Development of Multiplexed Real-Time Quantitative PCR Assay for Detecting Human Adenoviruses

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Abstract

Adenoviruses (AdV) have been associated with a wide variety of human disease and are increasingly recognized as viral pathogens that can cause significant morbidity and mortality in immunocompromised patients. Early detection of AdV DNA in plasma and sterile fluids has been shown to be useful for identifying patients at risk for invasive AdV disease. Due to the large number of existing Adv types, few real-time quantitative AdV PCR assays published effectively cover all AdV types. We designed a series of AdV PCR primers and probes and empirically multiplexed them into two separate real-time PCR assays to quantitatively detect all 49 species of human AdV (Types 1-49) available from ATCC. We then subsequently multiplexed all the primers and probes into one reaction. The sensitivity of these assays was determined to be less than 10 copies per reaction (500 copies/ml plasma). In a retrospective evaluation we detected all 84 clinical AdV isolates isolated in cell culture from patients undergoing HSCT between 1981 and 1987. Prospective analysis of 50 consecutive clinical samples submitted for adenovirus testing showed greater sensitivity and equal specificity of the AdV PCR than viral culture. This real time PCR assay allows rapid, sensitive and specific quantification of all currently defined adenoviruses into either two or one multiplex assay for clinical samples.

Key Words: Adenovirus, multiplex, real-time, PCR

Introduction

Adenoviruses (AdV) are nonenveloped, double stranded DNA viruses. Fifty-one human AdV have been identified on the basis of their resistance to neutralization antibodies. The different serotypes are classified into 6 species, A-F, based on their ability to agglutinate red blood cells and their DNA homology. The DNA sequence homology within adenovirus species ranges from 50% to almost 100%. The sequence homology between subgroups can be as low as 4% (Horwitz MS 2001; Schilham MW et al 2002; Shenk TE 2001)

The diseases caused by AdVs include respiratory illness, gastroenteritis , hemorrhagic cystitis and meningoencephalitis. In the immunosuppressed patients, disseminated AdV infections are well described and frequently result in death (Erard V et al 2007; Hierholzer JC 1992). Recent surveys suggest that AdV infections are increasing; especially among patients undergoing T depleted and unrelated cord transplantation. Most recognized AdV infections are due to viruses with A, B and C subgroups (Horwitz MS 2001). However, the other serotypes viruses have also been shown to cause diseases and the association of specific serotypes and patient groups or clinical diseases is not clear-cut (Horwitz MS 2001; Hierholzer JC 1992).

The current standard laboratory methods for diagnosis of AdV infection have limitations. Viral culture is the most common approach, cytopathic effect and identification takes several days and several adenoviruses are fastidious and do not replicate in commonly used cell cultures (Horwitz MS 2001). Antigen detection techniques such as direct immunofluorescence, detection of viral antigen by agglutination of antibody-coated latex beads and dot blot hybridization tend to have low levels of sensitivity and are not amenable to high throughput.

Several PCR-based methods have been developed to measurement of the AdV load in respiratory specimens, tissues and body fluids (Schilham MW et al 2002; Echavarria M et al 1998; Cooper RJ et al 1999; Vabret A et al 2004; Leruez-Ville M et al 2004; Lion T et al 2003). Real-time AdV PCR to detect and quantify AdV in peripheral blood has been shown to be useful in the characterization of the clinical risk status of immunosuppressed patients, the determination of appropriate treatment regimens, and the assessment of the therapeutic responsiveness (Schilham MW et al 2002; Vabret A et al 2004; Leruez-Ville M et al 2004; Lion T et al 2003). Number of real-time quantification Adenovirus PCR methods have been published, using degenerate/consensus primers and probes (Lion T et al 2003; Heim A et al 2003; Miura-Ochiai R et al 2007) or multiplexing multiple primers and probes into one or two different reactions (Chmielewicz B et al 2005; Gu Z et al 2003; Ebner K et al 2006; Ebner K et al 2005). The degenerate or consensus primers may amplify different viruses with different efficiency, and multiplexing PCR tends to be less efficient and prone to inhibition. In this study we designed group specific primers and probes first, optimized and multiplexed these primer/probe sets into 2 reactions to detect and quantify all pathogenic human adenoviruses. Lastly, we also evaluated the possibility to multiplex all 5 sets of primers and probes into one reaction (penta-plex) using commercially available PCR master mixture designed for multiplexing PCR.

