TITRATION OF AAV8 RSM VECTOR GENOMES USING QUANTITATIVE REAL TIME PCR

Materials

DNase digestion buffer [13 mM Tris-Cl, pH7.5 / 5 mM $MgCl_2$ / 0.12 mM $CaCl_2$]
DNase I (10units/mL)
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 optical plate sealer sheet
Distilled water DNAse/RNAse free (NF H_20)
qPCR mastermix
ABI Prism Sequence Detector or equivalent
37 °C water bath
rAAV control sample (previously titrated rAAV sample)
10X GeneAmp PCR buffer (Applied Biosystems, Cat # N8080006)
10% Pluronic F68 (PF68) (Invitrogen, Cat # 24040032)
Sheared Salmon Sperm (SSS) DNA (Ambion, Cat# 9680)
Eppendorf tubes

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. The suggested format is shown below:

	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 5 µL of rAAV preparation and controls (PBS and rAAV control) in the 1.5ml eppendorf tube.
Prepare a DNase digestion mix:

		[Final]
5µl	10x DNase I buffer	1X
0.5µl	100X PF68 (5% w/v)	0.05%
2µl	DNase I (10u/mL)	400U/mL
37.5µl	NF H_20	

Add 45 µL of DNAse digestion mix per tube. Incubate 60 minutes at 37°C.

Prepare Samples

Prepare Sample dilution buffer:

		[Final]
1mL	1X GeneAmp PCR Buffer	1X
0.1mL	100X SSS DNA 200µg/mL	2µg/mL
0.1mL	100X pluronic F68 (5%)	0.05%
8.8mL	NF H_20	

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

Prepare standard plasmid
Prepare a 10-fold serial dilution of RSS rAAV plasmid (pTR-UF11) in sample dilution buffer (10 to 10^8 copies/20µL).
See appendix A

Prepare PCR reactions
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 (H_2O).

Reagent	**Vol. per 50ul PCR reaction**	**[Final]**
TaqMan Universal PCR Mix (2X)	25µL	1X
Forward Primer (10µM)	1µL	0.2µM
Reverse Primer (10µM)	1µL	0.2µM
Fluorescent Probe (10µM)	0.5µL	0.1µM
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.

Place the optical caps or optical plate sealer sheet over the plate. Centrifuge briefly before placing in the machine.

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

Analyzing Data

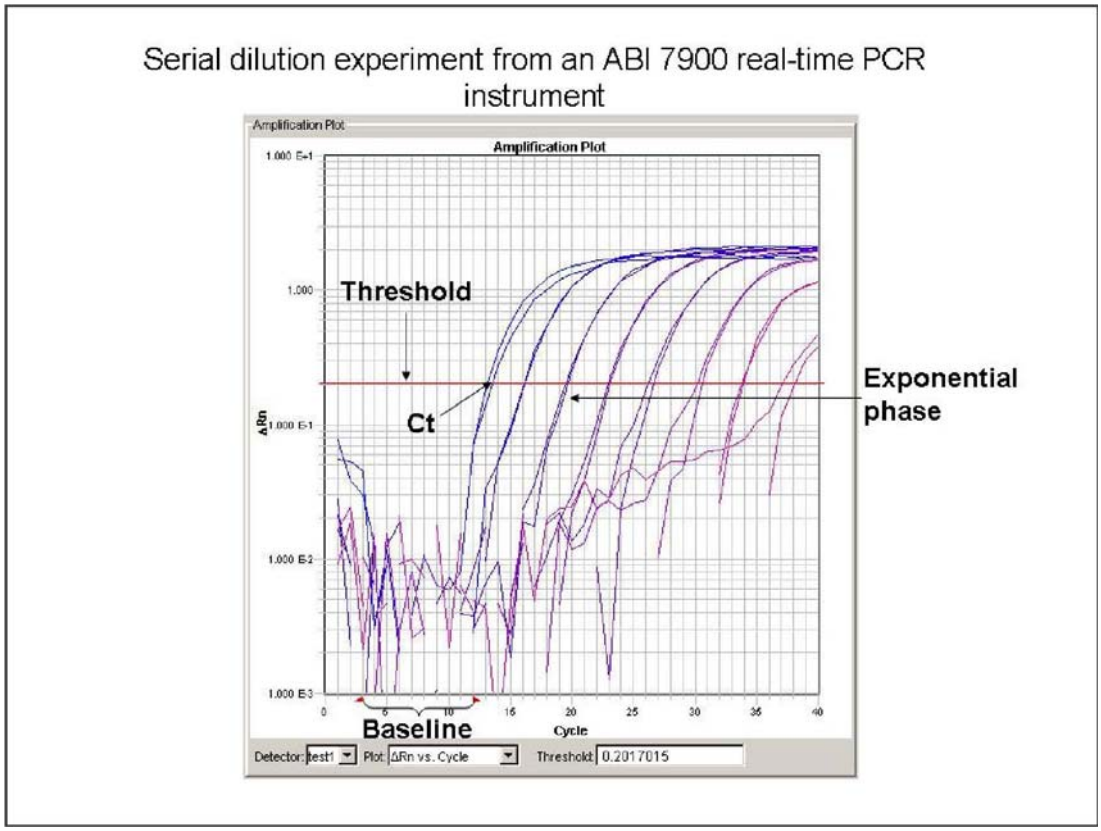
Refer to the appropriate instrument user guide for instructions on how to analyse your data.
The general process for analyzing the data involves the following procedures:

1. View the amplification plots
2. Set the baseline

During early PCR cycles, the background signal in all wells is used to determine the baseline fluorescence. This is usually cycles 3-12.

