Cat. # 6233

For Research Use

TaKaRa

AAVpro® Titration Kit (for Real Time PCR) Ver.2

Product Manual

We have begun the process of changing the names for Takara Bio's intercalator-based real-time PCR (qPCR) products to the "TB Green series".
These products can be used the same way as before, as only the names are changing.
Catalog number and product performance are unaffected by this transition.

The long-term storage temperature of TB Green® *Premix Ex Taq*™ II (Tli RNaseH Plus) in this product has been changed to -20℃ since Lot. #AH97918A. See section III. Storage.

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Safety & Handling of Adeno-Associated Virus Vectors

The protocols in this User Manual require the handling of adeno-associated virus vectors. It is imperative to fully understand the potential hazards of and necessary precautions for laboratory use of these vectors.

Viruses produced with AAV-based vectors could, depending on your gene insert, be potentially hazardous. Similar vectors have been approved for human gene therapy trials, attesting to their potential ability to express genes *in vivo*. For these reasons, due caution must be exercised in the production and handling of any recombinant viruses.

Follow all applicable guidelines for research involving recombinant DNA. Take appropriate safety measures when producing or handling recombinant adeno-associated viruses, including working in a biological safety cabinet and wearing protective laboratory coats, face protection, and gloves.

Available AAVpro Products

AAVpro® Helper Free System (AAV2)	Cat. #6230
AAVpro® Purification Kit (AAV2)	Cat. #6232
AAVpro® Purification Kit Maxi (All Serotypes)	Cat. #6666
AAVpro® Titration Kit (for Real Time PCR) Ver.2	Cat. #6233
AAVpro® Extraction Solution	Cat. #6235
pAAV-ZsGreen1 Vector	Cat. #6231
AAVpro® Helper Free System (AAV2-CRE Recombinase)	Cat. #6652
AAVpro® Helper Free System (AAV2-LacZ)	Cat. #6655
AAVpro® Tet-One ™ Inducible Expression System (AAV2)	Cat. #634310

I. Description

The AAVpro Titration Kit (for Real Time PCR) is a kit for determining the titer of adeno-associated virus (AAV) using real-time PCR. This kit contains all of the reagents necessary for extraction of AAV particles from AAV vector-producing cells and real-time PCR assay of virus genome. This kit allows more precise and rapid quantification than conventional DNA blotting or ELISA methods. Quantification is based on amplification of the ITRs (inverted terminal repeats) of AAV, and therefore titer measurement is possible for various serotypes of AAV. The stability of Positive Control has been improved for Ver.2.

AAV Vectors

AAV is a non-enveloped virus that belongs to the *Parvovirus* family of the *Dependovirus* genus. The AAV genome is a linear, single-strand DNA molecule of approximately 4.7 kb that has terminal hairpin structures called inverted terminal repeats (ITRs) at both ends.

Recombinant AAV particles exploit the properties of AAV for transduction of genes to cells and organisms. AAV vectors are used as research tools and also as vectors for gene therapy.

The AAVpro Helper Free System (AAV2) (Cat. #6230) can be used to prepare recombinant AAV2 particles by transfecting HEK293 or other cells with plasmids that express the necessary components required for AAV production.

Measurable AAV Serotypes

The serotype of AAV is determined by the type of Cap gene on the pRC plasmid used to prepare viral particles. The viral genome encapsulated within AAV particles typically contain the ITRs of AAV2, regardless of serotype. This kit uses the ITR sequence of AAV2 as a target to quantify the amount of viral genome. Therefore, it can be used regardless of serotype as long as the ITR domain of the vector genome is of AAV2 origin. Prior to using this kit, confirm that the ITR domain of the AAV vector being used is derived from AAV2.

Simplified AAV Genome Extraction

(A) AAV Extraction using AAV Extraction Solution
Extraction of AAV particles from AAV-producing cells is conventionally performed using freeze-thaw or sonication methods; however, these methods are time consuming and require special equipment. This kit includes AAV Extraction Solutions＊ that allow simple and efficient AAV particle extraction while minimizing protein and nucleic acid contamination.

＊ Also sold separately (AAVpro Extraction Solution, Cat. #6235).

(B) Extraction of AAV Genome from Extracts Containing Viral Particles
Extracts containing AAV particles also contain proteins and nucleic acids from the producer cells. This kit includes DNase I for removal of free nucleic acids and Lysis Buffer for heat-denaturation of proteins in extracts containing AAV particles.

