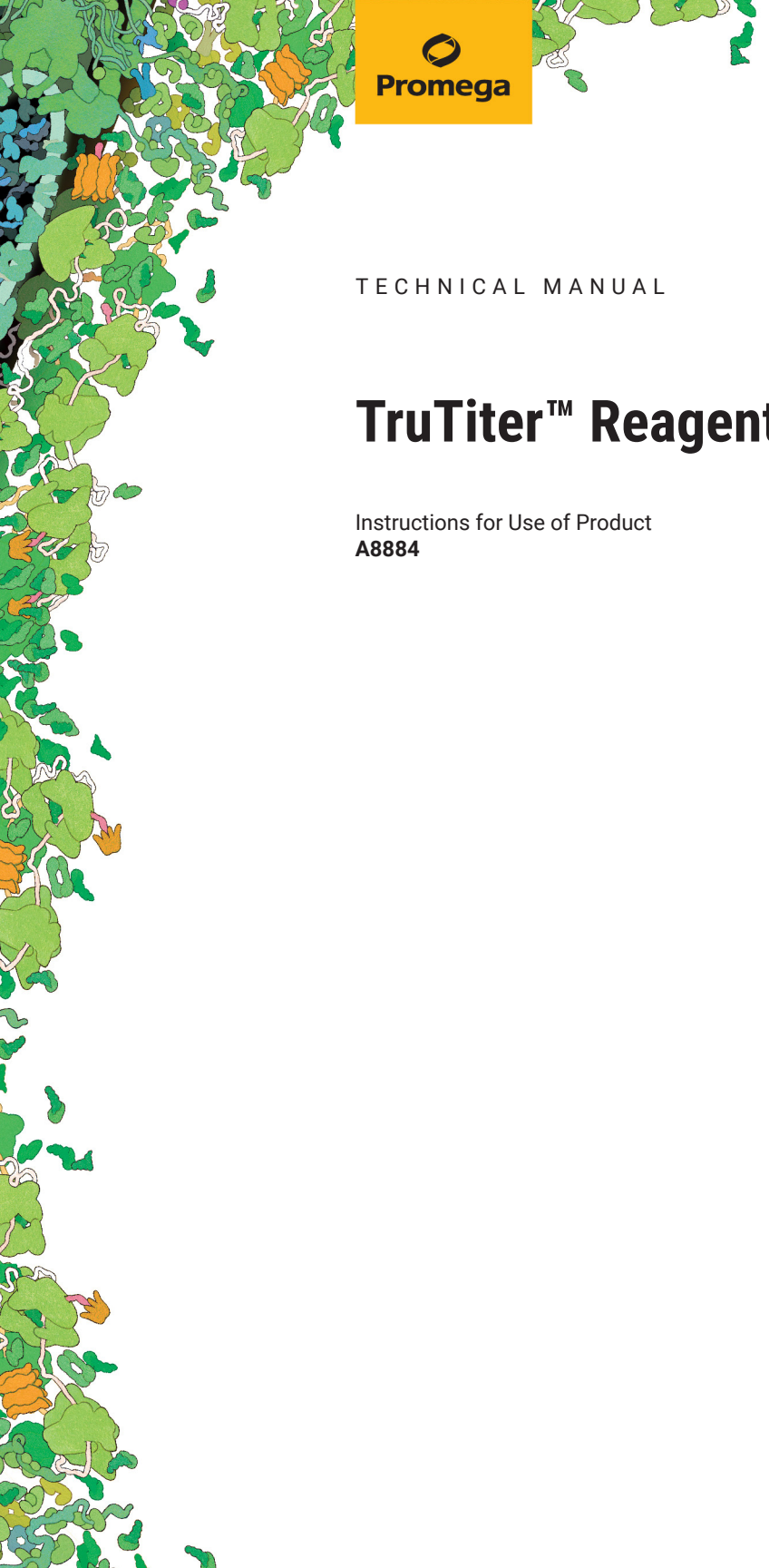

TECHNICAL MANUAL

TruTiter™ Reagent System

Instructions for Use of Product
A8884

1/25
TM742

TruTiter™ Reagent System

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

Adeno-associated virus (AAV) is one of the most commonly used vectors for gene therapy delivery. One of the main challenges for AAV gene therapy is the development of effective quality control and quality assurance protocols, as accurate quantification and characterization of AAV production batches is essential for proper clinical dosing. Current protocols are often inadequate, leading to a high degree of lot-to-lot variability, often resulting in nonoptimal trial outcomes due to inaccurate determination of functional gene dosage.

