Quantitative real-time PCR titering of infectious AAV particles

Calculation of plasmid copies for standard curve

1. Calculate the weight of one plasmid

$$m_{plasmid} = n\,[bp] * \frac{1\,mole}{6.023*10^{23}} * \frac{660g}{mole} = n\,bp * 1.096 * 10^{-21}\frac{g}{bp}$$

2. Calculate the weight that x copies of your plasmid have

$$m_{copies} = m_{plasmid} * copies$$

3. Scheme:

Copies	m_{copies}	Amount of DNA for qPCR [µl]	Final concentration of DNA [g/µl]
$1,3 * 10^1$			
$1,3 * 10^2$			
$1,3 * 10^3$			
$1,3 * 10^4$			
$1,3 * 10^5$			
$1,3 * 10^6$			
$1,3 * 10^7$			

Experimental outline of dsDNA preparation

1. Seed $5*10^4$ HT1080 cells / well in a 24 well plate
2. Infect cells 1 h later with ___**ml** of virus stock (**be sure not to infect your negative control cells!)**
3. Incubate for 24 h (infection start date_____ time _____ | end date_____ time_____)
4. Put plate on ice
5. **Carefully** remove cell medium (Saugflasche)
6. Wash cells **carefully** with 1 ml PBS
7. **Carefully** remove PBS (Saugflasche)
8. Trypsinize cells by adding 100 µl Trypsin
9. Stop reaction by adding 1 ml DMEM & transfer cell suspension to a 2ml Eppendorf tube
10. Harvest cells by centrifugation for 5 min @500 g
11. Wash pellet once with 1ml PBS
12. Harvest cells by centrifugation for 5 min @700 g
13. Resuspend cells in 50 µl of PBS

14. Add 10 µg of proteinase K
15. Incubate for 1 h @50 °C with occasional mixing
16. Heat inactivate enzyme via incubation @97 °C for 15 min
17. Clear lysate by centrifugation for 10 min @13000 g & transfer supernatant to a fresh tube
18. Supplement 10µl of cell lysate with S1 nuclease buffer & 20 U S1 nuclease & incubate for 30 min @37 °C (____µl S1 nuclease, _____µl buffer)
19. Heat inactivate enzyme by incubation @97 °C for 15 min

Experimental outline of quantitative real time PCR

1. **Remember to calculate everything for duplicate samples!**
2. Dilute prepared dsDNA 1:100
3. Pipetting scheme for qPCR

Component	Volume per reaction	Volume per reaction
2x QuantiFast SYBR Green pCR Mix	12,5	12,5
Primer for	0,4µM	1µl 10µM
Primer rev	0,4µM	1µl 10µM
Template	5µl	5µl
Water	ad 25µl	5,5µl
Total Volume	**25µl**	**25µl**

4. **Remember to prepare duplicates & a NTC (non – template – control)!**
5. PCR protocol

Step	Time	Temperature
Initial activation	5 min	95 °C
Two-step cycling		
Denaturation	10 sec	95 °C
Annealing / Extension	30 sec	60 °C
Number of cycles	45	

6. Calculate the number of infectious particles per ml:

$$inf.part._{/ml} = \frac{(copy\ number\ from\ qPCR) * a * b * c}{d}$$

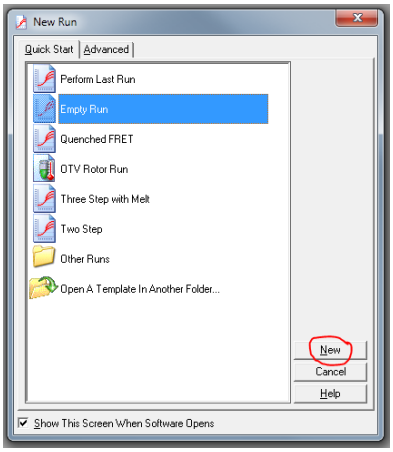
Where: a = dilution of whole cell lysate (100)

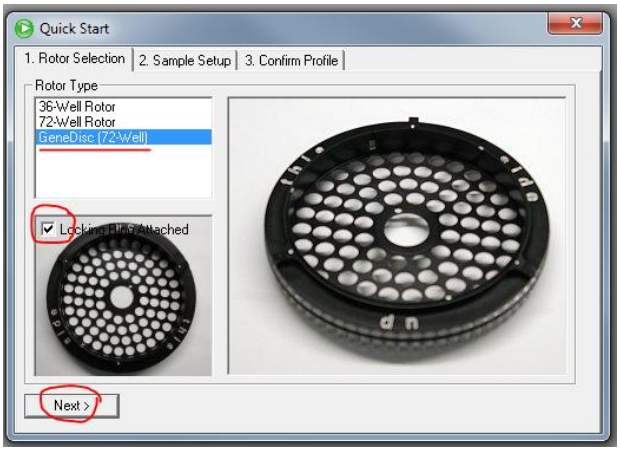
b = final volume of lysate (amount of proteinase K, buffer)