Materials and Methods

Viral strains and clinical samples

Forty-nine prototype strains of Adenoviruses (Table 1) purchased from the American Type Culture Collection (ATCC, Manassas, VS.) and 84 consecutive clinical isolates of adenovirus recovered from FHCRC Virology Lab (mid 1980s) were tested in the initial evaluation of the assays. Fifty consecutive clinical samples submitted to Virology Laboratory of University of

Washington in 2003 for adenoviruses testing were then tested to compare the sensitivity of PCR assays with culture and dFA. We also tested the primers and probes for cross reactivity from prototype clinical strains of HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, HHV-8, RSV-A, RSV-B, BK, JC, CoxB5, all known enteroviruses (as previously described), the human cell lines (HSB-2, HL, and 293) (Schilham MW et al 2002; Bruno B et al 2004; Zerr DM et al 2000; Limaye AP et al 2001; Pauk J et al 2000; Limaye AP 1999; Marra CM et al 2002; Lai KK et al 2003). Two hundred forty DNA from T cell-replete hematopoietic cell transplantation (HCT) plasma samples (Erard V et al 2007) were re-analyze to test the sensitivity of penta-plexing PCR.

Titration of Adenoviruses

The 10 group D adenovirus stocks from ATCC (serotypes 9, 38, 42, 43, 44, 45, 46, 48 and 49) were cultured on the HL cell line. The culture stocks were harvested and the titers of the cultured stocks were then measured by culturing 200 ul of each 10-fold dilution point. The titer was determined by the last dilution demonstrating CPE. To determine the viral DNA titer in each stock, adenovirus DNA was extracted from 5 ul of each cultured stock, 1/10 of the eluted DNA (0.5 ul equivalent viral stock) were used for each PCR.

DNA extraction

We utilized the DNA extraction methods in our previously published studies of PCR detection of human herpesviruses, BK and JC viruses (Marra CM et al 2002; Wald A et al 2003; Erard V et al 2004). In Brief, DNA was extracted from 5 ul of the stock viral isolates and from 200 ul of clinical samples using either QIAamp 96 DNA blood kits manually or using QIAamp Robot kits with BioRobot 3000. The DNA was then eluted into 100ul AE buffer (Qiagen Inc.). Ten ul of the DNA was then used for each 50 ul PCR reaction.

Primer and probe Design

Sequences from hexon and penton genes of each subgroup were aligned among subgroup members using Sequencher program (Gene Codes Co., MI). Primer Express program from ABI was used to design primers and probes. The accession numbers of sequences used to design the primers and probe are described as following and arranged according to the subgroup, and serotypes: subgroup A: Adv12 (X73487, complete genome), Adv18 (DQ149610, hexon), Adv 31 (DQ149611, hexon); subgroup B: Adv 3 (X76549, hexon; Z29487, penton), Adv7 (X76551, hexon; AD001675, penton), Adv 11 (AY598970, complete genome), Adv 14 (AY803294, complete genome); subgroup C: Adv 5 (AY339865, complete genome), Adv 6 (DQ149613, hexon); subgroup D: Adv 8 (Y17246, hexon; AJ249343, penton), Adv 19 (Y17240, hexon; AF217409, penton), Adv 37 (Y17252, hexon; AF217408, penton); subgroup E: Adv 4 (AY487947, complete genome); subgroup F: Adv 40 (L19443, complete genome), Adv 41 (D13781, hexon; AF105145, penton). Multiple consensus primer and probe sets from each gene of each subgroup were designed and tested against the adenovirus obtained from ATCC. We empirically designed primers and probes and tested these against serial dilution of the prototype viruses. The best performing primer/probe sets (i.e., most sensitive) from each subgroup were then used to test different combination and conditions of multiplexing PCRs. Our initial strategy was to develop a separate PCR assay for each major adenovirus serotype (A-F). However, as described below, our initial studies indicated that primer sets to Adenovirus of subgroups B and E detected all prototype subgroup D viruses as efficiently as Adenovirus group D type-specific primers. From these preliminary experiments, which tested 10 sets of primers and probes, we selected 5 primer/probe sets for further evaluation (Table 1).

PCR Conditions for the 2 Mixture Multiplexing Assay

The PCR conditions were 50°C (2 min), 95°C (2 min), followed by 45 cycles of 95°C (20 sec) and 60°C (1 min). Amplifications were carried out using ABI HT7900 Sequence Detector (Applied Biosystems, Foster City, CA). Each 50 ul PCR mixture contained 5ul 10X buffer II (Applied Biosystems, Foster City, CA), 10 mM $MgCl_2$, 0.275 ug of TaqStart antibody (Clontech, Palo Alto, CA), 2.5 U of AmpliTaq (Applied Biosystems, Foster City, CA), and 0.05 units of UNG, 8% of glycerol and 0.5 ul ROX (Invitrogen, Carlsbad, CA), 200 uM dNTPs, 400 uM dUTP and EXO internal control (Pauk J et al 2000). All the AdV probes are labeled with FAM at 5'end and TAMRA at 3'end. The concentration of each primer for Adeno-AF is 416 nM, and for Adeno-BEC assay is 267nM. The probe concentration for Adeno-AF PCR reaction is 100 nM. The probe concentrations for the 3 different probes in Adeno-BEC reaction are 66 nM for HexonB-P and PentonC-P and 33 uM for HexonE-P**.**

To detect inhibition of the PCR assay all reactions were spiked with internal control EXO; a 130 base pair region of jellyfish DNA. All negative PCR results required the detection of EXO DNA to be considered valid.