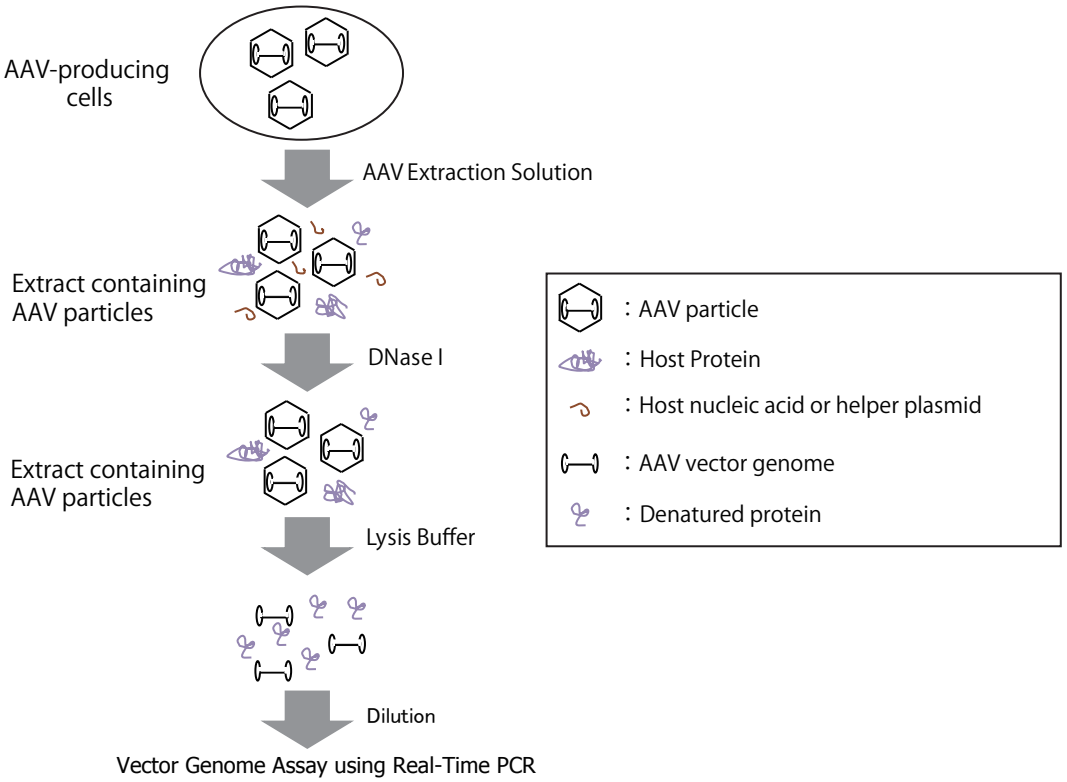

Figure 1. Overview of the AAV particle and vector genome extraction.

Real-Time PCR

Real-time PCR can be used for rapid and sensitive detection of viral genomic DNA. In this method, PCR amplification is monitored in real time using a fluorescent DNA intercalator. The reagent in this kit, TB Green *Premix Ex Taq* II, includes a hot start PCR enzyme and TB Green for fluorescent detection.

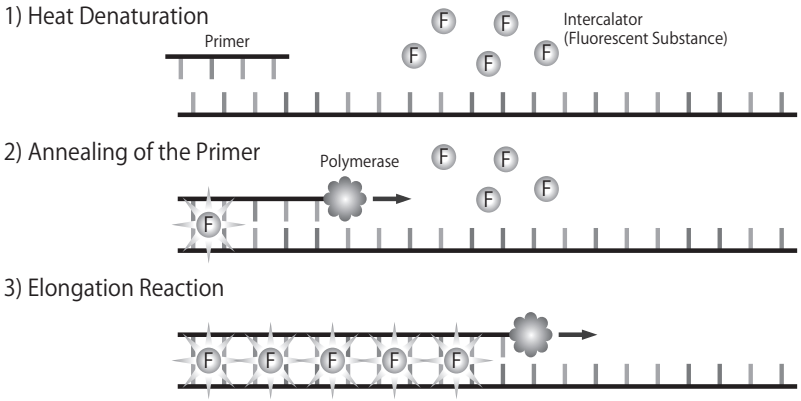

Figure 2. Fluorescent intercalator detection method.

