ABSTRACT

Title of Document:	RESIDUAL DNA IN COMMERCIAL *TAQ* DNA POLYMERASE AS A SOURCE OF INTERFERENCE WITH IMMUNO-PCR ASSAY
	Jake Juyoung Guag, MPH, 2012
Directed By:	Professor and Director, Dr. Donald K. Milton, Maryland Institute for Applied Environmental Health

Polymerase Chain Reaction (PCR) was developed for a broad range of purposes. As part of developing a very sensitive Immuno-quantitative PCR (iqPCR) assay, we attempted to reproduce two of the published papers, almost always experienced false-positive amplification. Based on personal communication from one of the authors, we suspected that impure reagents were responsible for the false-positive amplification. However, PCR can amplify a small number of DNA into enormous numbers of copies and the possibility of environmental contamination cannot be excluded. In this paper we show that our primers appeared to amplify residual DNA in the *Taq* DNA polymerase, and induced false-positive results. This finding is not in the published methods papers for iqPCR.

RESIDUAL DNA IN COMMERCIAL *TAQ* DNA POLYMERASE AS A SOURCE OF INTERFERENCE WITH IMMUNO-PCR ASSAY

By

Jake Juyoung Guag

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Advisory Committee:
Professor Donald K. Milton, Chair
Professor Michael L. Grantham
Professor Amy R. Sapkota

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Chapter 1: Introduction

Immuno-PCR (IPCR) uses antibody-antigen interaction principle used in the Enzyme-linked immuno sorbent assay (ELISA), and obtains enormous signals through the PCR amplification. Instead of the enzyme, DNA is labeled to detection antibody, and then the labeled DNA functions as the marker. The labeled DNA is amplified through PCR, and IPCR's limit of detection is theoretically 100 to 10,000 fold better than the ELISA[1], and allows for detecting even an extremely small amount of the target agent. However, IPCR is primary used for research purposes, because there is no commercial kit available for common immuno assay.

In IPCR, amplicon can be measured through different techniques, such as gel electrophoresis. These readout techniques require additional procedures or experimental equipment. Among those readout techniques, Real-time PCR can directly measure quantification of amplicon. Immuno-quantitative PCR (iqPCR) is combining of IPCR and Real-Time PCR, and there are previous studies that used this[2,3]. Because there is no commercial kit for iqPCR to detect cytokines, we needed to develop our own iqPCR. There are different approaches to assemble the iqPCR detection system. We used the simplest assembling method which is a similar method used in Barletta et al, 2009. We decided to follow two published papers[4,5]. One of them used pUC19 plasmid vector, and the other used Lambda phage DNA as labeling DNA which is the template for PCR reaction. A Number of other references also used

them as standard sequences. We detected cytokines as antigen by using cytokines specific antibody with labeled DNA. After PCR, if we observed amplification of the labeled DNA, then it indicates we detected cytokines from the sample. Before immuno assay, we tested the PCR to test if it had a clean background. However, we found false-positive problems. This problem is common in our iqPCR because of the unusually large number of PCR cycles (total 70 cycles split into two rounds: 20 cycle in first round of PCR, and 50 cycles in second round of PCR) compared to other conventional PCR (usually less than 50 cycles in single round of PCR). We only observed the False-positive in the second round of PCR. The literature on iqPCR does not discuss this problem. Because of the false-positive problem, we contacted one of the authors and received a message about frequent contamination of *Taq* DNA polymerase (Barletta J. Personal Communication (2012))[6].

PCR can amplify a single copy of a DNA sequence into millions of copy products[7]. Thus, contamination can be induced significant problem. To prevent environmental contamination, a previous study published multiple strategies to prevent environmental contamination[8]. In addition, contamination can come from the PCR reagent itself. Previous studies reported *Taq* DNA polymerase as a contamination source[9] [10] [11]. Although various decontamination treatments have been proposed[12] [13] [14] [15], they were not successful in decontamination without reducing the assay sensitivity[16] [17]. In addition, small DNA fragments, especially smaller than 200bp, are not easily eliminated[18] [19].

Although contamination is very concerning, it has been reported related to PCR but not to iqPCR. The information about contamination of *Taq* DNA polymerase was not on any published papers about IqPCR, and it is a significant problem to replicate the result. We cannot rule out probability of environmental contamination. Because of that, we set out to experiment and to test where the contamination comes from, and to identify which small DNA fragments would be amplified through PCR. If contamination comes from *Taq* DNA polymerase, it is recommended that researchers should include all required and necessary information onto their papers. In addition, it is necessary to address that the PCR agent can possibly induce false positive problems at a very high sensitivity iqPCR assay.

