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AAV Titration by qPCR Using SYBR Green Technology

Addgene the nonprofit plasmid repository[1]

[1]Addgene

1 Works for me dx.doi.org/10.17504/protocols.io.47kgzkw

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ABSTRACT

This protocol goes through AAV titration by qPCR using SYBR Green Technology. To see the full abstract and additional resources, visit the Addgene protocol page.

Sample Data

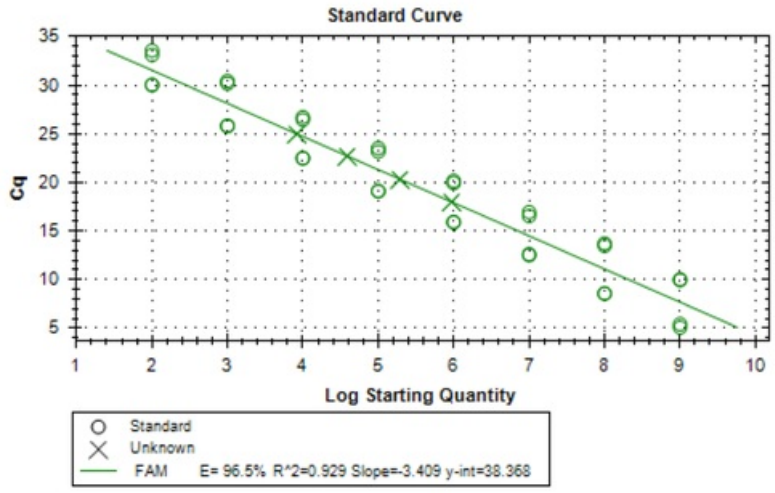

Figure 1: Example of a valid 8-point standard curve.

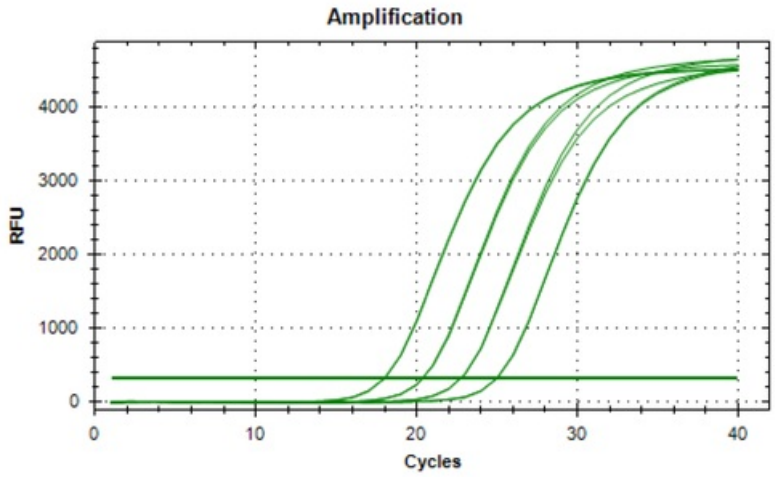

Figure 2: Example of the amplification plots obtained from an AAV sample. Each curve represent a dilution.

References

Aurnhammer C, Haase M, Muether N, Hausl M, Rauschhuber C, Huber I, Nitschko H, Busch U, Sing A, Ehrhardt A, Baiker A. Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Hum Gene Ther Methods. 2012 Feb;23(1):18-28.PMID: 22428977

EXTERNAL LINK

https://www.addgene.org/protocols/aav-titration-qpcr-using-sybr-green-technology/

GUIDELINES

General Considerations

- Always run standards and samples in duplicate at least
- If possible, include an AAV reference sample (AAV2 Reference Material or internal reference)
- Always include a No Template Control (NTC), i.e master mix + water
- Whenever possible use a multichannel pipet to minimize pipetting error and variability
- Mix samples very well by pipetting back and forth multiple times at each step

Workflow Timeline

- **Plate set-up:** 2 hours
- **qPCR run:** 1.5 hours
- **Data analysis:** 30 minutes

MATERIALS TEXT

Equipment

- qPCR instrument
- Heating plate
- Pipettors
- Multi-channel pipette (optional, but strongly recommended)