This method involves the addition of an intercalating reagent that emits fluorescence when bound to double-strand DNA in the reaction mixture. This enables the detection of amplified DNA by monitoring fluorescence.

II. Components

1. Extraction of the AAV Vector

AAV Extraction Solution A	1.5 ml x 2
AAV Extraction Solution B	300 µl

2. Simplified AAV Genome Extraction (for 100 samples)

DNase I	100 µl
10X DNase I Buffer	200 µl
Lysis Buffer	1 ml x 2

3. Real Time PCR (For 100 reactions; 25 µl volume)

TB Green *Premix Ex Taq* II (Tli RNaseH Plus)＊1	625 µl x 2
AAV Forward Titer Primer	20 µl
AAV Reverse Titer Primer	20 µl
dH_2O＊2	1 ml x 3
ROX Reference Dye＊3	50 µl
ROX Reference Dye II＊3	50 µl
Positive Control	50 µl
EASY Dilution (for Real Time PCR)	1 ml x 5

＊1 The name of intercalator-based qPCR reagent has been changed.

＊2 dH_2O is also used in the DNase I reaction.

＊3 Use when reactions will be performed on an Applied Biosystems real-time PCR device or another instrument that uses between-well correction of the fluorescent signal.

◆ Add ROX Reference Dye
- StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Add ROX Reference Dye II
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ No ROX Reference Dye
- Thermal Cycler Dice™ Real Time System series (Cat. #TP900/TP960, etc.)＊4

＊4 Not available in all geographic locations. Check for availability in your area.

III. Storage

- TB Green *Premix Ex Taq* II
  Store at 4℃ for up to 6 months. Protect from light and avoid contamination.
  ＊ Keep at -20℃ for long term storage. Once thawed, store at 4℃ and use within 6 months.
- AAV Extraction Solution A and B and Lysis Buffer
  -20℃; store at room temperature after thawing.
- Other components
  -20℃. Use the components other than TB Green *Premix Ex Taq* II within 2 years.

IV. Materials Required but Not Provided

- General equipment for cell culture
- Transfection reagent (e.g., CalPhos™ Mammalian Transfection Kit, Cat. #631312; Xfect™ Transfection Reagent, Cat. #631317)
- 0.5 M EDTA (pH 8.0) (EDTA Buffer Powder, pH 8.0 (Cat. #T9191))
- Heat block (set to 37℃, 70℃, 95℃), or a thermal cycler
- Real-time PCR instrument and tubes
  Thermal Cycler Dice Real Time System *II* (Cat.#TP900/TP960)＊
  Thermal Cycler Dice Real Time System *Lite* (Cat.#TP700/TP760)＊
  Applied Biosystems 7500 Fast Real Time PCR System (Thermo Fisher Scientific)
  StepOnePlus Real Time PCR System (Thermo Fisher Scientific), etc.

＊ Not available in all geographic locations. Check for availability in your area.

V. Precautions for Use

The following are precautions regarding the use of this kit. **Read before use**.

1. This kit includes reaction reagents for the extraction of AAV particles from AAV-producing cells, the extraction of the vector genome from AAV particles, and real-time PCR.
2. Store the primer in the kit at -20℃ without mixing or dilution, and prepare the primer mix immediately before use.
3. For the PCR reaction mixture, prepare a master mix that contains dH_2O, buffer, and enzyme. Preparing a master mix will decrease the loss due to pipetting and increase accuracy and reproducibility.
4. Briefly centrifuge TB Green *Premix Ex Taq* II before use to bring all of the reagent to the bottom of the tube. Pipette slowly and carefully, as it is highly viscous.
5. When dispensing reagents, be sure to use a new disposable tip, and avoid between-sample contamination.
6. When preparing reaction solutions and samples and adding samples to the reaction solution, physically separate the areas and avoid opening and closing tubes that contain amplification products. Because the amplification reaction and detection take place in real time, there is no need for electrophoresis on the amplification products after the reaction is complete.

VI. Protocol

Note: The following procedures include the process of extracting AAV particles from AAV-producing cells. To measure the titer of AAV that has already been isolated, begin the protocol at step VI-2.