Chapter 2: Materials and Method

We purchased pUC19 (Fermantas, #SD0061, Waltham,MA) and Lambda phage DNA (Takara, Code 3010, Mountain View, CA) for our template. After we observed the false positive problem, we needed new primers for our investigation. Because of that, we designed new primers for the pUC19 and the Lambda phage that do not overlapped to previously made PCR products or previously used primers (Appendix A). Both of the new primers were designed to bind far downstream of the template DNAs, and avoid overlapping of the previously made PCR products. To prevent contamination from the previously used primers or previously made PCR products, any opened PCR materials were discarded. The entire experiment process was performed in 3 physically separated labs to prevent any cross contamination. PCR master mixes were made and commercial PCR-grade water (Ambion, #9935G, Grand Island, NY) was added instead of the DNA template in Lab-1. The DNA template was added in Lab-2, and PCR was performed in Lab-3. 1% agarose gels were made (Mo Bio, #15003-50, Carlsbad, CA) and run, and DNA was extracted through a commercial extraction kit (Promega, #328269, Madison, WI) in different work space in Lab-3. Each lab has its own PCR-grade water, lab coats, and pipets, thus no lab coats or pipets were transferred between the labs. Lab-3 contained one set of lab coats and pipets for the PCR, and another set for the agarose gel and DNA extraction but were stored in different places. All working spaces and pipets were wiped down with 10% (v/v) bleach before and after each experiment.

Master mixes: We purchased commercial PCR reagents (Invitrogen. Cat#12339-024, Grand Island, NY). Master mixes followed the methods of Barletta, et al (2009) with minor modification. Master mix A contained 10x AccuPrime PCR Buffer-1 with 2.0mM MgCl2 (final 3.5mM MgCl2) and 0.4 M of the forward and reverse primers. Master mix B contained 19 µL of the Master mix A with 1µL of AccuPrime *Taq* polymerase. We made sufficient amounts of the Master mixes for a second round of PCR and preloaded the PCR tubes for the second round and closed the caps in Lab-1 at the start of the experiment. We then carried closed the tubes for the second round of PCR until we reached Lab-3 and stored them at -20C in Lab-3 until the end of the first round of the PCR. All the Master mixes were prepared in Lab-1 prior to the start of the experiment to prevent any aerosol contamination during the sample loading, especially before the second round of PCR at the Lab-3, and to prevent carrying back of the product to Lab-1.

We performed all PCR in the MyCycler Thermal cycler (Bio-rad, Hercules, CA). Our PCR amplification cycles were 1 x (95C for 30 s) for denaturing; 20 x for the first round and 50 x for the second round (95C for 30 s; 55C for 1 min, and 68C for 30 s), then 75C for 4 mins and maintains the reaction at 4C after the PCR cycling ends. 5µL of PCR-grade water or DNA template (total 5fg) were added to the sample for the first round of PCR. After the first round, we transferred 5µL of the first round PCR product to new PCR tubes, and ran second round of PCR. For example, 5µL of the Negative control-1 PCR products was transferred to a new Negative control-1 tube. After the second round of PCR, we ran the second round of PCR products on 1%

agarose gels and observed the presence and locations of the bands on the agarose gel with DNA ladder (Bio-rad, #170-8202, Hercules, CA).

Samples: Three different negative controls (Negative control-1, 2 and 3) and the No-DNA Polymerase-control (NPC) were designed to detect contamination arising from several different sources. Negative controls contained all PCR materials but no DNA template. Instead of the template, the same amount of the PCR-grade water was added. Negative control-1, 2 and 3 have the same materials, but have different names because materials are added in different labs. We made each control in duplicate in Lab-1. We carried Negative controls-2 and 3 throughout the experiment in duplicate. For Negative control-1 and the Positive control, after the first round of PCR, we split each duplicate into four samples for the second round of PCR to give two sets of quadruplicates for a total of 8 replicates of Negative Control-1 and the Positive control. This provided additional material for DNA extraction and sequencing. We made all of the replicates on the same day. The experimental procedure is diagrammed in Figure i.

Positive control: contains all PCR required materials. 20μL of the Master mix B was loaded in Lab-1, and the cap was closed. It was opened in Lab-2 where the template was added and cap was reclosed before it was transferred to Lab-3. There was no additional action performed until the second round of PCR.

Negative control-1: This sample is same as our negative control for routine PCR. 20μL of the Master mix B was loaded and the 5μL of PCR-grade water was added as a negative template control in Lab-1. The cap was closed and no more action was performed until the second round of PCR.

Negative control-2: This sample was designed to test if environmental contamination occurs during the sample transfer from Lab-1 to Lab-2 and during the template adding. After 20μL of the Master mix B was loaded in Lab-1, we kept its cap open until the 5μL of PCR-grade water was added as a template in Lab-2. In Lab-2, the Negative control-2 was handled last and its cap closed after the Positive control was loaded with template.

Negative control-3: This sample was designed to test if aerosol contamination occurred during the transfer between the first and second rounds of PCR products. 20μL of the Master mix B were loaded and the 5μL of PCR-grade water that was added as a template in Lab-1. Then the cap was kept closed. We did not open it after the first round of PCR, and ran second round of PCR without transferring any PCR products.

NPC: This sample was designed to confirm the primers do not produce any major PCR products without the *Taq* DNA polymerase. 19μL of the Master mix A, and 1μL of PCR-grade water was added instead of the DNA polymerase in Lab-1. Then, 5μL of the DNA template added in the Lab-2.

Work-flow and PCR program: This experiment started in Lab-1 and moved to Lab-2 and then into Lab-3 without going back into earlier labs on the same day to prevent any cross contamination. The detailed steps are shown in Figure i.