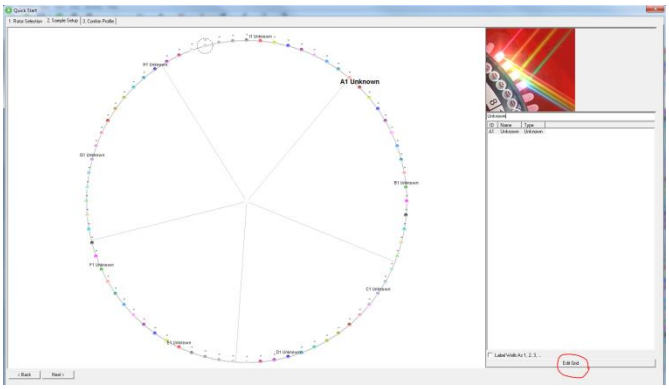
c = factor to yield infectious particles per ml (= 1 ml/x ml infection volume)

d = template volume in qPCR

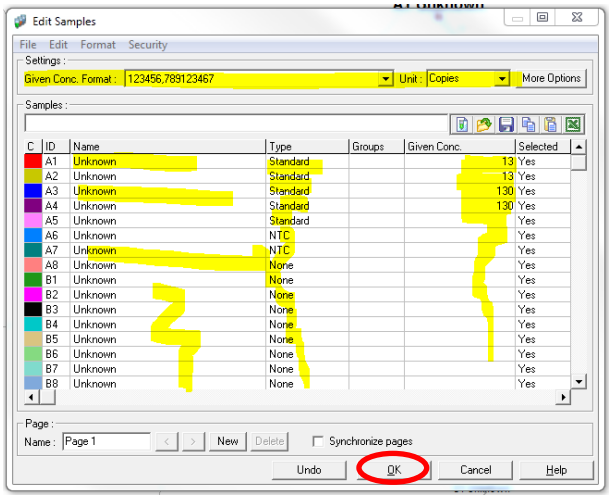
Rotor Gene Software

1. 

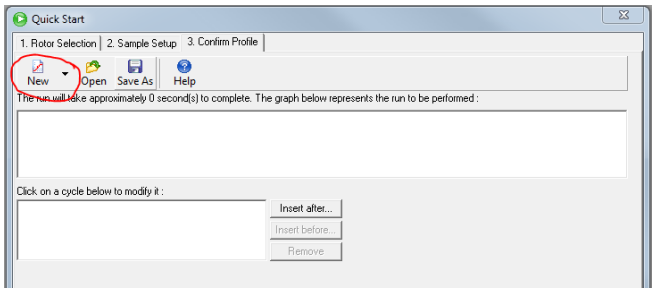
2. 

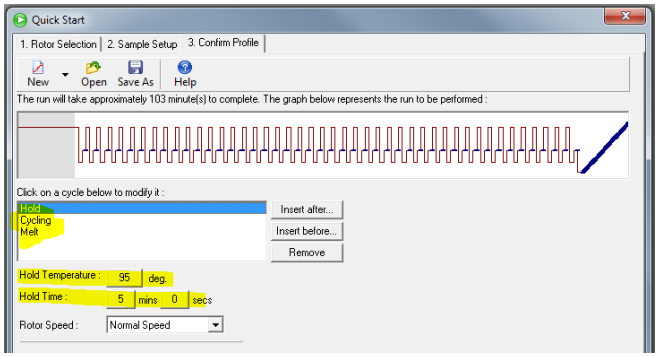
3. 

4.

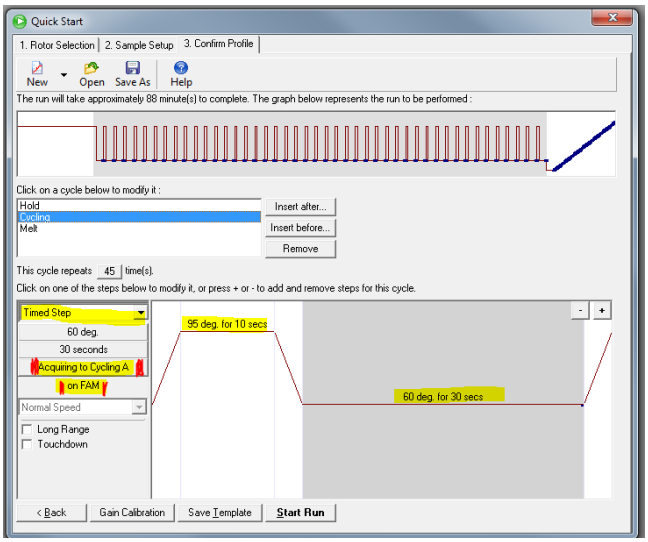
a. Adjust number format
b. Adjust unit
c. Fill out table with name / type…etc (leave Groups-field blank)
d. 

e. Back on last screen, click “Next”

5. 

6. Select “New --> Cycling & Melt”
7. Activation 

8. Cycling



9. Melt