Present methods of AAV characterization rely on a combination of mammalian infectivity assays and digital PCR coupled with DNase pretreatment. These approaches are often cumbersome and lack the precision needed for accurate viral titer determination. The workflow described here uses a novel small molecule (Viability PCR Reagent) pretreatment approach to estimate the functional AAV titer in a viral preparation. The Viability PCR Reagent, with its small size, has enhanced accessibility to exposed nucleic acids from damaged viral and nonviral sources. Furthermore, use of this reagent suppresses the PCR signal more consistently from such exposed nucleic acids, allowing a more precise functional AAV titer determination. This new platform, the TruTiter™ Reagent System[a], enables users to quantify AAV preparations in a more robust and accurate manner.

The TruTiter™ Reagent System is compatible with existing PCR viral quantification protocols, allowing direct amplification using a variety of qPCR or digital PCR platforms.

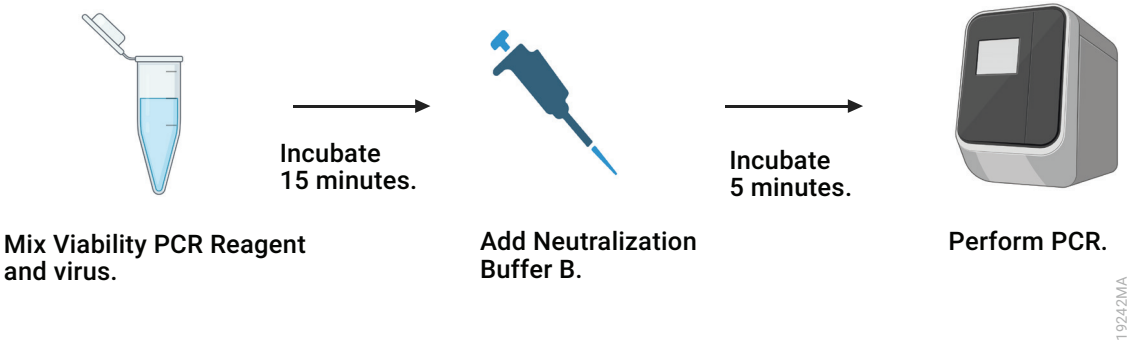

Figure 1. TruTiter™ Reagent System workflow. (Image created with BioRender.com.)

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
TruTiter™ Reagent System	**200 reactions**	**A8884**

For Research Use Only. Not for Use in Diagnostic Procedures. Includes:

- 1 × 250µl Viability PCR Reagent (1mM)
- 1 × 500µl Neutralization Buffer B (100X)

Storage Conditions: Store all components at –30°C to –10°C, protected from light.

Safety Note: Wear proper laboratory personal protective attire (gloves, lab coat, safety glasses) while handling the Viability PCR Reagent to reduce direct exposure risk. See the Safety Data Sheet (SDS) for more information.

3. Protocol

Materials to Be Supplied By the User

- 1.5ml microcentrifuge tubes
- micropipettes and tips
- heat block or static incubator
- vortex mixer
- **optional:** 1X PBS
- **optional:** Proteinase K (PK) Solution (e.g., Cat.# MC5005); **Note:** Proteinase K (PK) Solution can increase consistency in results when working with recombinant AAV capsids or AAV samples in crude lysates. See optional protocol, Steps 1 and 2 in Section 3.B.
- **optional:** Viability Direct Neutralization Buffer (Cat.# A8885); **Note:** The Viability Direct Neutralization Buffer kit contains additional Neutralization Buffer B for use when treating larger volumes of AAV-containing sample.

3.A. Viability PCR Reagent Treatment

Viability PCR Reagent treatment concentrations may need to be optimized for the specific virus serotype being treated. A Viability PCR Reagent concentration that's too high may inhibit intact virus PCR signal. Treatment should be optimized using intact virus as a baseline. Optimal treatment concentration should not significantly inhibit intact virus PCR signal. See the optimization protocol in Section 7.B for details.

A 10µM Viability PCR Reagent final concentration example is shown here. You may need to modify the volume of Viability PCR Reagent added to reactions. Suggested final treatment concentrations are 1µM–50µM. If lower concentration working stocks of Viability PCR Reagent are needed, dilute Viability PCR Reagent in 1X PBS and dispose of unused Viability PCR Reagent appropriately after experiments. Diluted Viability PCR Reagent is not stable for long-term storage (more than two hours) in aqueous solution.

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3.A. Viability PCR Reagent Treatment (continued)

1. Add 100µl of each test sample into two separate 1.5ml microcentrifuge tubes.
2. Add 1µl of 1mM Viability PCR Reagent to one of the tubes (10µM final), omitting Viability PCR Reagent from the remaining tube of the pair. Vortex to mix and incubate at 37°C for 15 minutes.
3. Add 1µl of 100X Neutralization Buffer B to each tube. Vortex to mix.
4. Incubate at room temperature for 5 minutes.