PCR Conditions for the Penta-plexing Assay

To evaluate if we could use a single "Penta-plexing assay", we re-analyzed the DNA extracted from all 49 cultured strains and leftover DNA from a previous study (Erard V et al 2007) with QuantiTect Multiplex PCR Master Mix (Qiagen, Inc.) without changing the concentration of primers and probes. The thermo-cycling conditions used for this 45 cycle PCR reaction were as recommended by the manufacturer; they are 52°C for 2 minutes 95°C for 15 minutes and followed by 45 cycles of 94°C for 1 minute and 60°C for 1 minute. The PCR efficiency of this Penta-plexing reaction on the culture strains from ATCC and standard curves were compared to the 2 mixture multiplexing PCR using laboratory made master mix.

TA cloning and plasmid standards

PCR products from strains 1C, 4E, 7B, 11B, 12A and 40F were cloned into plasmid using TOPO TA cloning Kit (Invitrogen, Carlsbad, CA). Plasmid DNA was purified by either Qiagen plasmid Midi Kit or Qiagen plamid Maxi Kit and quantified by OD_{260} reading. The copy number of each plasmid with amplicon was then calculated, linearized by restriction enzymes, and serial dilutions were made to test the PCR efficiency.

Results

Specificity of AdV primer/probe sets

All 5 primer/probe sets were tested individually against DNA extracted from 39 adenovirus species purchased from ATCC individually (Table 2). The adenovirus group A Hexon primer/probe set amplified only AdV subtype A strains (12, 18 and 31). The AdV group B Hexon primer/probe set amplified all 8 group B AdVs and all 21 group D adenoviruses tested. This set also amplified 4E with less efficiency. AdV C Penton primer/probe set amplified all 4 group C strains only. AdV E Hexon primer/probe set amplifies AdV 4 (group E) and most group D strains (AdV species 8,10,13,15,17,19, 22, 23, 24, 25, 26, 28, 32, 33,17, 39) effectively and amplifies AdV 30 (serotype D) less effectively. This set did not amplify any of sub-groups A, B, C or F. AdV F Hexon primer/probe set specifically amplified both 40F and 41F, and weakly amplified adenovirus type 12 (group A).

Multiplexing of the Adenovirus Assays

The initial multiplexing of all 5 sets of primers and probes into one reaction using laboratory made master mix resulted in both inhibition/interference in combination testing and failure of amplification of our EXO DNA internal control (data not shown). We then initiated a series of studies using several PCR conditions and different primer/probe set combinations. In

general, optimal sensitivity was achieved with mixtures of 2 or 3 of the primer/probe sets (data not shown). The 2 combinations that produced the best PCR efficiency without interfering the amplification of internal control were the mixture containing primers for the A, F group (mixture 1) and the mixture containing primers for B, E and C group (mixture 2). These primer probe combinations were then evaluated further.

DNA extracted from all 49 adenoviruses purchased from ATCC was analyzed with these 2 separate mixtures (Table 2). Adenoviruses 50 and 51 were not included because they are currently not available through ATCC. As shown in Table 2, the AdV serotype B, C & E mixture amplified all subgroups of B, C, D and E adenovirus efficiently except strain 9D and amplified 40F weakly. The AF mix amplified seroptypes A, 9D and F. We plotted the sensitivity as measured by CT (Threshold cycle) with the single primers versus the mixed primers on 39 strains that we have data on single primer PCR. For the A-F mixture as compared to A & F primers the R^2 was 0 .96; for the B, C, E subtype mixture R^2 was 0.88 (Figure 1A and 1B). Overall the PCR efficiency of single primer reactions was similar to the primer mixtures ($P > 0.20$).

The same DNAs were then re-analyzed by the penta-plexing PCR. Cycle threshold values of penta-plexing PCR using QuantiTect Multiplex PCR Master Mix are very similar to the two mixture assays and PCR with single primers (Table 2, Figure 1C).

