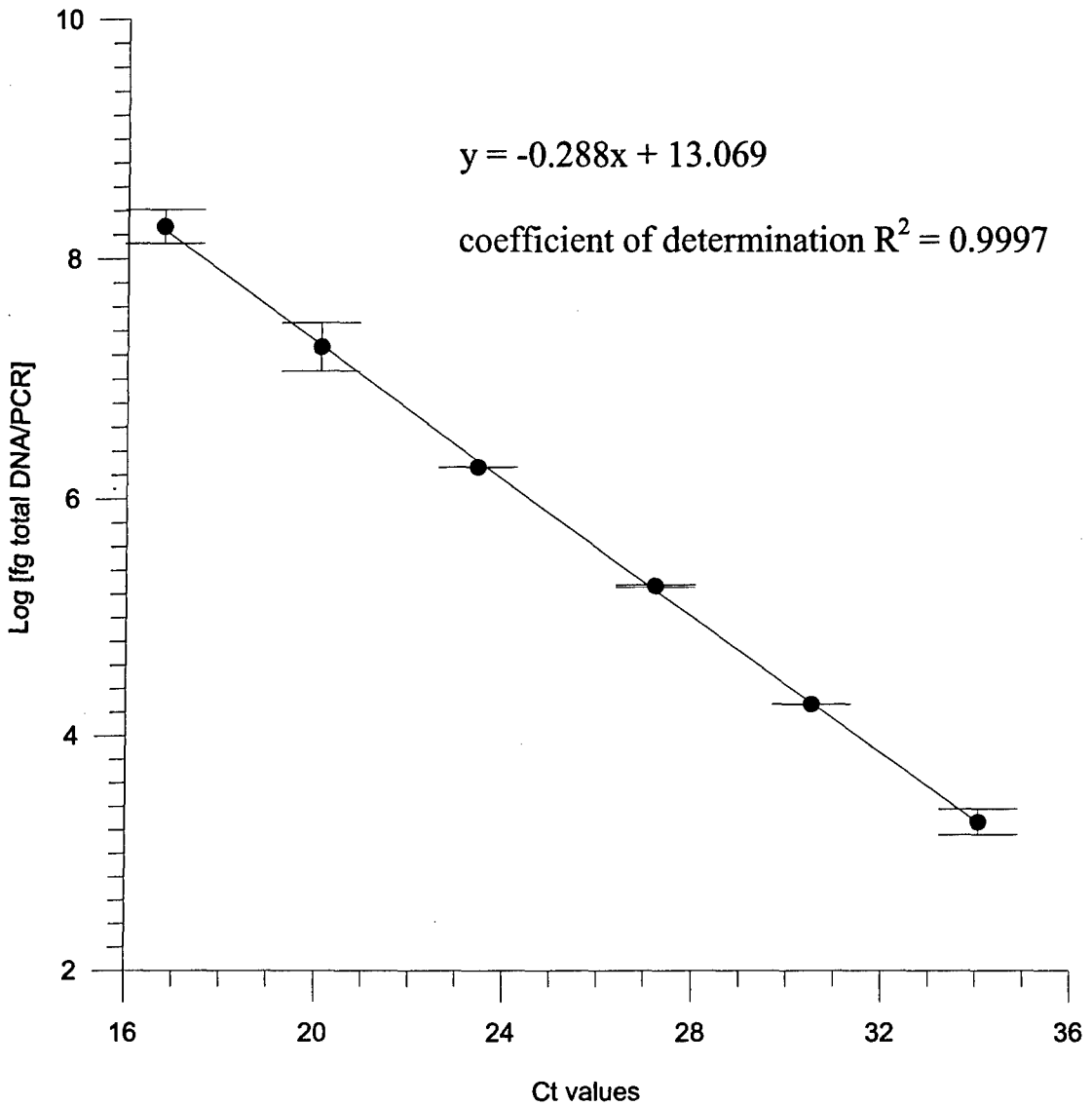

Figure 1. Standard Curve for the *cap*B Assay using *B. anthracis* Ames DNA in solution