VI-1. Extraction of AAV Particles

The following protocol is for extraction from one 10-cm dish containing AAV vector-producing cells. Refer to Table 1 for the amount of solution that should be used for different types of culture vessels.

1. Add 125 µl of 0.5 M EDTA (pH 8.0) (1/80 volume of the culture media) to a 10-cm dish containing AAV vector-producing cells (10-ml culture volume). Refer to Table 1 for the amount of EDTA for different types of culture vessels. Allow to stand at room temperature for 10 min.
2. Transfer the detached cells to a sterile 15-ml centrifuge tube.
3. Centrifuge at 1,750*g* at 4℃ for 10 min. Completely remove the supernatant and collect the cell pellet.

   Note 1: Confirm that the supernatant has been completely removed before proceeding; particle extraction may be affected by residual supernatant.
4. Loosen the cell pellet by tapping or vortexing the tube.

   Note 2: If the cell pellet has not been loosened sufficiently, the efficiency of extraction may decrease. Confirm that there are no clumps of cells before proceeding.
5. Add 0.5 ml of AAV Extraction Solution A.
6. Suspend the cell pellet by vortexing for 15 sec.
7. Allow to stand at room temperature for 5 min. Vortex for 15 sec.
8. Centrifuge at 2,000 - 14,000*g* at 4℃ for 10 min to pellet cell debris.

   Note 3: If the titer of the recovered AAV vector is low, the efficiency may be increased by repeating steps 6 - 8.
9. Collect the supernatant in a new sterile centrifuge tube. Add 50 µl of AAV Extraction Solution B and mix by pipetting.

   Note 4: The mixture can be stored at -80℃. Thaw quickly in a 37℃ water bath before use.

   Note 5 : The supernatant may change to a pink color after AAV Extraction Solution B is added.

Table 1. Amount of solutions required for various culture vessels.

	Volume of Culture	0.5 M EDTA (pH 8.0)	AAV Extraction Solution A	AAV Extraction Solution B
6-cm dish	4 ml	50 µl	200 µl	20 µl
10-cm dish	10 ml	125 µl	500 µl	50 µl
15-cm dish	26 ml	325 µl	1,300 µl	130 µl
T25 flask	4 ml	50 µl	250 µl	25 µl
T75 flask	13 ml	162.5 µl	650 µl	65 µl
T225 flask	40 ml	500 µl	2,000 µl	200 µl

VI-2. Extraction of AAV Vector Genome

1. Treat the AAV particle solution with DNase I and incubate at 37℃ for at least 15 min to digest free genomic DNA and plasmid DNA derived from host cells.

AAV Particle Solution	2 μl
10X DNase I Buffer	2 μl
DNase I	1 μl
dH_2O	15 μl
Total	20 μl

2. Inactivate DNase I by heat treatment at 95℃ for 10 min.
3. Add an equal amount of Lysis Buffer (20 μl).
4. Incubate at 70℃ for 10 min.
5. Dilute the AAV vector genome solution at least 50-fold using EASY Dilution (for Real Time PCR) and use directly as the template for real-time PCR.

VI-3. Real-Time PCR

Use 5 μl of the AAV vector genome solution prepared in VI-2. as the template for real-time PCR. At the same time, use the Positive Control to prepare the standard curve.

1. Sample Preparation for Standard Curve

   Dilute the Positive Control using EASY Dilution to obtain the samples for standard curve preparation. (Use 5 μl of each solution as a template for real-time PCR.)

   (1) 2×10^7 copies/μl (Positive Control solution)
   (2) 2×10^6 copies/μl (5 μl of Positive Control solution + 45 μl of EASY Dilution)
   (3) 2×10^5 copies/μl (5 μl of (2) + 45 μl of EASY Dilution)
   (4) 2×10^4 copies/μl (5 μl of (3) + 45 μl of EASY Dilution)
   (5) 2×10^3 copies/μl (5 μl of (4) + 45 μl of EASY Dilution)
   (6) 2×10^2 copies/μl (5 μl of (5) + 45 μl of EASY Dilution)

2. Preparation of 50X Primer Mix

   Prepare the 50X primer mix as indicated below. Prepare the necessary amount of 50X primer mix immediately prior to use. Do not store.