The specificity of the mixtures was retested against human T-cell line HSB-2, and HL cells. In addition, DNA extracted from clinical studies of HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, HHV-8, JC, RSV-A, RSV-B, BK, CoxB5, and over 50 other enterovirus species were all tested against each mixture; no cross-reactivity was noted. We also analyzed

DNA from 293 cell-lines, a permanent line of primary human embryonic kidney transformed by sheared human adenovirus 5 DNA, again no signal was detected.

Quantitative linearity and sensitivity

To assess the sensitivity of our adenovirus PCR mixtures, 10 fold dilution series from linearized known copy number plasmid DNA of the amplicon from adenovirus strains 1C, 4E, 7B, 11B, 12A and 40F were tested. We compared the sensitivity of each of these amplicons in single versus the multiple and penta-plex PCR mixtures; similar PCR efficiency was present in all situations. Representative amplification plots for Ad 12A, Ad4E and 11B are shown in Figure 2. For continued standardization of the assay we subsequently selected the plasmid Ad11B for standardizing the AdV-BEC PCR mix and strain 12A for the Adv-AF PCR mix and penta-plexing PCR reaction. All three assays detected all samples at 10 copies/reaction (minimum sensitivity of 500 copies/ml of plasma); 80% sensitivity is seen at between 3-5 copies/reaction (150-250 copies/reaction) (data not shown).

Comparison of titer in viral culture with copy number by PCR

Although our PCR can detect D group viruses well, none of our 5 primer sets were designed specifically for this serogroup. As such, a comparison between viral culture and PCR on 10 culturable group D agents (Table 3). The viral DNA titers detected by PCR were consistently higher than end point dilution titer in the cells (Table 3).

Evaluation of the Multiplexed PCR assays on clinical isolates and clinical samples

To evaluate the potential clinical utility of the assays, we analyzed culture harvest aliquots from 84 consecutive adenovirus isolates obtained in the University of Washington Clinical Virology Laboratories from clinical samples submitted from patient undergoing hematopoietic stem cell transplantation (HSCT) at the FHCRC from 1981 to 1987. The culture

harvests were thawed, and an aliquot of supernatant fluid sent to the PCR laboratory for extraction and testing. Negative samples consisting of culture supernatant from uninoculated Hep2 cells were also sent and mixed blindly. Adenovirus DNA was detected by PCR in all 84 samples; 75 were detected in the AdV-BEC mixture, 9 in the AF mixture, and none in both. We then evaluated 50 consecutive clinical samples submitted to Virology Laboratory of University of Washington in 2003 for adenovirus culture or direct FA assays for adenovirus using monoclonal antibody testing. DNA was extracted from the sample and run in the multiplex PCR assays. Adenovirus was detected in culture or FA and PCR in 12 of the 50 samples (Table 4). Two additional samples were positive by PCR but negative by culture or FA; in each sample another viral pathogen was detected (CMV in a sigmoid biopsy by shell vial culture and RSV in a nasal wash sample by DFA).

We also re-extracted the DNA from plasma for the penta-plexing assay from 240 T cell-replete Hematopoietic cell transplantation plasma samples which had been analyzed by the two mixture PCR (Hierholzer JC 1992). The penta-plex assay detected all of the positive samples previously detected by the 2 mixture assay (data not shown).

Discussion

This paper describes the development of the multiplexed PCR assays that is both sensitive and specific and is capable of detecting all known human adenovirus species in either a single or two separate reactions. Due to the genetic heterogeneity among different serotypes of adenoviruses, the development of a real time PCR assay to detect and quantify all pathogenic human adenoviruses has been difficult. Moreover, clinical manifestations between adenoviruses overlap and there is no definable prevalence of species, it is difficult to predict what adenovirus species will be detected in clinical samples. In addition, many different adenovirus serotypes are

being developed as vaccines or gene therapy vectors. These issues spurned our desire to develop a broad ranging PCR that would be diverse in its strategy for detecting adenovirus. Real time PCR assays have been developed to detect multiple adenoviruses using degenerate or consensus primers and probes (Lion T et al 2003; Heim A et al 2003; Miura-Ochiai R et al 2007). Multiplexing primers and probes into one reaction or 2 reactions have been published also (Chmielewicz B et al 2005; Gu Z et al 2003; Ebner K et al 2006; Ebner K et al 2005). All these assays have been proved to be useful and successful in detecting AdV in clinical and environmental specimens. In this study we designed primers and probes for each subtype specifically and then optimized the multiplexing condition to make sure the PCR efficiency among the different strains were equivalent to the single primer-probe sets. Our assays were broad ranging in serotype, and were linear over a fairly wide range of detection. In certain clinical situations, identification of AdV type is important. Molecular methods for typing AdV viruses such as PCR-microarray, PCR-sequencing and PCR-fragment length analysis have been published (Miura-Ochiai R et al 2007; Ebner K et al 2006; Blyn LB et al 2007; Lin B et al 2004; Lu B et al 2004). The purpose of this study is to develop a stable, reliable and easy to perform real-time AdV assay for clinical monitoring or to screen for the presence of adenovirus in a specific sample or tissue. Although our multiplex PCR assays are not designed to identify the specific adenovirus serotype, our assays provide the quantification information which is very important in monitoring disease progress and efficacy of any potential treatment.