3. Set the threshold

The Threshold should be placed in the region of exponential phase. To standardize threshold placement we recommend you adjust the threshold until the 10^8 copies standard reads ~13.08Cts. The replicates should be tight, within 0.5Cts (Threshold Cycle).



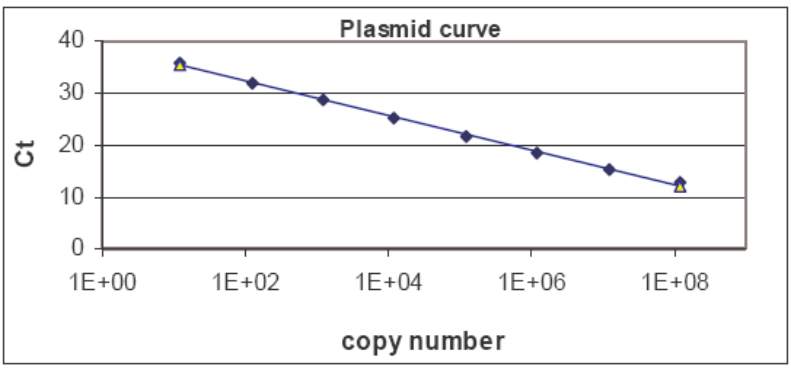
Plasmid range analysis

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 H_2O).

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

Materials

- 20% Lysol
- 70% Ethanol
- Plasmid
- Restriction Enzyme with Buffer
- Sample dilution buffer (from the protocol above)
- 15mL polypropylene tube
- Vortex mixer
- Biosafety cabinet with UV capabilities
- QIAquick PCR Purification Kit (Qiagen, Cat# 28104)
- QIAmp DNA Mini Kit (Qiagen, Cat# 51304)
- Spectrophotometer

Procedure

Note: Work inside the biosafety cabinet to avoid potential contamination from the laboratory environment.

- Clean the biosafety cabinet work surface and pipette shafts with 20% Lysol, followed by 70% Ethanol. Irradiate with UV light for 10-15 minutes.
- Linearize the standard plasmid with a restriction enzyme that cuts outside your intended PCR target.
- Purify the linearized plasmid using the QIAquick PCR purification kit or similar if it is less than 10kb in size. For larger plasmids, use the QIAmp DNA mini kit or similar. In both cases, pre-warm the elution buffer to 70^{o}C before use. Also, pre-warm the columns loaded with elution buffer at 70^{o}C for 5 minutes. Carry out two elutions in a minimum volume to maximize recovery and concentration of the plasmid.
  Note: Do not carry out a phenol:chloroform: isoamyl purification of the digest as any contamination with phenol will affect the accuracy of the spectrophotometric reading.
- Determine the concentration of the linearized plasmid by spectrophotometry and convert the concentration readout to grams per Liter (g/L).
  Note: Make sure the spec reading falls within the linear range of the instrument. Dilute the sample less if necessary. Blank the reading against an equal dilution of elution buffer and use the background correction feature of the instrument (read at 320nm).
- Calculate the formula weight (F.W.) of your standard plasmid: F.W.= Plasmid size (in base pairs) x 662 grams/mole.bp **(For TRUF11, this is 4766400)**
- Calculate the molar concentration (M) of your linearized plasmid. M = mole/L = (mass (in grams)/F.W.)/1L.
- Determine copy number per microliter of your linearized plasmid based on molar concentration. 1M is equivalent to about 6.0221415 x10E23 copy numbers.
- Make the first dilution, typically with a final concentration of 1x10E10 copies per 20µL (5x10E8 copies/µL). Use the $C_1V_1=C_2V_2$ relationship, where V_1 is the unknown *volume*, C_1 is the stock linearized plasmid *concentration* (copies/µL), V_2 is the final volume (typically 100µL) and C_2 is the final concentration (5x10E8 copies/µL). Add V_1 volume of stock linearized plasmid to enough Sample dilution buffer for 100µL of solution.
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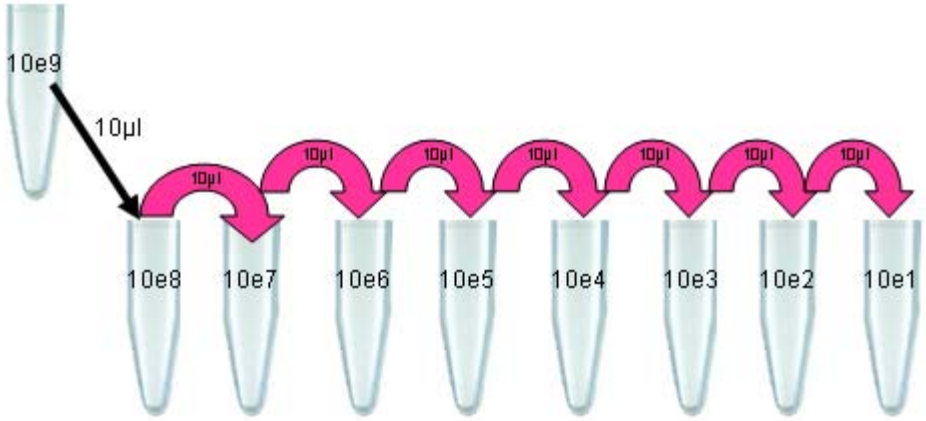
- Carry out a serial dilution of 1x10E10 copies/20µL of the linearized standard using sample dilution buffer. A volume of 90µL of diluent is used per dilution



B) AAV8 Reference Standard Material Test Record Packet.