Materials
DNase digestion buffer [13 mM Tris-Cl, pH7,5 / 5 mM MgCl2 / 0,12 mM CaCl2]
RSS plasmid pTR-UF11
SV40pA Forward primer (10µM) AGC AAT AGC ATC ACA AAT TTC ACA A
SV40pA Reverse Primer (10µM) CCA GAC ATG ATA AGA TAC ATT GAT GAG TT
SV40pA Probe (10µM) 6FAM- AGC ATT TTT TTC ACT GCA TTC TAG TTG TGG TTT GTC -TAMRA
96-well Optical Reaction Plate
Optical Caps or plate sealer sheet
Distilled water DNAse/RNAse free
DNase I
qPCR Mastermix
ABI Prism Sequence Detector or equivalent
37 °C water bath
rAAV control sample (previously titrated rAAV sample)

Procedure:
Digest virus particles to release DNA
Prepare an experimental plan for the position of sample, controls and DNA standards in duplicate in a 96 well format.
Place 5 µL of rAAV preparation and controls (PBS and rAAV control) in the 1.5ml eppendorf tube.
Add 45 µL of DNAse digestion buffer and 10u of DNAse I per tube. Incubate 30 minutes at 37°C.
Keep tubes on ice.

Prepare Samples
In duplicate, prepare a serial dilution of extract sample and controls: Dilutions 1 and 2: 90 µL H2O + 10µL sample (10-fold dilution) Dilutions 3 to 7: 40 µL H2O + 10µL sample (5-fold dilution)

Prepare standard plasmid
Prepare a 10-fold serial dilution of RSS rAAV plasmid (pTR-UF11) in H2O (10 to 10^8 copies/µL).
See appendix A

Prepare PCR
Prepare enough mix for 2 reactions of each DNA sample, a standard curve, two control samples (PBS + rAAV control), plus 2 reactions for no template control (H2O). For a whole 96-well plate, consider preparing the equivalent of 102 reactions in total.

Reagent	Vol. per 50ul PCR reaction	Final Concentration
TaqMan Universal PCR Mix (2X)	25µL	1X
Forward Primer (10µM)	1µL	2µM
Reverse Primer (10µM)	1µL	2µM
Fluorescent Probe (10µM)	0.5µL	1µM
Nuclease-free water	2.5µL	
Sample DNA	20µL	

NB: Lower reaction volumes can be used to accommodate your real-time PCR machine – reagents should be scaled down appropriately.

Load 96 well optical plate (see below) adding DNA last: 9 µl reaction mix per well 9 µl DNA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Plasmid 10^8	Plasmid 10^8		rAAV control d2	rAAV control d2		RSS d2	RSS d2			PBS d2	PBS d2
B	Plasmid 10^7	Plasmid 10^7		rAAV control d3	rAAV control d3		RSS d3	RSS d3			PBS d7	PBS d7
C	Plasmid 10^6	Plasmid 10^6		rAAV control d4	rAAV control d4		RSS d4	RSS d4				
D	Plasmid 10^5	Plasmid 10^5		rAAV control d5	rAAV control d5		RSS d5	RSS d5				
E	Plasmid 10^4	Plasmid 10^4		rAAV control d6	rAAV control d6		RSS d6	RSS d6			H_20	H_20
F	Plasmid 10^3	Plasmid 10^3		rAAV control d7	rAAV control d7		RSS d7	RSS d7			H_20	H_20
G	Plasmid 10^2	Plasmid 10^2										
H	Plasmid 10	Plasmid 10										

Place the optical caps over the plate. Centrifuge briefly before putting on machine.