In Lab-1, we performed following steps:

1. Made Master mix A and B;

2. Added Master mixes to the PCR tubes for the first and second rounds of PCR, and closed all caps except for Negative control-2 first round PCR tubes;

3. Added PCR-grade water as a template in the following order: Negative control-1 and Negative control-3, then closed the caps;

4. Added additional PCR-grade water to the NPC instead of the DNA polymerase, and closed the cap.

In Lab-2, we performed following steps:

1. Added a template in the following order: Positive control and NPC, and then closed the caps;

2. Added the PCR-grade water as a template to the Negative control 2, and then closed the cap.

In Lab-3, we performed following steps:

1. Ran first round of the PCR (20 cycles);

2. Transfer 5 l of the first round of the PCR product to the new tubes. The transferring order was as following: Negative control-1, Positive control, NPC, Negative control-2;

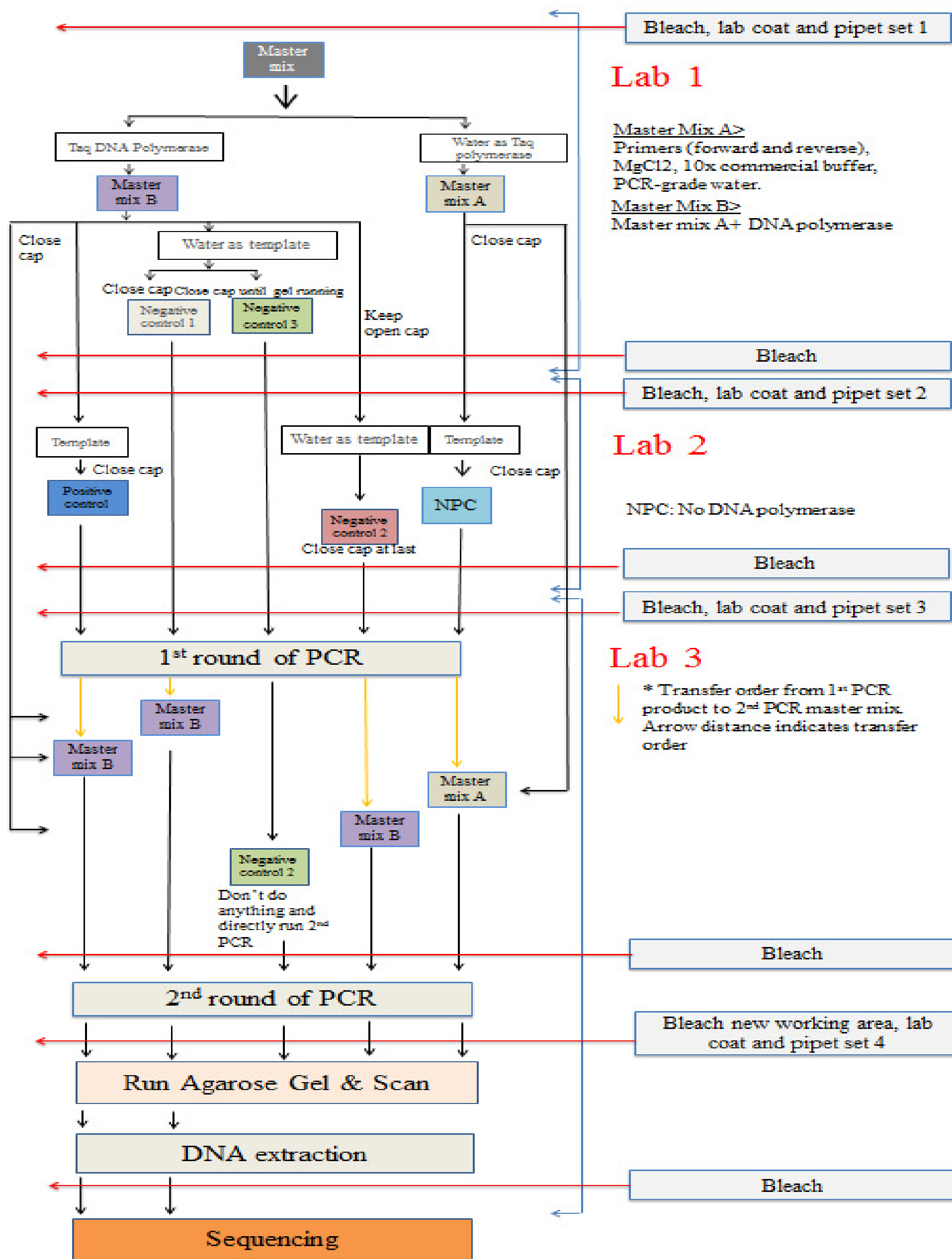
3. Ran second round of the PCR (50 cycles);

4. Took 20 l of the second round of PCR products and ran on agarose gel;

5. Gel purified DNAs (Positive control and Negative control-1);

6. Sent the purified DNAs for sequencing.

Figure i. Work-flow procedure chart



After we checked the bands of the second round PCR products on agarose gels, we gel purified DNA from the Positive control and Negative control-1s. We sequenced these DNAs to identify which DNA sequences were amplified in the bands. We sent DNAs to the Institute for Bioscience & Biotechnology Research (IBBR) at University of Maryland. The IBBR used an Applied Biosystems DNA sequencer (model 3730) and BigDye Terminator v3.1 Cycle Sequencing Kit for DNA sequencing.