Viral culture has been the gold standard for adenovirus diagnosis because of its broad ability to detect a large variety of adenovirus species. However, culture may take days to weeks to detect the viral replication depending on the specimen source and the concentration of the virus in the specimen. Moreover, while adenoviruses are felt to be fairly stable, temperature

lability and alterations in pH, and humidity may alter the ability to isolate virus as well as the condition and types of tissue culture cells and media used in isolation. Certain sample types, such as serum and CSF, may contain low level of virus that is easily missed by culture and yet very critical for diagnosis and patient care. The increased sensitivity of PCR over culture is shown by the dilution series experiments we performed with a wide variety of serotypes and perhaps more importantly, by the two culture negative PCR positive clinical specimens we detected in our initial comparison of PCR with adenovirus culture. Interestingly, co-pathogens were identified in the cultures, perhaps masking for the detection of adenovirus in these cases. Importantly, the assay demonstrated the detection of dual Adeno and CMV and Adeno and RSV in clinical samples, underscoring the importance of complete detection of viral pathogens in the immunocompromised host.

Our initial studies in developing the assay illustrated the complexities in multiplexing real time PCR assays. Inhibition was common when too many primer probe sets were put together. Only through a series of systematic "checkerboard" experiments did we optimize the eventual combination of primer sets. The sensitivity of both our mixed sets (mixture A-F) and mixture B, C, E was demonstrated to <10 viral genome copies per reaction. Both assays exhibited a wide dynamic linear range and no evidence of cross-amplify other viruses or human genome tested. Using the 2-mixture AdV assay we have successfully detected AdV in serial of plasma samples collected from HCT patients and identified the patients at risk for invasive AdV diseases (Erard V et al 2007). We have successfully multiplexed these 5 sets of primers and probes into one reaction to detect AdV in respiratory samples qualitatively (Kuypers J et al 2006). Moreover, the penta-plexing procedure uses commercial reagents and can also accurately provide the quantification information in the clinical samples. Our penta-plexing PCR mix proved to be

sensitive and may be the most usefully way for laboratories to translate our work into clinical utility as it uses a commercial master mix, which provides uniformity to the procedure between laboratories.

As adenovirus infections are being identified more frequently in the HSCT setting, the availability of a technology that allows rapid detection of the virus in clinical samples is needed. Our assay and those developed by other groups should prove useful for identifying adenovirus infection in humans in a wide variety of clinical settings (Leruez-Ville M et al 2004; Lion T et al 2003; Heim A et al 2003; Miura-Ochiai R et al 2007; Gu Z et al 2003; Ebner K et al 2005; Myers GD et al 2005; Leen AM et al 2006).

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FIGURE LEGENDS:

Figure 1 Comparison of the threshold cycle number of single primer set with mixture of primer sets using DNA extracted from prototype virus obtained from ATCC

Figure 2. Comparison of amplification plots of real time AdV PCR reactions with single primer set, multiplex or penta-plex primer set. The10-fold dilution series (from 10e1 to 10e5) of 3 representative amplicon plasmid clones from a Group A (AdV12); Group B (AdV11), and Group A (AdV4).were amplified by both PCR reactions with single primer set or mix primer sets.

Table 1. Primers and probes for the multiplexing Adenovirus real-time PCRs

Virus type	Target	Primer and Probe Sequences (forward, probe, reverse)	Primer and Probe Concentration*	Viruses detected
AdV-AF PCR Mixture				
AdV A	Hexon	CCGGKCTGGTGCAATTCG	416nM	Subgroups
		CCACGGACACCTACTTCACCCTGGG	100nM	A and F
		CGATCCACGGGCACAAA	416nM	and 9D
AdV F	Hexon	TGTTYGAAGTTTTCGACG TYGT	416nM	
		CGCATCCACCAGCCSCACC	100nM	
		SAGGTAGACGGCCTCGATGA	416nM	
AdV-BEC PCR Mixture				
AdV B	Hexon	TGGACATGACYTTTGAGGTGGAT	267nM	Subgroups
		CCATGGATGAGCCCACCCTGCTTT	66 nM	B, C, D**
		CGTCGAARACTTCGAARAGAAGA	267nM	and E
AdV E	Hexon	TTAACCACCACCGCAATGC	267nM	
		TACCGCTCCATGCTCCTGGGCA	33 nM	
		TGGATGTGGAATGGCACGTA	267nM	
AdV C	Penton	TCGACACCACCCGTGTGTAC	267nM	
		TGGACAACAAGTCAACGGATGTGGCA	66 nM	
		TGCTGTGGTCGTTCTGGTAGTT	267nM	

- * The concentration of each primer and probe in the PCR reaction mix was optimized separately to minimize the interference of the amplification efficiency of the other set(s) in the same mix.
- ** The AdV-BEC PCR Mixture can't detect 9D.

