qPCR for the Detection and Quantification of Adeno-Associated Virus Serotype 2 (AAV2) ITR-Sequences

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Introduction:

Viral vectors based on different naturally occurring adeno-associated virus (AAV) serotypes belong to the most promising tools in human gene therapy. For the production of recombinant AAV (rAAV) vectors, researchers are focusing predominantly on cross-packaging an artificial AAV genome based on serotype 2 (AAV2) into the capsids derived from other serotypes. Within the packaged genome the inverted terminal repeats (ITRs) are the only *cis*-acting viral elements required for rAAV vector generation and depict the lowest common denominator of all AAV2 derived vector genomes (Fig. 1).

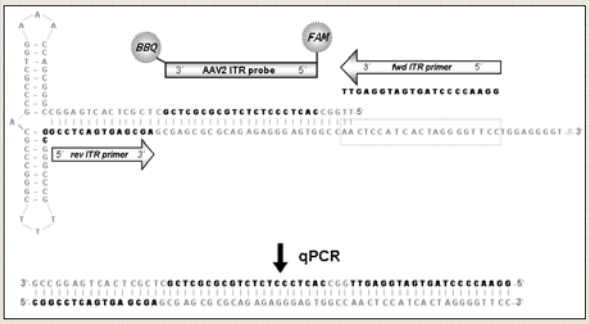

Figure 1. Design of AAV2-ITR-qPCR Primers and Probe. The upper panel illustrates the 5´-ITR secondary hairpin structure of (wildtype) AAV2 and the localization of the AAV2-ITR-qPCR specific primers and probe. The corresponding 62bp PCR product is depicted below.

Up to now, no qPCR for the detection and quantification of AAV2-ITRs could be established due to their extensive secondary hairpin structure formation. We established a molecular biological method that allows accurate and reproducible quantification of AAV2 genomes based on an AAV2-ITR sequence-specific qPCR. Primers and labelled probe are located within the ITR sequence and have been designed to detect both, wild type AAV2 and AAV2-based vectors. This method is suitable for detecting both, single-stranded DNA derived from AAV2 vector particles and double-stranded DNA derived from vector plasmids. In conclusion, this method describes the first qPCR system facilitating the detection and quantification of AAV2-ITR sequences with a limit of detection at 50 ITR sequence copies per reaction [1].

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Validation using a plasmid standard

For the establishment of the AAV2-ITR-qPCR the AAV2 vector plasmid pZACmSB was used as template. This plasmid comprises 4 AAV2-ITR target sites for qPCR amplification (Fig. 2A).

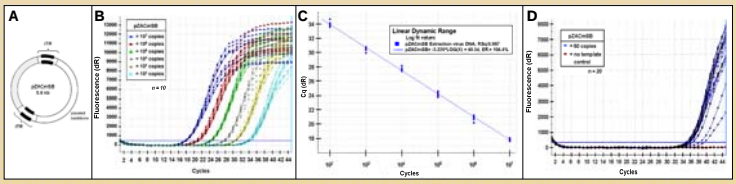

Figure 2. Performance of AAV2-ITR-qPCR within quantification of AAV2 vector plasmids. (A) pZACmSB. (B, C) Linear dynamic range of the AAV2-ITR-qPCR was determined as 10^2 to 10^7 plasmid copies, n=10. Within this range the AAV2-ITR-qPCR exhibited a linear correlation coefficient (RSq) of 0.997, a slope of -3.220 and an efficiency of 104.4%. (D) LOD, 50 plasmid copies.

The intraassay variance (repeatability) could be determined as or 0.7% and the interassay variance (reproducibility) could be defined as 14,8% (data not shown). To investigate robustness of this method, the performance of the AAV2-ITR-qPCR was assayed using three qPCR kit systems: Quantitect Multiplex No Rox Kit (Qiagen, Germany), Brilliant III Ultra-Fast QPCR Master Mix Kit (Agilent Technologies, USA) and the TaqMan Universal PCR Master Mix (Applied Biosystems, USA) in combination with the Stratagene MX3000 Real Time PCR device (Agilent Technologies, USA), respectively. All qPCR reaction kits yielded similar amplification results. Additionally, the Quantitect Multiplex No Rox Kit (Qiagen, Germany) worked in combination with 7900 HT Sequence Detection System (Applied Biosystems, USA) and the Light Cycler 480 device (Roche, Germany) (data not shown). Establishment and validation of this qPCR method were performed according to [2].