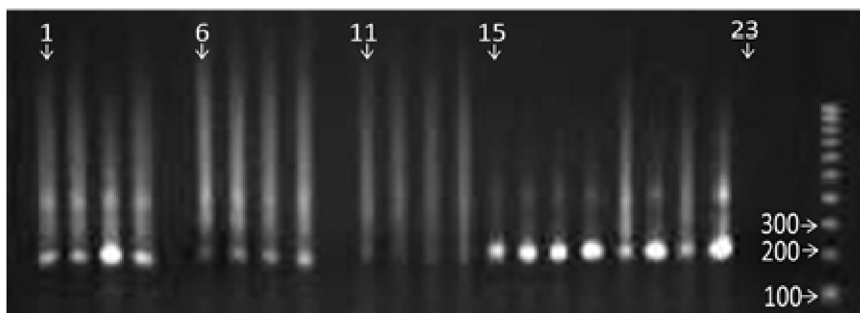
Chapter 3: Result

We performed the pUC19 experiment once. We repeated the full experiment twice with Lambda, and repeated four more times for the Positive control and Negative control-1.

pUC19

The second round of the PCR product on agarose gel (Figure ii) was smeared from one set of quadruplicates the pUC19 Negative control-1 (set A) and the NPC showed no product. However, we observed bands from all replicates of the other controls: pUC19 Positive control, Negative control-1 (set B), Negative control-2, and Negative control-3. All of the bands were located at very similar locations (Figure ii). We observed that all bands were located slightly lower than 200 base pair band of the ladder as we expected for the 179 base pairs pUC19 product.

Figure ii. 1% Agarose gel with second round PCR product from the pUC19 Positive control, Negative control (1, 2, and 3), and No DNA-Polymerase (NPC) control

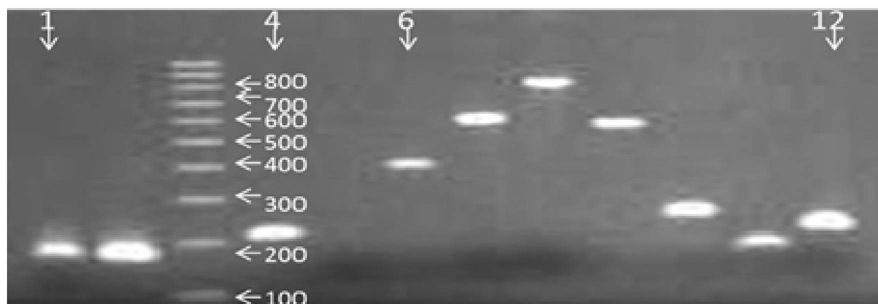


Description (Left (1) to right (24) Well):
Well 1-4: pUC19 Positive control set A
Well 5: blank
Well 6-9: pUC19 Positive control set B
Well 10: blank
Well 11-14: pUC19 Negative control-1 set A

Well 15-18: pUC19 Negative control-1 set B
Well 19-20: pUC19 Negative control-2 A, B
Well 21-22: pUC19 Negative control-3 A, B
Well 23-24: pUC19 NPC control A, B
Well 25: DNA ladder

We gel purified DNAs from the Positive controls and Negative control-1, confirmed purified DNAs had a clean and single band (Figure iii), and then sent them for DNA sequencing. After we obtained the DNA sequences, we aligned the pUC19 plasmid vector DNA (Genbank Accession number #L09137.2), the Positive control extracted DNA and the Negative control-1 extracted DNA using the ClustalW, and found that over 130 base pairs are quite similar, and base pairs near 3' are not really close (Figure iv). However, when we did BLAST the Positive control and the Negative control-1, we found they are more closely matched to varieties of cloning vectors (Table i)

Figure iii. Extracted DNAs from the pUC19 and Lambda Positive control and Negative control-1 on 1% Agarose gel run



Description (Well left (1) to right (12)):
Well 1: DNA extracted from the pUC19 Positive control
Well 2: DNA extracted from the pUC19 Negative control-1 Set B (well 6-9 on the Figure ii)
Well 3: Ladder
Well 4: DNA extracted from the Lambda Positive control
Well 5: Blank
Well 6-11: DNAs extracted from the Lambda Negative control-1.
Well 12: DNA extracted from the Lambda Positive control 2

Figure iv. ClustalW DNA alignment of the pUC19 plasmid vector DNA (Ref), extracted DNA from the pUC19 Positive control (Positive-pUC19), and extracted DNA from the pUC19 Negative control-1 (Negative-pUC19)

```
Ref               ATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGC
Positive-pUC19    -------------------------------------TGANTGGTA-CTCTTGATCCGGC
Negative-pUC19    ------------------------------------GAANNTGGTAGCTCTTGATCCGGC
                                                           ***** *************

Ref               AAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGA
Positive-pUC19    AA-CAA-CCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGA
Negative-pUC19    AA-CAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGA
                  ** *** *****************************************************

Ref               AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC
Positive-pUC19    AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC
Negative-pUC19    AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC
                  ************************************************************

Ref               GAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATC
Positive-pUC19    GAGGNCTGCCGCCCNNGTGNAGC--N-ANGTCTNCNNCCCCCTGAANCNNACNCTGANTG
Negative-pUC19    GACGGNGNCTGNCCCTCNGTGTC--CCNTCGNCTTTTCCACNCCNATCTGACGCTCANTG
                  **       *                              *       * *    **   *
```