3.B. Direct Amplification Sample Preparation

The steps below are a suggested protocol for preparing AAV samples to be used in a dPCR/qPCR direct amplification setup without a nucleic acid purification step.

Note: Proteinase K Solution treatment (Steps 1 and 2) is optional. In certain situations, Proteinase K Solution can improve efficiency of downstream direct amplification workflows using PCR.

1. Add 1µl of Proteinase K Solution to each AAV sample from Section 3.A, if Proteinase K treatment is desired. If Proteinase K treatment will not be performed, proceed to Step 3.
2. Incubate samples with Proteinase K Solution at 55°C for 30 minutes.
3. Incubate samples at 95°C for 10 minutes to denature capsids.
4. Cool samples on ice.
5. Proceed to PCR setup. Alternatively, store samples at –30°C to –10°C for later analysis.

3.C. Quantitation

Follow your preferred digital or quantitative PCR protocol for the target of interest, for the samples prepared using Sections 3.A and 3.B. Input may need to be optimized, depending on the DNA volume.

4. Representative Data

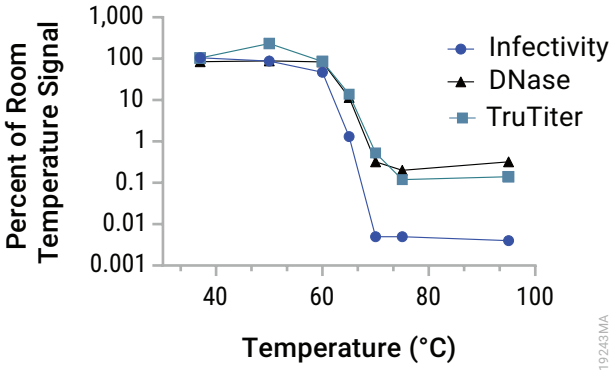

Figure 2. TruTiter™ Reagent System provides similar results to DNase-dPCR and mammalian infectivity assays. Adeno-Associated Virus (AAV2-NanoLuc) viral capsids (10^7 GU/ml) were treated at the indicated temperature for 10 minutes. Then AAV was treated with DNase or Viability PCR Reagent followed by dPCR. In parallel, a 24-hour infectivity assay was performed using HEK293 cells. To aid interpretation, data are represented as percent of signal achieved with the room temperature treatment.

4. Representative Data (continued)

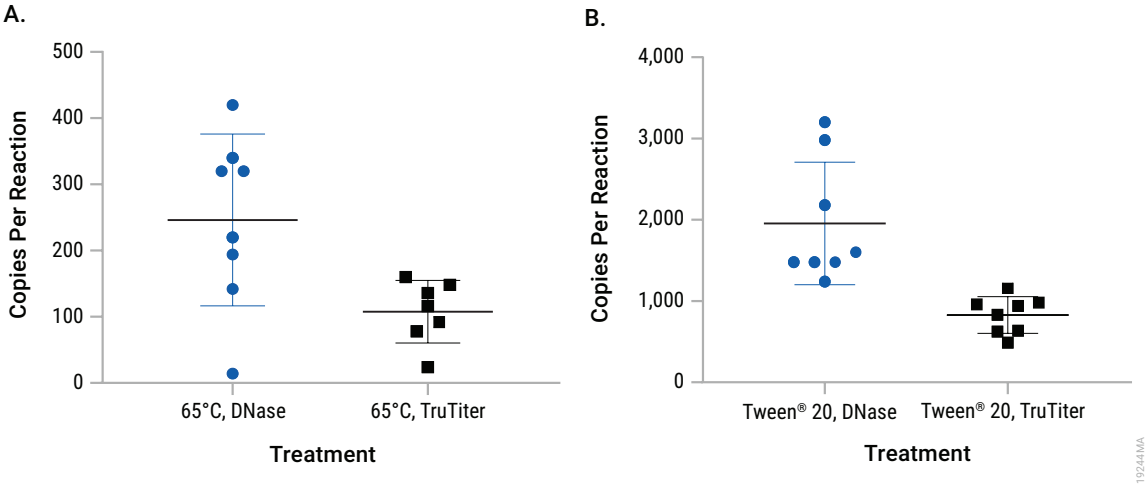

Figure 3. TruTiter™ Reagent System titer results are more precise than DNase-based dPCR workflow. Adeno-associated virus (AAV2-NanoLuc) viral capsids (10^{10} GU/ml) were treated at the indicated temperature (**Panel A**) or with detergent (Tween® 20; **Panel B**) for 10 minutes. Following treatment, AAV particles were treated with DNase or Viability PCR Reagent (TruTiter) followed by dPCR. Eight technical replicates of each treatment were performed.

5. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

For general information and best PCR practices, please visit:
www.promega.com/resources/student-resource-center/lab-essentials/nucleic-acid-amplification/

Symptoms	Causes and Comments
Significant suppression of intact AAV PCR signal	Reduce final concentration of Viability PCR Reagent added to samples.
	If diluting Viability PCR Reagent, ensure that working solutions are diluted in PBS. DMSO can artificially permeabilize viruses.
	If performing resuspension or washing of samples, make sure to use an isotonic buffer (e.g., PBS), as Milli-Q® or deionized water results in a hypotonic environment that can cause capsid lysis.
	Ensure that the proper amount of Neutralization Buffer B is added to sample before nucleic acid direct amplification.
Insufficient dead/nonviable AAV PCR signal reduction	Increase final concentration of Viability PCR Reagent added to samples.
	If possible, dilute complex samples 1:10 before treatment with Viability PCR Reagent. Samples with a very high bioburden may reduce effectiveness of Viability PCR Reagent.
	Ensure that aqueous working Viability PCR Reagent solution is discarded after use, as long-term reagent stability in aqueous solution is not guaranteed.
Unexpectedly low AAV titer in direct-amplification PCR	Ensure that proper amount of Neutralization Buffer B is added to sample before nucleic acid direct amplification.
	Perform optional Proteinase K Solution digestion (Section 3.B, Steps 1 and 2).

6. Related Products

Product	Size	Cat.#
Proteinase K	4ml	MC5005
	16ml	MC5008
Viability Direct Neutralization Buffer	500µl	A8885

7. Appendix: Example Optimization Protocol for TruTiter™ Reagent System

In this example protocol, the starting material is an AAV sample (in-process or final purified form).

7.A. Preparing Reagents

Prepare Viability PCR Reagent working solutions from 1mM Viability PCR Reagent stock provided in the kit as follows:

1. Dilute 1mM Viability PCR Reagent stock 1:10 in 1X PBS to create a 100µM Viability PCR Reagent working solution.
2. Dilute the Viability PCR Reagent 100µM working solution 1:10 in 1X PBS to create a 10µM Viability PCR Reagent working solution.

   Note: Do not store diluted working concentrations, as diluted reagent is not stable for long-term storage in aqueous solution.

7.B. Optimization Protocol

1. Aliquot 100µl of AAV sample into the twelve tubes listed in Table 1.

Table 1. Sample List for AAV Optimization with TruTiter™ Reagent System. RT: Room temperature; VR: Viability PCR Reagent.

Sample #	Temperature	VR Concentration	DNase Treatment
1	RT	–	–
2	RT	0.1µM	–
3	RT	1µM	–
4	RT	5µM	–
5	RT	25µM	–
6	RT	–	+
7	65°C	–	–
8	65°C	0.1µM	–
9	65°C	1µM	–
10	65°C	5µM	–
11	65°C	25µM	–
12	65°C	–	+

2. Heat-inactivate indicated samples (#7–12) by incubating at 65°C for 10 minutes, then cool to room temperature.
3. Hold samples #1–6 at room temperature.
4. Follow the standard TruTiter™ workflow protocol in Section 3, using volumes and concentrations of Viability PCR Reagent listed in Table 2.

Table 2. Volumes of Working Solution to Add to Result in Indicated Final Concentration of Viability PCR Reagent.

Final Viability PCR Reagent Treatment Concentration	Viability PCR Reagent Working Solution Concentration and Volume
0.1µM	1µl of 10µM solution
1µM	1µl of 100µM solution
5µM	5µl of 100µM solution
25µM	2.5µl of 1mM solution

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7.B. Optimization Protocol (continued)

5. For samples #6 and #12, treat with DNase according to your laboratory protocol.
6. Perform direct-amplification PCR as outlined in Sections 3.B and 3.C or according to your laboratory protocol.

7.C. Data Analysis and Interpretation

1. Using your choice of analysis software, calculate the AAV titer for each treatment condition listed in Table 1.
2. The optimal Viability PCR Reagent treatment condition will:
    - Exhibit minimal difference between Viability PCR Reagent-treated and -untreated samples when incubated at room temperature.
    - Exhibit maximal difference between Viability PCR Reagent-treated and -untreated samples when incubated at 65°C.
    - Ensure more consistent, robust and precise measurements when compared to DNase treatment.

[a]Patents Pending.