Cycles:				
	Hold		95	10:00
	Cycle	40	95	00:15
			60	01:00

Analyzing Data

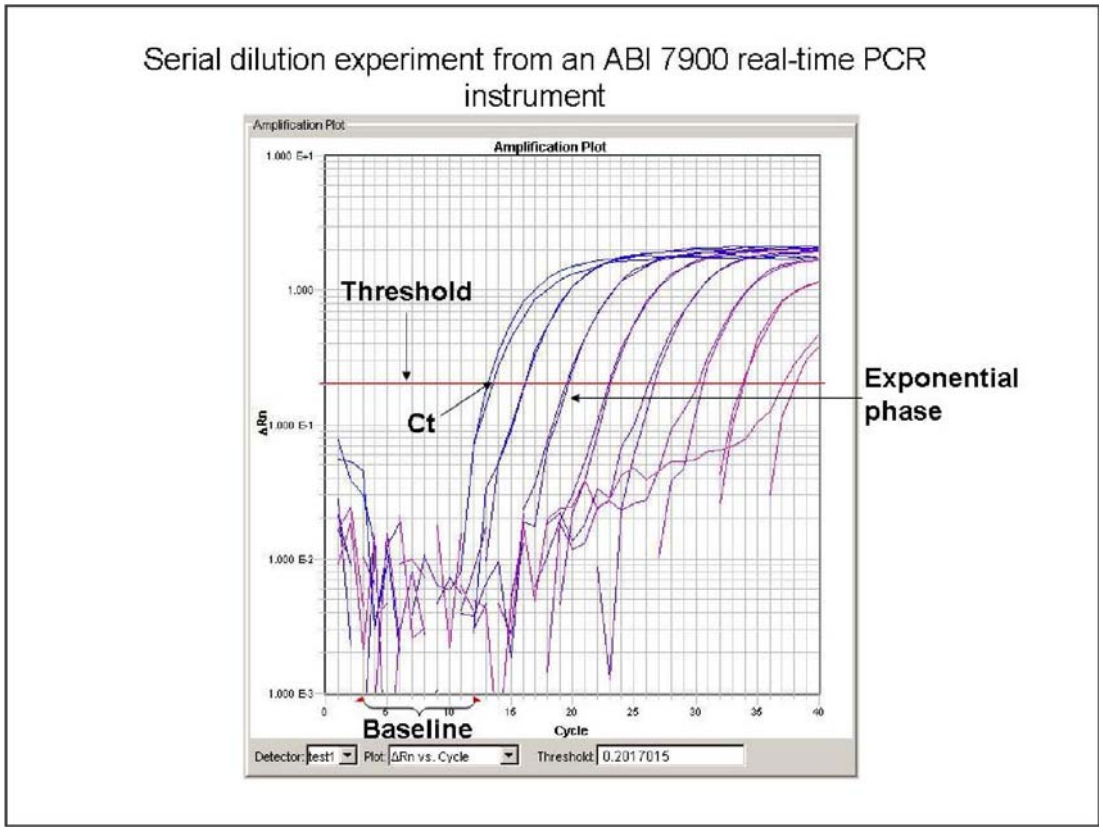
Refer to the appropriate instrument user guide for instructions on how to analyse your data. The general process for analyzing the data involves the following procedures:

1-View the amplification plots

2-Set the baseline and threshold values

During early PCR cycles, the background signal in all wells is used to determine the baseline fluorescence. The Threshold should be placed in the region of exponential phase

3-View Ct value. The replicates should be tight, within 0.5Cts (Threshold Cycle).



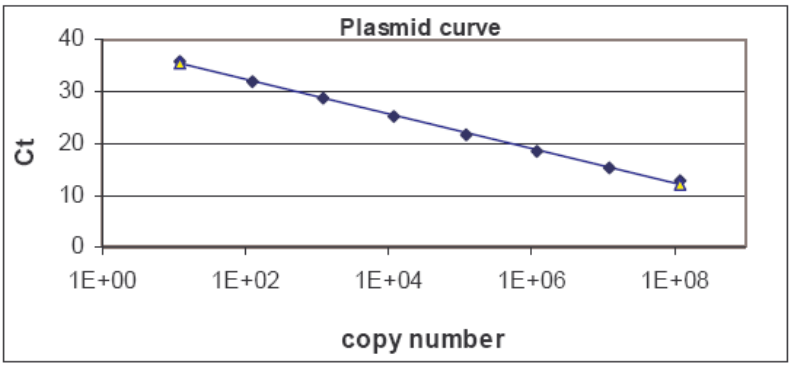
Plasmid range analysis

Plot the Cts of your standard plasmid (Y axis) versus the log of your initial quantity (X axis) to generate a standard curve. The standard curve should be linear over the entire range of where you expect your unknowns to fall. Standard curve specifications should be:

-Correlation Coefficient ≥ 0.99
-Efficiency of amplification 95-105%

Example:

Copy number	ln (copy number)	Ct1	Ct2	Ct average
9,00E+07	18,32	12,8	12,9	**12,9**
9,00E+06	16,01	15,7	14,7	**15,2**
9,00E+05	13,71	18,2	18,5	**18,4**
9,00E+04	11,41	21,7	21,7	**21,7**
9,00E+03	9,10	25,2	25,3	**25,2**
9,00E+02	6,80	28,6	28,6	**28,6**
9,00E+01	4,50	32,0	31,9	**31,9**
9,00E+00	2,20	35,6	35,7	**35,6**



Regression line: y = -a ln(x) + b

Negatives controls:
Cts of controls should be above 35 (PBS and H2O).

Determination of titer:
1-Determine number of copies per PCR reaction for each dilution of test article using the standard curve . This feature is automated in most real time PCR software packages. For those performing the calculations manually, determine the number of copies of each sample by applying the equation of the regression line (plasmid range).

Copy number = e^[(Ct-b)/a]

2- According to the dilution of the sample and volume of sample loaded, calculate the number of copies per ml (vg/ml). NB : since real time PCR only targets one of the two strands packaged within the AAV capsid, a multiplication factor of 2 is also required.

3- Carry out the average of the titers obtained for each dilution to arrive at the final

4-rAAV control : Check that the titer obtained is included in the interval of data previously obtained..

Appendices

A) Preparation of Standard Plasmids for Real-Time PCR
B) Test Record
C) Summary Test Record Form