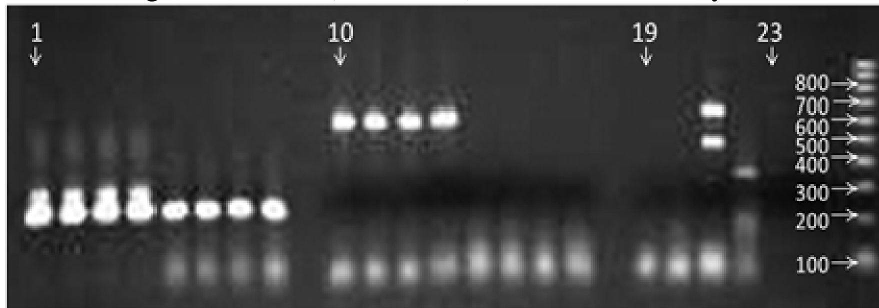
Table i. pUC19 BLAST result from the pUC19 Positive control and Negative control-1 extracted DNA (Some matched BLAST results)

pUC19	Description	Accession number	Max ident (%)
Positive control	Cloning vector pNHG, complete sequence	JQ585717.1	98
	Plasmid vector pFKm4, complete sequence	JX504717.1	98
	Plasmid vector pEXGm5B, complete sequence	JX504716.1	98
	Allelic replacement vector pAMG270, complete sequence	JX477172.1	98
	Gateway recycling vector pCON-R5R6 DNA, complete sequence	AB752387.1	98
Negative control-1	Cloning vector pME-lama4-NS, complete sequence	JX217820.1	99
	Cloning vector p5E-twhh, complete sequence	JX261972.1	99
	Cloning vector pME-ILK-NS, complete sequence	JX261971.1	99
	Plasmid vector pFKm4, complete sequence	JX504717.1	99
	Gateway recycling vector pCON-R5R6 DNA, complete sequence	AB752387.1	99

Lambda

After we ran the second round of PCR products on the agarose gel, we observed no bands from the NPC (Figure v). However, compared to the pUC19, Lambda controls showed strange results. The Positive controls showed strong bands and their locations seemed correct (Figure v). The Positive control bands were located slightly above the 200 base pair band of the ladder as we expected for the 218 base pairs for Lambda product. However, Negative controls showed inconsistent results. The Lambda Negative control-1 set A showed bands at about 600 or 700 base pairs, while the Negative control-1 set B did not show any bands. Neither set of Negative control-2 showed bands. One of the Negative control-3 set showed two bands, while the other Negative control-3 had very weak bands at another location. No lambda Negative controls produced bands that migrated the same distance as the Positive control. Because of the inconsistent results, we repeated the Lambda experiment (data not show), and observed consistent results for the Positive control but inconsistent result for the Negative controls once again. Because of the inconsistent results, we repeated the Lambda experiment several times for the Positive control and Negative control-1. We continuously observed consistent results from the Positive control, but inconsistent results from the Negative control-1 (data not shown).

Figure v. 1% Agarose gel with second round PCR product from the Lambda Positive control, Negative control (1, 2, and 3), and No DNA-Polymerase Control (NPC)



Description (Left (1) to right (24) Well):
Well 1-4: Lambda Positive control set A
Well 5-8: Lambda Positive control set B
Well 9: Blank
Well 10-13: Lambda Negative control-1 set A
Well 14-17: Lambda Negative control-1 set B
Well 18: Blank
Well 19-20: Lambda Negative control-2 A, B
Well 21-22: Lambda Negative control-3 A, B
Well 23-24: Lambda NPC control A, B
Well 25: DNA Ladder

Because we performed multiple experiments for the Positive control and the Negative control-1, we gel purified 2 DNAs from the Positive control and multiple DNAs from the Negative controls. After we confirmed all the purified DNA had a clean and single band (Figure iii), we sent all of them (2 Positive control DNAs, 6 Negative control-1 DNAs) for DNA sequencing. Through the BLAST, we found that both of the extracted Positive control DNAs were matched (99% for one and 98% for another) to the Enterobacteria phage lambda, a complete genome (GenBank accession number: J02459.1) (Data not shown). We found that the extracted DNAs from the Negative control-1 had a very different result. We tested all of the Negative control sequences, and found many of them matched the broad ranges of BAC (Bacterial artificial chromosome) clones; One of the Negative control sequences matched the Propionibacterium acnes genome (Table ii). However, none of the Negative control-1

DNAs were matched to the Lambda phage DNA. We aligned the Negative control-1 DNAs, Positive control, and the Lambda phage DNA by the ClustalW. Some extracted Lambda Negative control-1 DNAs showed some similarity to the Lambda phage DNA and the Lambda Positive control (Figure vi).