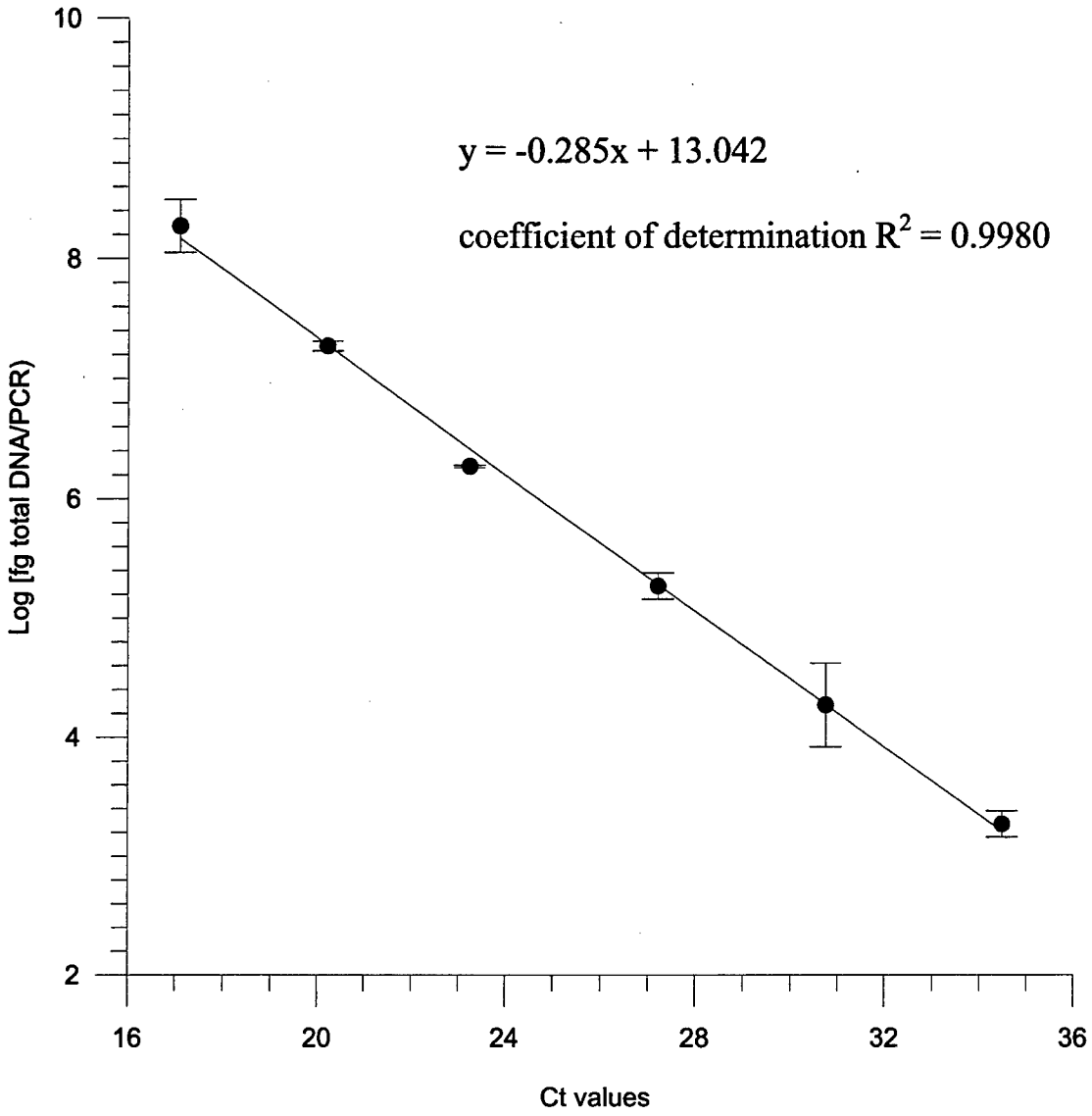

Figure 2. Standard Curve for the *lef* Assay using *B. anthracis* Ames DNA in solution

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A commercially available solid-phase DNA binding matrix (FTA® cards) was evaluated for its ability to capture and release DNA for downstream gene amplification and detection assays using polymerase chain reaction (PCR) analysis, as part of a project to determine the utility of FTA® cards for sampling, archiving, and transport of samples that may contain biowarfare (BW) or bioterrorist (BT) agents. PCR was performed using assays designed to amplify and detect two different *Bacillus anthracis* virulence genes, namely the lethal toxin gene (*lef*) found on plasmid pXO1, and the capsule B gene (*cap*B), located on plasmid pXO2. PCR assays were conducted using real-time, Taqman fluorescent probe detection on a Cepheid Smart Cycler® instrument. A baseline detection limit of about 300 gene copies was observed for each assay prior to DNA-FTA® card binding studies using *B. anthracis* Ames DNA. Direct PCR of DNA bound to FTA® discs resulted in a loss of sensitivity compared to DNA in solution, however, this method proved better than the heat-elution method since PCR signals were observed at earlier cycles. Furthermore, FTA® bound DNA generated a reproducible PCR signal for 12 of 12 replicate tests (100%), compared to 11 of 12 (92%) for a single heat elution treatment, and 5 of 12 (42%) replicates for a double heat elution treatment. Even after the FTA® bound DNA discs were heat-treated twice, the DNA was still detected directly from the discs in every replicate (12/12) and only 2.5 cycles later than untreated discs. This indicated that even after heat elution, considerable DNA was retained on the FTA® discs for subsequent PCR analysis. This study demonstrated that although there is a loss in PCR sensitivity for DNA captured on FTA® Whatman solid phase media, it still serves as a potentially useful media for the storage, archiving and transport of genetic material for downstream PCR analysis.

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Bacillus anthracis DNA, genetic analysis, polymerase chain reaction, PCR, solid-phase DNA binding media