AAV Forward Titer Primer	5 μl
AAV Reverse Titer Primer	5 μl
TE or dH_2O	15 μl
Total	25 μl

3. Preparation of the Reaction

Prepare the reaction mixtures shown below on ice.
Prepare a master mix containing all of the components except for the template. Prepare sufficient master mix for the required number of tubes plus a few extra, and dispense 20 μl into each reaction tube and close the caps lightly.

(For Thermal Cycler Dice Real Time System)

[1 reaction]

TB Green *Premix Ex Taq* II (2X conc.)	12.5 μl
50X Primer mix	0.5 μl
dH_2O	7.0 μl
Template	(5.0 μl)
Total	25.0 μl

(For Applied Biosystems Real Time PCR Equipment)

[1 reaction]

TB Green *Premix Ex Taq* II (2X conc.)	12.5 μl
50X Primer mix	0.5 μl
ROX Reference Dye or ROX Reference Dye II＊	0.5 μl
dH_2O	6.5 μl
Template	(5.0 μl)
Total	25.0 μl

＊ For StepOnePlus, use ROX Reference Dye, and for Applied Biosystems 7500 Fast Real-Time PCR System, use ROX Reference Dye II.

4. Addition of the Template

Add 5 μl of dH_2O to one tube as the negative control and close the cap of the reaction tube tightly.

Add 5 μl of the template (the sample DNA solution and standards) to the reaction solutions and close the caps tightly. Wear gloves to prevent getting the tubes dirty when closing the caps, as fluorescence will be measured. Centrifuge briefly and place in the real-time PCR amplification device.

5. Start the Real-Time PCR Reaction

Use the following PCR conditions.

＊ Please refer to the instruction manual for the real-time PCR amplification device for specific operation methods.

Initial Denaturation
95℃ 2 min

2 Step PCR
35 Cycles
95℃ 5 sec
60℃ 30 sec (Detection of Fluorescence: FAM)

Melt Curve Analysis

VII. Quantification

The Positive Control in this kit is a plasmid DNA that includes the ITR, adjusted to 2×10^7 copies/μl based on the value at OD_{260}. When serially diluted, it can be used to produce a standard curve for absolute quantification. Because the control contains the same ITR copy number as the AAV vector genome, it is possible to convert copy number to vector genome number (vg/ml and vg/cell). This kit is improved at stability of Positive Control.

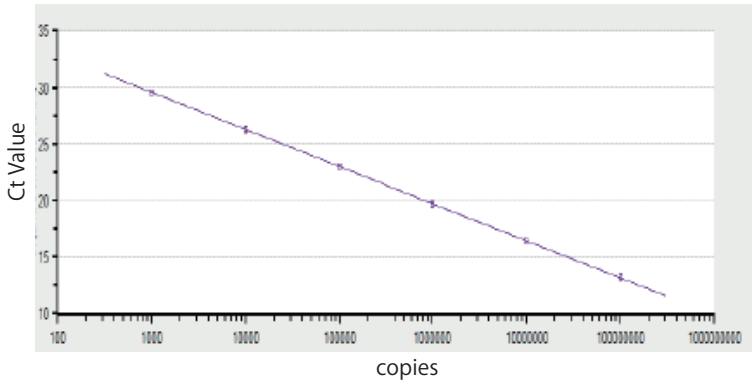

Figure 3. Standard Curve generated with the Positive Control.

VIII. Experimental Examples

Experiment 1: Vector Genome Quantification using ITR and Other Regions

Real-time PCR amplification of either the ITR domain (target in this kit) or the CMV promoter region of the AAV vector genome was performed to quantify vector genomes.

(Methods)
HEK293 cells producing AAV1, AAV2, and AAV6 vectors were prepared. AAV vectors were extracted from these cells using AAV Extraction Solution and quantitative analysis was performed using real-time PCR. The ITR and the CMV promoter regions of the AAV vector genome were amplified and the titer was calculated (Figure 4).