Table ii. Lambda BLAST result from the Lambda Positive control and multiple Negative control-1 extracted DNAs (Some matched BLAST results)

Lambda	Description	Accession number	Max ident (%)
Negative control-1 (well 6 on figure iii)	Homo sapiens BAC clone RP11-327N17 from 2, complete sequence	AC007041.3	97
	Pan troglodytes BAC clone CH251-29B11 from chromosome 7, complete sequence	AC198714.4	91
Negative control-1 (well 7 on figure iii)	Homo sapiens 3 BAC RP11-234A1 (Roswell Park Cancer Institute Human BAC Library) complete sequence	AC073861.29	100
	Callithrix jacchus BAC clone CH259-69H12 from chromosome unknown, complete sequence	AC187715.2	98
Negative control-1 (well 8 on figure iii)	Homo sapiens chromosome X sequence from BAC CEPHB197N14 region PHKA1-DXS227 map Xq13, complete sequence	AL135749.3	98
	Homo sapiens BAC clone CTD-2007N20 from chromosome 2, complete sequence	AC145029.2	98
Negative control-1 (well 9 on figure iii)	Propionibacterium acnes C1, complete genome	CP003877.1	99
	Propionibacterium acnes TypeIA2 P.acn33, complete genome	CP003195.1	99
Negative control-1 (well 10 on figure iii)	Human DNA sequence from clone RP3-340G1 on chromosome 6q16, complete sequence	Z84719.1	98

Negative control-1 (well 11 on figure iii)	Pan troglodytes BAC clone CH251-329N17 from chromosome 7, complete sequence	AC211562.4	100
	Human chromosome 14 DNA sequence BAC C-2509G16 of library CalTech-D from chromosome 14 of Homo sapiens (Human), complete sequence	AL355076.5	100