Reagents

- Universal SYBR Master Mix 2X
- Primer pair targeting AAV2 ITR (Aurnhammer et al., 2012)
- fwd ITR primer, 5'-GGAACCCCTAGTGATGGAGTT
- rev ITR primer, 5'-CGGCCTCAGTGAGCGAITR-containing plasmid for standard curve
- RNase-free DNase
- 10X DNase buffer
- Nuclease-free water
- Microcentrifuge tubes
- 96-well optical plate
- Pipettes
- Pipette tips

Reagent Preparation

Master Mix: count the number of samples (n) and prepare master mix for an additional 10 samples (n+10 - the additional amount will ensure that there is enough master mix for all samples). Each sample requires 15 µL of master mix.

Pro-Tips

- Use a “Universal” SYBR master mix which contains a a high- quality DNA polymerase and a blend of dTTP/dUTP to minimize carryover contamination. The master mix should also contain an internal passive reference (typically ROX dye), to normalize non-PCR–related fluorescence fluctuations and to minimize well-to-well variability that result from a variety of causes, such as pipetting error and sample evaporation.
- Make the master mix after all the samples have been added to the qPCR plate. Start by adding water, then SYBR master

mix, then the forward and reverse primers. Vortex briefly, immediately before use.

- Use a reservoir and a multichannel pipette to dispense the master mix into the wells.

Reagent	Amount for ONE Reaction	Amount for 100 reactions (1 x 96 well plate)
Unversal SYBR Master Mix 2X	10 µL	1,000 µL
100 µM Forward Primer	0.15 µL	15 µL
100 µM Reverse Primer	0.15 µL	15 µL
Nuclease Free Water	4.7 µL	470 µL

SAFETY WARNINGS

See SDS (Safety Data Sheet) for safety warnings and hazards

Procedure

1 Prepare a plasmid stock of $2x10^9$ molecules/µl to generate a standard curve:

One option is to use plasmid #59462 from Addgene. The values highlighted below in red were calculated using this plasmid, but will change if you use a different plasmid.

Sample Calculations	
Size in bp of Addgene plasmid #59462:	6208 bp
Concentration of Addgene plasmid #59462:	1.07 µg/µL
Molecular Weight:	6208 bp x 650 daltons/bp (g/mole)= 4.03 x 10^6 grams/mole
Moles/µl:	1.07 µg/µL x 1g/10^6 µg x1 / 4.03x10^6 g/mole = 2.65 x10^{-13} moles/ µL
Molecules/ µl:	2.65 x 10^{-13} moles/ µL x 6.022145 x 10^{23} molecules/mole = 1.59x 10^{11} molecules/µL

To obtain a solution at $2x10^9$ molecules/µL:

- 1.59 x 10^{11} / $2x10^9$ = 79.8x dilution 100 µL / 79.8 = **1.25 µl**
- Therefore we need to dilute **1.25 µl** stock into **98.74 µl** H_20

Pro-Tips

- Once a validated standard curve is obtained, make a small aliquot of each standard (enough for 1 or 2 plates) and store at **-20 °C** . Once a standard is thawed do not freeze it again but store at **4 °C** and use within 1 week.
- Keep track of the Ct value for each standard over time. They should remain within 0.5 Ct of their initial value. If the Ct value of the standard starts to drift, it's time to make a new one.
- When developing the assay multiple plasmids containing ITR were tested. Plasmid #59462 is one plasmid that gave reliable and consistent results. Use the recommended plasmid, or test multiple plasmids to find a suitable one.
- Some labs have reported better results when the plasmid is linearized.

2 Treat the purified AAV samples with DNase I to eliminate any contaminating plasmid DNA carried over from the production process (DNase does not penetrate the virion).

1. **5 µl** sample + **39 µl** H_2O + **5 µl** 10x DNase buffer + **1 µl** DNase
2. Gently mix sample (do not vortex)
3. Incubate **00:30:00** at **37 °C** .
4. Transfer to ice

**** Critical: do NOT treat your plasmid standard with DNase ****

3 Make 6 serial dilutions, in duplicate, of your standard curve plasmid ($2x10^9$stock made in step #1):

Volume of $2x10^9$ stock or previous dilution (µL)	Volume of nuclease-free water (uL)	Molecules per µL
10	90	$2x10^8$
10 of $2x10^8$ dilution	90	$2x10^7$
10 of $2x10^7$ dilution	90	$2x10^6$
10 of $2x10^6$ dilution	90	$2x10^5$
10 of $2x10^5$ dilution	90	$2x10^4$
10 of $2x10^4$ dilution	90	$2x10^3$

Pro-Tip
To help stabilize the standards add carrier DNA to a final concentration of 4 ug/mL to each standard dilution.