Table 2. PCR detection as defined by the cycle number for detection of different subgroups of human AdV using 5 different subgroup specific primer/probe sets and 2 multiplexing PCR and penta-plexing all primers. Ct = cycle number in which threshold of positivity was detected.

Subgroup specific p/p sets[a]		A p/p	F p/p	AF mix	B p/p	C p/p	E p/p	BEC Mix	Penta-plex[f]
Subgroup	Serotypes[b]	Ct[c]	Ct	Ct	Ct	Ct	Ct	Ct	Ct
A	12	16.30	30.84	16.77	-	-	-	-	14.92
	18	13.23	-	14.82	-	-	-	-	12.78
	31	22.13	-	22.06	-	-	-	-	21.76
B	3	-[d]	-	-	16.70	-	-	17.78	16.48
	7	-	-	-	15.25	-	-	16.34	13.89
	11	-	-	-	16.57	-	-	18.02	17.53
	14	-	-	-	17.47	-	-	18.41	16.63
	16	-	-	-	18.58	-	-	19.22	17.93
	21	-	-	-	14.90	-	-	16.45	13.96
	34	-	-	-	15.04	-	-	15.83	14.18
	35	-	-	-	17.29	-	-	18.38	16.45
C	1	-	-	-	-	21.78	-	20.99	22.28
	2	-	-	-	-	18.69	-	17.86	17.90
	5	-	-	-	-	18.33	-	18.06	18.25
	6	-	-	-	-	23.44	-	23.38	23.40
D	8	-	-	-	26.22	-	26.14	27.82	28.60
	9	ND[e]	ND	21.4	ND	ND	ND	-	22.4
	10	-	-	-	20.12	-	18.70	20.49	21.08
	13	-	-	-	18.73	-	18.87	19.87	19.71
	15	-	-	-	22.65	-	21.71	22.18	22.35
	17	-	-	-	18.13	-	16.81	17.60	17.30
	19	-	-	-	22.49	-	23.28	23.88	24.29
	20	-	-	-	19.21	-	-	20.02	17.99
	22	-	-	-	19.72	-	19.81	21.33	19.74
	23	-	-	-	19.27	-	19.40	20.84	18.22
	24	-	-	-	17.53	-	17.17	17.45	16.30
	25	-	-	-	20.99	-	19.79	20.62	21.21
	26	-	-	-	18.42	-	18.14	18.65	17.84
	27	-	-	-	20.64	-	-	22.03	20.38
	28	-	-	-	20.16	-	20.59	20.59	20.39
	29	-	-	-	18.67	-	-	20.26	18.62

	30	-	-	-	16.36	-	30.86	18.63	16.94
	32	-	-	-	19.73	-	18.28	19.72	19.00
	33	-	-	-	18.44	-	18.40	19.68	18.46
	36	-	-	-	17.06	-	17.05	17.58	15.92
	37	-	-	-	19.92	-	31.32	21.77	19.90
	38	ND	ND	-	ND	ND	ND	21.5	23.2
	39	-	-	-	20.45	-	23.17	21.98	20.53
	42	ND	ND	-	ND	ND	ND	16.4	15.2
	43	ND	ND	-	ND	ND	ND	22.5	23
	44	ND	ND	-	ND	ND	ND	22.2	23.5
	45	ND	ND	-	ND	ND	ND	33.9	24.1
	46	ND	ND	-	ND	ND	ND	21.7	22.6
	47	ND	ND	-	ND	ND	ND	20.5	21.1
	48	ND	ND	-	ND	ND	ND	21.2	21.8
	49	ND	ND	-	ND	ND	ND	21.6	21
E	4	-	-	-	26.64	-	17.02	17.84	16.85
F	40	-	21.72	23.09	-	-	-	33.98	22.00
	41	-	15.51	15.61	-	-	-	-	14.94

a: primer and probe sets
b: all serotypes of Adenoviruses were purchased from ATCC
c: threshold cycle
d: negative
e: not done
f: Qiagen multiplex PCR kit

Table 3. Comparison of viral titers in HL cell culture and viral DNA titers by PCR reaction Among Group D Adenoviruses