Figure vi. ClustalW Alignment of the Lambda phage DNA (Ref), extracted DNA from the Lambda Positive control (Positive-Lambda), and DNA extracted from the Lambda Negative control (DNA from well 10 from Figure iii) (Negative-Lambda)

```
Ref.                    CACACCATTGATTTTTATCAATAGTCGTAGTCATACGGATAGTCCTGGTATTGTTCCATC
Positive-Lambda.        -----CATGGATTTTN-TCAATAGTCGTAGTCATACGGATAGTCCTGGTATTGTTCCATC
Negative-Lambda.        ATTATATTCTTTTTATACCAAAATTCACAGCCTTTTAAATGTACTTAATAACAAAAACTT
                               *   ***    *** * **  ** * *    **   * *  **        *

Ref.                    ACATCCTGAGGATGCTCTTCGAACTCTTCAAATTCTTCTTCCATATATCACCTTAAATAG
Positive-Lambda.        ACATCCTGAGGATGCTCTTCGAACTCTTCAAATTCTTCTTCCATATATCACCTTAAATAG
Negative-Lambda.        CCATACTACCAAAG-TAGAATAGCCAAGCTAGTGACTCAGTAATATTCCTTGAAGAATAA
                         *** **    * * *     * *    * * *   **    ****  *      ****

Ref.                    TGGATTGCGGTAGTAAAGATTGTGCCT----GTCTTTTAACCACATCAGGCTCGGTGGTT
Positive-Lambda.        TGGATTGCGGTAGTAAAGATTGTGCCT----GTCTTTTAACCACATCAGGCTCGGTGGTT
Negative-Lambda.        TATATGCTATTAGTGTACACTAGTCCTTGAAGAATAGTATAAGCAGTGGGCTCGGTGGTT
                        *  **     ****  * * *   ***    *  *  **    **   ************

Ref.                    CTCGTGTACCCCTACAGCGAGAAATCGGA-TAAACTATTACAACCCCTACAGT-TTGATG
Positive-Lambda.        CTCGTGTACCCAAACCGAGNNGGATGTGN-TGTAAAACNACAGGNNCNNT--C-TTTACT
Negative-Lambda.        CTCGTGTACCCAAACCGNGNNTNCTGNTTCTTCTAGGCNTCGGGTACTGTGATACCTNAT
                        ***********  ** * *     *     *         *     *

Ref.                    AGTATAGAAATGGATCCACTCGTTATTCTCGGACGAGTGTTCAGTAATGAACCTCTGGAG
Positive-Lambda.        ACCGCAN-------TCCACTATTTA-----AGGNGNATATATGNNAAGAAGAATTTGAAG
Negative-Lambda.        ANCATTCNTNANGCTTCAGGNNTANTNCGNGCTCGCTNNCTTGGCTTTGCTACTTTGGGA
                        *             * **    *           *                  * **

Ref.                    AGAACCATGTATATGATCGTTATCTGGGTTGGACTTCTGCTTTTAA-GCCCAGATAACTG
Positive-Lambda.        AGNTNNAANAGCNTCTCNG--ATGNGNANGGNAACANNACNGGNACTNTCCNNATGACTA
Negative-Lambda.        GGATGGTNGGTTTTTGTTAT--TANGNACNTTNANNGGGCTGTGANGTTTGGTATAANNN
                         *           *        *  *             *    *        ** *
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Chapter 4: Discussion

We observed that both the pUC19 and Lambda Positive controls' from the second round of PCR products gave off strong bands in the right location. We designed the different Negative controls to test where the contamination came from. If we observe the bands from the Negative control-1 and 2, then it would indicate that the contamination came from the Lab-3. If we observe the bands from the Negative control-2 only, then would indicate that the contamination came from the Lab-2. If we observe the bands from all three of the Negative controls, then it would indicate either the contamination came from the Lab-1 or from the PCR reagent itself. However, the contamination of the Lab-1 had a low probability. Because A) we designed new primers that were not overlapped to the previously made PCR products or previously used primers to prevent contamination: B) we cleaned the entire working area in the Lab-1 with bleach (10% v/v): C) we have never put neither the previous nor new PCR products back to the Lab-1. Because the probability of contamination from Lab-1 is very low, PCR reagent - especially *Taq* DNA polymerase - was highly suspected as a contamination source.

pUC19

From the agarose gel experiment, we observed smear bands from the Negative control-1 of set A through two rounds of PCR. Over the course of numerous PCR experiments (using different primers), the Negative control-1 set A was the only

negative control that had smeared gels and no bands, so an experimental error cannot be ruled out. The smeared Negative control-1 of set A would be the weakness of the study. Except for the Negative control-1 of Set A, we observed bands from the pUC19 Negative control-1 of set B, 2, and 3, and observed strong bands in the same locations as the pUC19 Positive control. We observed bands from all Negative controls, except Negative control-1 of Set A, and it indicates contamination would have come from Lab-1. In particular, the amplification of both replicates of Negative control-3 strongly supported that the contamination was not from the environment in Labs-2 and 3 but came from Lab-1. We have never put PCR products back into the Lab-1. In addition, we have never stored or used pUC19 complete DNA in the Lab-1. These support contamination of the Lab-1 has low a probability and the contamination would have come from the PCR reagent, especially *Taq* DNA polymerase itself.

We identified which DNA fragment was amplified from the Negative control-1. We found that the pUC19 plasmid vectors completed DNA, the extracted pUC19 Positive control DNA, and the extracted pUC19 Negative control-1 DNA had very close DNA sequences. However, although both the Positive control and Negative control sequences were very close to the pUC19 complete sequence, we found they did not match to the pUC19 sequence through the BLAST. We aligned the Positive control's and the Negative control's sequences to the pUC19 complete sequence, and found that both of the control sequences were aligned to the region that overlaps to the origin of replication on the pUC19. A high portion of the pUC19 complete sequences, including the matching region originated from pBR322. From the BLAST, we found

that the both the Positive control and Negative control-1 were matched to a variety of sequences and cloning vectors, and most of them contained an origin of the replication from pBR322 or pUC (Data not shown). Any sequences or cloning vectors that contained the origin of replication from either the pBR322 or pUC were very close. This would occur because the pUC19 primer binds sequences that other cloning vectors shared rather that binding within a pUC19 specific sequence.

All of these results strongly support that contamination can come from the *Taq* DNA polymerase, the *Taq* DNA polymerase contains pUC related residual DNA, and our primers can bind and amplify without the template.

<u>Lambda</u>

We repeated the full experiment twice, and observed bands from one set of the Negative control-1 and both of Negativecontrol-3 from one experiment (Figure v), and observed bands from all Negative controls, except for one of the Negative control-2 set from the other experiment (Data not shown). Both experiments provided inconsistent results in terms of the presence and the location of the bands from the Lambda Negative controls' second round of PCR products. Not all of the Lambda Negative controls showed bands. This could be possible considering that no bands of Negative controls indicate the detection of environmental contamination. However, we observed this phenomenon from the previous Real-Time PCR test. From our previous PCR test, our Lambda Negative controls were occasionally amplified and occasionally did not amplify after the Real-Time PCR. A possible explanation could