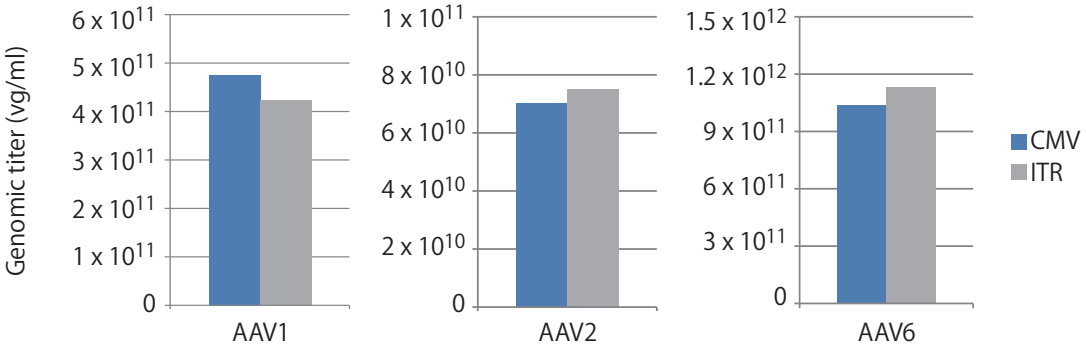

Figure 4. Titer measurement of AAV by real-time PCR using ITR and CMV promoter targets.

(Results)
The titer was roughly identical for both the ITR and CMV promoter targets.

Experiment 2: Comparison of AAV Extraction Solution with the Freeze-Thaw Method

AAV vector was extracted from AAV-producing cells using either the AAV Extraction Solution in this kit or the conventional freeze-thaw method.

(Methods)
HEK293 cells producing AAV1, AAV2, and AAV6 vectors were prepared, and AAV vector was extracted from the cells using the AAV Extraction Solution or the freeze-thaw method. Quantitative analysis of virus titer was performed by real-time PCR using this kit (Figure 5).

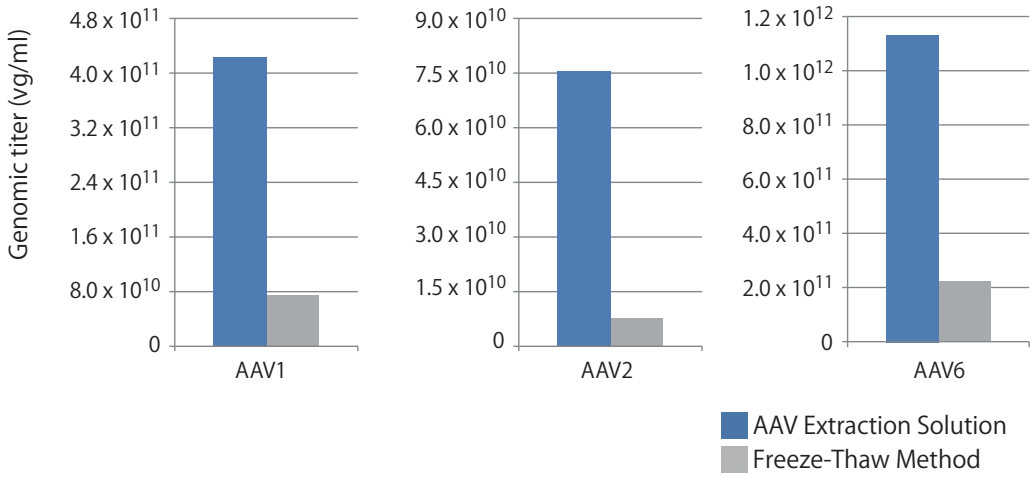

Figure 5. Titer of AAV particles prepared with AAV Extraction Solution or the freeze-thaw method.

(Results)
The use of AAV Extraction Solution for AAV preparation provided higher extraction efficiency than the freeze-thaw method, generating >5X higher titers in this experiment.

IX. Related Products

AAVpro® Helper Free System (Cat. #6230)
pAAV-ZsGreen1 Vector (Cat. #6231)
AAVpro® Purification Kit (AAV2) (Cat. #6232)
AAVpro® Purification Kit Maxi (All Serotypes) (Cat. #6666)
AAVpro® Extraction Solutions (Cat. #6235)
AAVpro® Helper Free System (AAV2-CRE Recombinase) (Cat. #6652)
AAVpro® Helper Free System (AAV2-LacZ) (Cat. #6655)
AAVpro® Tet-One™ Inducible Expression System (AAV2) (Cat. #634310)
Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960)*
Thermal Cycler Dice™ Real Time System *Lite* (Cat. #TP700/TP760)*
EASY Dilution (for Real Time PCR) (Cat. #9160)

* Not available in all geographic locations. Check for availability in your area.

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