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Quantification of AAV2 vector genome copies:

We could utilize our AAV2-ITR-qPCR method for the quantification of vector genomes of two different AAV2 vector (Fig. 3A) preparations, AAV2.8 mTRIF (Fig. 3B) and AAV2.8 EGFP (Fig. 3C). For pre-analytics, each AAV2 vector sample was tenfold serially diluted and viral nucleic acids were extracted in triplicates from 5 µl of each dilution. Both qPCR-based AAV2 titers appeared within the same order of magnitude compared to the respective titers determined by dot blot analyses [3].

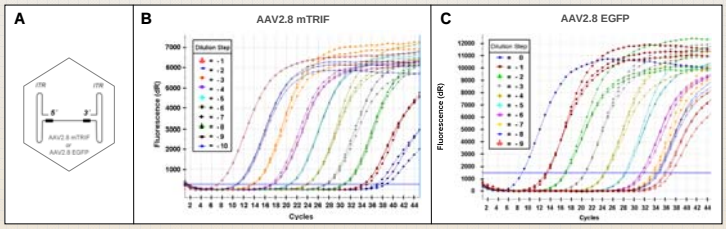

Figure 3. Performance of AAV2-ITR-qPCR in quantification of AAV2 vector genome copies. (A) AAV2 vector sample (AAV2.8 mTRIF or AAV2.8 EGFP). For qPCR quantification 5 µl AAV2 nucleic acids (10% of the nucleic acid elution volume) were assayed in duplicates using defined serial dilutions of pZACmSB for the calculation of a standard curve. **(B)** Amplification plots for tenfold dilution series of AAV2.8 mTRIF. In case of AAV2.8 mTRIF the AAV2-ITR-qPCR exhibited a RSq of 0.999, a slope of -3.405 and an efficiency of 96.6%. **(C)** Amplification plots for tenfold dilution series of AAV2.8 EGFP. In case of AAV2.8 EGFP, a RSq of 0.969, a slope of -3.056 and an efficiency of 112.9% could be observed. Cq: quantification cycle; Eff: efficiency; RSq: linear correlation coefficient.

For assessing the cross-reactivity of the AAV2-ITR-qPCR with other AAV serotypes, the AAV2 amplicon sequence (62 nucleotides) was aligned with respective sequences of other known AAV serotypes. In order to practically study any potential cross-reactivity, all orthologue amplicon sequences were synthesized as 62mer oligonucleotides and subjected to AAV2-ITR-qPCR analysis using 5 x 10^4 molecules per qPCR reaction.

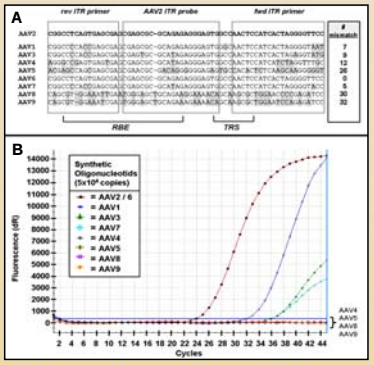

Figure 4: Cross-Reactivity of AAV2 qPCR with ITR Sequences Derived From other AAV Serotypes. (A) The sequence alignment of the AAV2 amplicon with AAV serotype 1 and 3-9. Grey squares depict sequence mismatches compared with the AAV2 amplicon. Numbers of mismatches are given on the right. Highest similarities could be shown for AAV6, AAV1, AAV3 and AAV7. Sequence alignment was performed using Husar sequence analysis software (http://genius.embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/). The three grey boxes depict the locations of the probe and the corresponding primers. The amplicon sequences of AAV6 and AAV2 is identical. **(B)** Specificity testing proved cross-reactivity to some extend for AAV1, AAV3, AAV6 and AAV7, but not for AAV4, AAV5, AAV8 or AAV9. *RBE*, rep binding element; *TRS*, terminal resolution site.

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Discussion

We could develop the first AAV2-qPCR system enabling the detection and quantification of AAV2-ITR sequences and is independent on respective insert sequences within vector genomes. This method is suitable for both, single-stranded DNA derived from AAV2 vector particles and double-stranded DNA derived from vector plasmids. Cross-reactivity which was verified for other AAV serotypes (AAV1, 3 and 7) occurred to considerable lower extend compared to AAV2-ITR values. AAV6-ITR-sequences appear to be detectable alike with AAV2-ITR sequences. Since this AAV2-ITR-qPCR can be used for all AAV2-genome based vectors in a one-for-all based manner, it will significantly simplify rAAV2 vector titrations in the future.

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References:

[1] Aurhammer et al., submitted.
[2] Bustin et al., "The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments". Clinical Chemistry, **55**, 611-622, 2009.
[3] Grieger et al., Production and characterization of adeno-associated viral vectors. Nature protocols, **1**, 1412-1428 (2006).

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