be related to the Limit of Detection (LOD). Real-time PCR's LOD is at least 100 folds more sensitive than that of the agarose gels'. Occasionally, we observed strange shapes of an amplification curve that had have very low amounts of amplification from the previous Real-time PCR. If a very low number of DNA copies were to be amplified, it would not have been detected or visualized on the agarose gel. Thus, it is possible that the Lambda Negative control was amplified but not observed on the agarose gel because the amounts of PCR products were lower than that of the agarose gel's LOD.

A different location of the Lambda Negative control bands can also occur because of non-specific amplification. Compared to the pUC19 plasmid vector DNA, the Lambda phage DNA was much larger, and would have more of a chance of non-specific binding. It is also possible that the new designed Lambda primers were not as specific as the primers used in Barletta et al (2009). If we had used the better designed primers, it may have reduced the detection of non-specific PCR products. However, we cannot guarantee that the probe would have been able to distinguish non-specific PCR products. From our previous Real-Time PCR, we observed amplification even though we used probe. Some of the Lambda Negative control had regions that were similar to the Lambda phage DNA and Lambda Positive control (figure vi). If we designed the primers and the probe was able to bind to certain similar regions, we would have obtained false information.

We tested all the extracted Lambda Negative control-1 DNAs through the BLAST, and found that some control sequences were matched to varieties of BACs, while

another sequence was matched to the bacterial genome. Due to the fact that the Lambda Negative control sequences are similar to very broad ranges of sequences, it is hard to conclude which sequences the Negative control matched.

Compared to the pUC19, the Lambda results did not strongly support the *Taq* DNA polymerase which contained the Lambda phage like DNA. However, this strongly supports that the *Taq* DNA polymerase would contain some residual DNAs that could be amplified by the Lambda primers without the template.

Conclusion

Taq DNA polymerase is an enzyme found in the Thermuc aquaticus bacterium. We contacted a company where we bought the *Taq* DNA polymerase system, but we could not obtain detailed information about how the company manufactured the *Taq* DNA polymerase system. It is likely that the Thermus aquaticus gene is cloned and expresses its protein in the Eschericia coli, and then the protein is extracted and stabilized with animal protein. Because of this, it is possible that the *Taq* DNA polymerase contains some residual DNA and pUC19 and Lambda primers could bind to some of the residual DNA in the *Taq* DNA polymerase or anything in the *Taq* DNA polymerase system, and could amplify without the template. We demonstrated that the commercial *Taq* DNA polymerase system would contain some residual DNA that were very similar to the pUC19 plasmid vector DNA, and DNA that could be amplified by the Lambda primers without the template. We only tested the pUC19 plasmid vector DNA and the Lambda phage DNA, and it was possible that the *Taq*

DNA polymerase system could contain other types of DNA as well. Therefore, it is highly recommended to test DNA polymerase if it contained any residual DNA before using the PCR tests. Although the false positive problem associated with the PCR reagent has been reported related to the PCR, there was no literature on iqPCR that discussed this issue. False positive DNA amplification in the iqPCR was not observed because other conventional PCR was usually not run in two rounds with a total of 70 cycles. Previously, in our Real-Time PCR with 50 cycles (1 round), neither the pUC19 nor the Lambda Negative controls ever detected amplification (Data is not shown). However, if an additional round of PCR was added, we almost always observed amplification of negative control. This implies that amounts of the residual DNA in the *Taq* polymerase would be very minimal and would not induce significant problems if the PCR ran only 50 or less cycles. If a very small amount of DNA amplification is observed because of contamination from manufacturing, it has a high probability that environment contamination is suspect. Thus, it is highly recommended to test their PCR reagent if it contains any residual DNA at the beginning, especially for a very sensitive iqPCR assay, or use of decontamination techniques to remove residual DNA.

Limitation

The Lambda DNA experiment did not demonstrate that the extracted Lambda Negative control DNAs matched that of the Lambda phage DNA. Although it has been proved that the *Taq* DNA polymerase contains some residual DNA that can be amplified through the Lambda primers, it is still required to know exactly which

DNA is in the *Taq* DNA polymerase. Better designed primers or improvement of the PCR condition could reduce the non-specific amplification. However, we cannot guarantee this because we tried to improve our PCR condition without success from the previous PCR experiment. Therefore a better decontamination technique would be the best method to solve this problem.

Although we used commercial PCR-grade water for this experiment, it is not a guarantee that the PCR-grade water was really contamination free. Even commercially purified water that was used for the PCR produced false positive results, and recommended autoclave the commercial PCR-grade water[20]. Thus, it would be preferred to use autoclaved PCR-grade water to eliminate the possibility of contamination from PCR-grade water. Commercial PCR reagents contain PCR materials in addition to the *Taq* DNA polymerase. The *Taq* DNA polymerase is highly suspected as contamination source. However, other PCR reagents, such as provided buffer - AccuPrime PCR Buffer-1, would be a contamination source.

Future Application as public health perspective

Many times, the amount of environmental factors or biomarkers is very minimal, and requires a very sensitive technique to detect. We wanted to develop iqPCR for the detection of cytokines, as a biomarker, from exhaled breath. The amount of cytokines in exhaled breath is very low, and iqPCR would be very useful. Because iqPCR is very sensitive, contamination would fail the proper performance of an assay. Prevention of contamination is definitely required, and we can prevent environmental

contamination through multiple strategies. However, contamination can come from PCR reagents itself, thus it is highly recommended to test PCR at the beginning if it has a clean background.

Appendices

Appendix A: Previously used and new primer sequences

<u>Previous primer sequences:</u>

Previous pUC19 primers (Designed in cooperate research lab, Dr, Ian White's research lab at UMCP)

Forward: 5'-Biot / TCC GAC CCT GCC GCT TAC -3'

Reverse Primer: 5'-GAC CTA CAC CGA ACT GAG ATA CC-3'

Previous Lambda Primers (Same primers used in Barletta, et al (2009) paper.

Forward: 5'-GGA TGA ACC TGT GGC ATT TGT GCT-3'

Reverse: 5'-GCC ATG TAC CCG CGT ATC GTT TCA-3'

<u>New primer sequences:</u>

New primer sequence for pUC19 (expected PCR products: 179 base pairs):

Forward: 5'-CTG CGC TCT GCT GAA GCC AGT TAC C-3'

Reverse: 5'-CGT TCC ACT GAG CGT CAG ACC CCG T -3

New primer sequence for Lambda (expected PCR product: 218 base pairs)

Forward: 5'-AGC TTG CAT CCA TTG CAT CGC TTG A-3'

Reverse: 5'-GGG TAC ACG AGA ACC ACC GAG CC-3'

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