4 Dilute DNase-treated and AAV reference samples according to the dilution scheme in the table below:

Dilution Series	Volume of sample(uL)	Volume of nuclease free water (uL)	Dilution factor	Total dilution
Dilution 1 (DNase step)	5uL AAV stock	45 uL	10x	10x
Dilution 2	5uL Dil. 1	95 uL	20x	200x
Dilution 3	20uL Dil. 2	80 uL	5x	1000x
Dilution 4	20uL Dil. 3	80 uL	5x	5000x
Dilution 5	20uL Dil. 4	80 uL	5x	25000x
Dilution 6	20uL Dil. 5	80 uL	5x	125000x
Dilution 7	20uL Dil. 6	80 uL	5x	625000x
Dilution 8	20uL Dil. 7	80 uL	5x	3125000x

Dilutions highlighted in green are the ones loaded onto the qPCR plate for most samples.

- If sample is expected to have a titer <1x10^{12} GC/mL, use dilutions 3-6
- If sample is expected to have a titer >3x10^{13} GC/mL, use dilutions 5-8

Note, at Addgene we use 2 different AAV reference depending on whether we expect our sample(s) to have a low (<1-3x10^{12}) or high titer (>7x10^{12}).

- Low titer reference: AAV RSM2, 3.28 x 10^{10} GC/mL, available from ATCC (cat# VR-1616)
- High titer reference: internal AAV vector, 3.0 x10^{13} GC/mL

Pro-Tips

- The quality of the sample dilution series is critical. Make sure to pipet each dilution up and down at least 10 times, and use at least half of the final volume (mix with > **50 µl** if your well contains **100 µl**)
- Use a multichannel pipette to load the standards and samples onto the qPCR plate

5 Set up and load the 96-well plate:

1. Load **5 µl** of each standard in duplicate
2. Load **5 µl** of each sample in duplicate. Do not forget to include a no template control (*NTC = master mix + water*).
3. Add **15 µl** of Master Mix per well and mix well by pipetting back and forth at least 5 times.
4. Seal plate with transparent film.
5. Centrifuge at 3,000 rpm for **00:02:00** to bring the sample to the bottom of the tube.
6. Run the following protocol in your qPCR instrument using SYBR detection:

98 °C for **00:03:00** / **98 °C** for **00:00:15** / **58 °C** for **00:00:30** / read plate/ repeat 39x from step 3 / melt curve

Example of plate set-up:

	1	2	3	4	5	6	7	8
A	1.00×10^9	1.00×10^8	1.00×10^7	1.00×10^6	1.00×10^5	1.00×10^4	empty	NTC
B	1.00×10^9	1.00×10^8	1.00×10^7	1.00×10^6	1.00×10^5	1.00×10^4	empty	NTC
C	AAV reference				Sample 3			
D								
E	Sample 1				Sample 4			
F								
E	Sample 2				Sample 5			
F								

6 Perform data analysis using the instrument's software. Determine the physical titer of samples (viral genomes (vg)/mL) based on the standard curve and the sample dilutions.

Pro-Tips

Make sure that the qPCR is valid by checking to the following:

- Standard curve: R^2(coefficient of correlation) ~ 1.0, E (efficiency of PCR) ~100% (90%-110% range is acceptable)
- Baseline removal: all samples will have some small amount of background signal that is most evident during initial PCR cycles. This background signal must be removed to accurately determine differences between samples.
- Melt curve analysis: a single peak should be seen. The presence of a second peak at a temperature of ~70-75°C usually indicates the presence of primer dimers which can increase background signal and alter the Ct values of your samples.
- Quality of your standard curve: you should observe differences in Ct values that make sense for your dilutions (~3.3 difference Ct for a 10-fold dilution is appropriate).
- Quality of duplicates: Exclude duplicates from analyses if there is more than a 0.5 Ct difference between them.