Serotype	*Titer by culture*	*DNA titer by AF-mix*	*DNA titer by BCE-mix*
9	$1x10^5$	$4.24\ x10^{10}$	Neg
38	$1x10^4$	Neg	$4.1x10^8$
42	$1x10^5$	Neg	$6.6x10^9$
43	$1x10^3$	Neg	$1.3x10^9$
44	$1x10^6$	Neg	$3.4x10^9$
45	$1x10^5$	Neg	$4.7x10^7$
46	$1x10^5$	Neg	$7.7x10^8$
47	$1x10^4$	Neg	$8.2x10^8$
48	$1x10^5$	Neg	$7.5x10^8$
49	$1x10^5$	Neg	$9.3x10^8$

Table 4. Detection of Adenoviruses by multiplexing PCR in clinical isolates .
Part A: Tissue culture isolates from HSCT patients 1981-1987
Part B: Consecutive clinical samples to University of Washington Virology Laboratory between January 1, 2003 and March 31, 2003

Part A. Detection of AdV isolates in HSCT 1981-1987

	Number of samples tested	Number samples AF mixture	Number samples positive BEC mixture
Total	84	9	75

Part B. Detection of AdV in consecutive clinical samples

Clinical samples positive either by culture or dFA[a]	Number of samples tested	PCR results	
		Number samples positive by AdV type AF mixture	Number samples positive by AdV types BEC mixture
None	5	0	0
RSV	24	0	1[b]
FluA	2	0	0
FluB	2	0	0
HSV	2	0	0
VZV	1	0	0
CMV	1	0	1[c]
Para 2	1	0	0
Adeno	11	0	11
Adeno+RSV	1	0	1

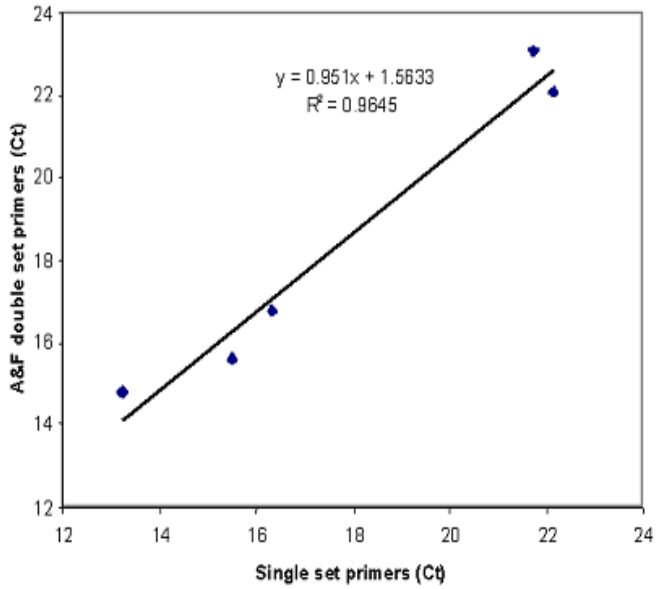
[a] Direct fluorescent antibody assay.

[b] Neither adenoviral culture or dFA was done on this sample.

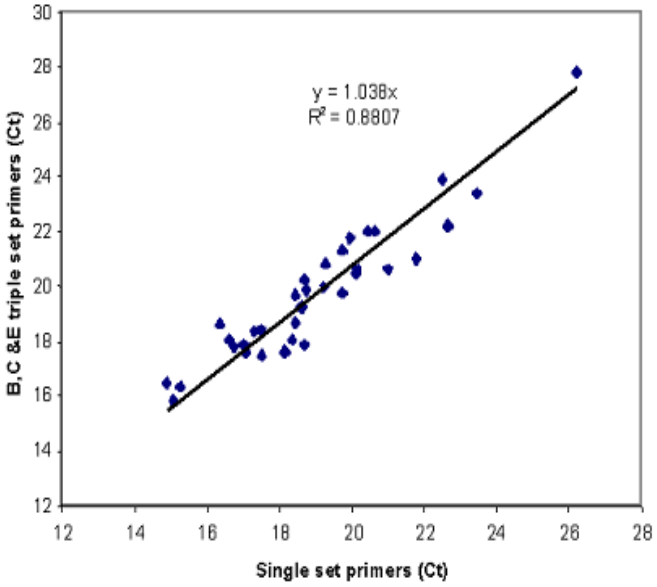
[c] Sigmoid biopsy sample is weakly positive with BEC AdV PCR. The titer is about 1 copy/reaction. Neither adenoviral culture or dFA was done on this sample.

Figure 1.

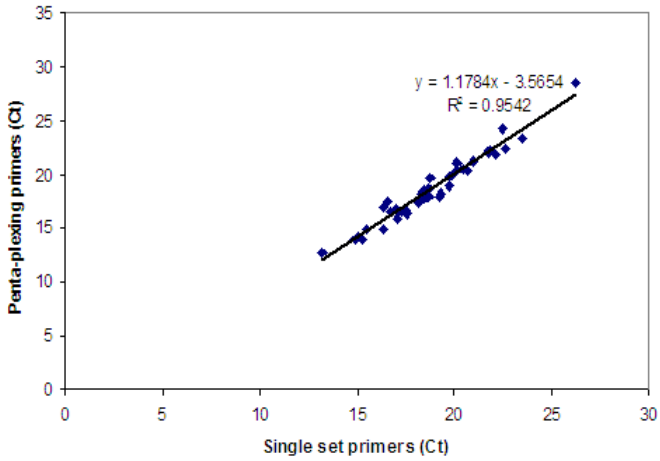
A. Ct for adenovirus types A and F



B. Ct for adenovirus types B, C & E assay.

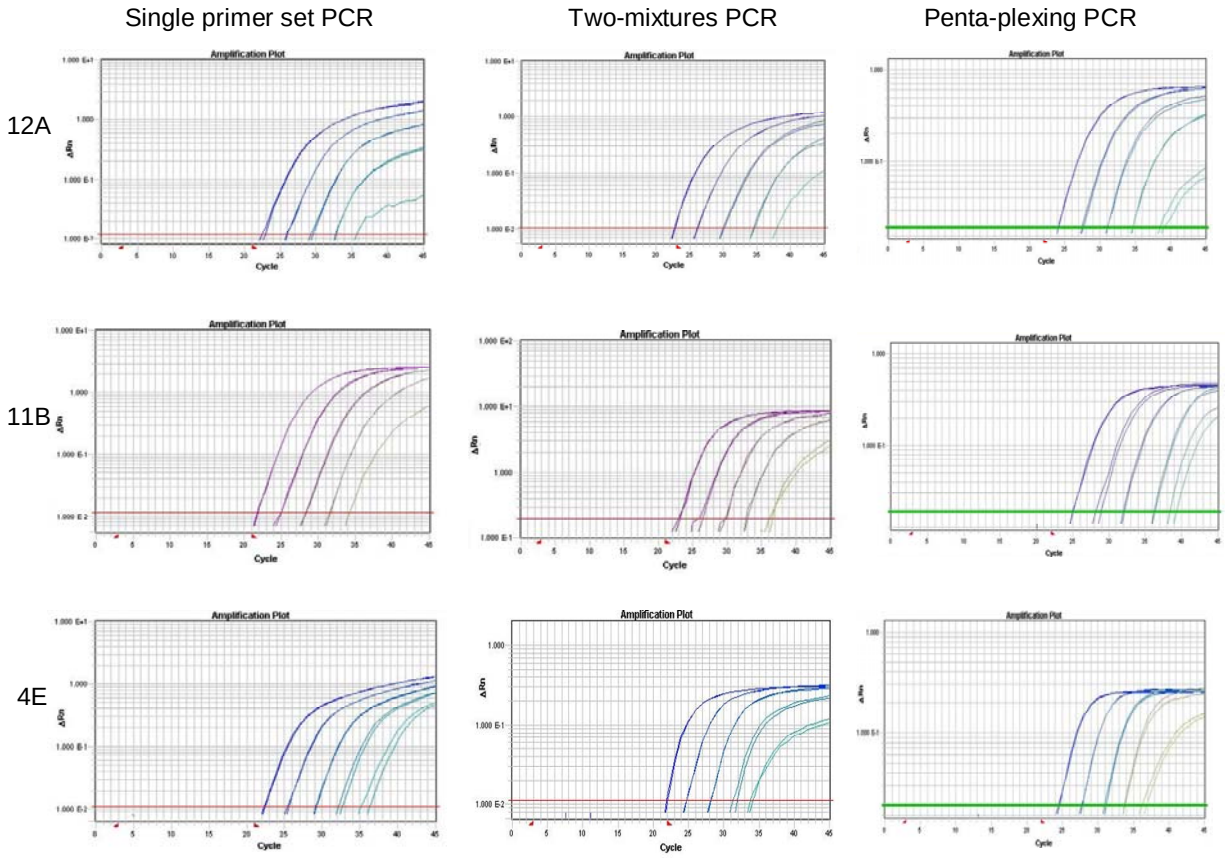


C. Ct for all adenovirus types



set

Figure 2.


Single primer set PCR
Two-mixtures PCR
Penta-plexing PCR
12A
11B
4E
Amplification Plot
Cycle