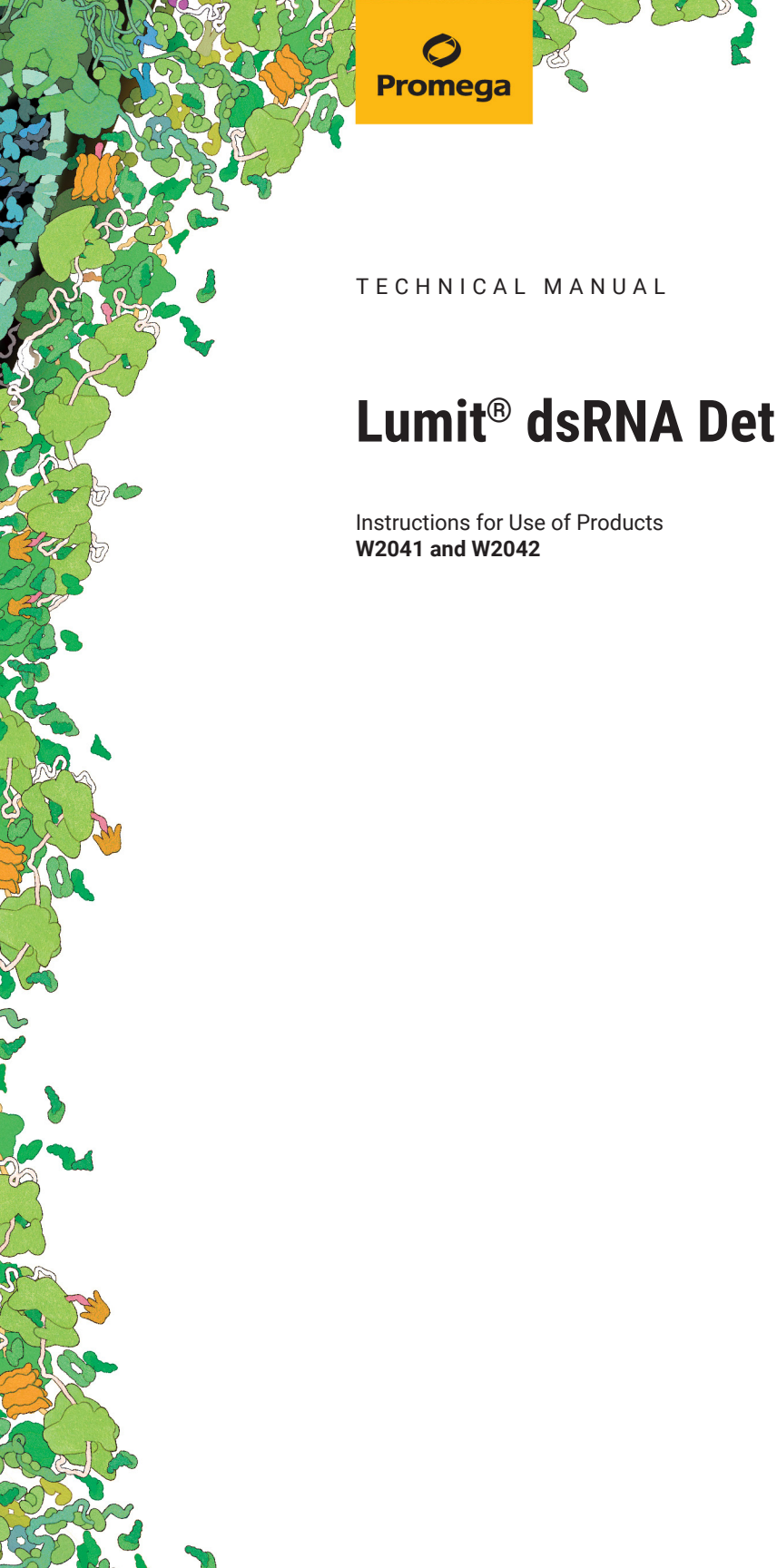

TECHNICAL MANUAL

Lumit® dsRNA Detection Assay

Instructions for Use of Products
W2041 and W2042

11/24
TM746

Lumit® dsRNA Detection Assay

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

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1. Description

Messenger RNA (mRNA) is a therapeutic modality designed to express a specific protein within target cells by using the cell's translation machinery. Large-scale mRNA production is typically achieved through in vitro transcription (IVT), a process in which T7 RNA polymerase transcribes a DNA template into single-stranded mRNA. However, this process often generates double-stranded RNA (dsRNA) as a byproduct, with its formation influenced by factors such as reaction conditions, the choice of RNA polymerase and the purification methods employed. dsRNA is highly immunogenic and readily detected by various cellular sensors, resulting in inflammation, inhibition of protein translation and cell death (1). Therefore, accurate dsRNA quantification is essential to ensure the safety and efficacy of mRNA-based therapies.

Traditional methods for quantifying dsRNA, such as dot blot or enzyme-linked immunosorbent assay (ELISA), rely on antibody-based detection. However, these methods are often limited by sequence bias, reduced sensitivity and labor-intensive protocols (2). In contrast, the Lumit® dsRNA Detection Assay[a–c] offers a homogeneous, bioluminescent approach for dsRNA quantification, eliminating the need for antibodies or wash steps. This assay uses NanoLuc® Binary Technology (NanoBiT), a split luciferase complementation system specifically designed for biomolecular interaction studies (3). NanoBiT comprises two subunits: Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acids), both of which are engineered for stability and minimal spontaneous association. In the Lumit® dsRNA Detection Assay, the sample containing dsRNA is incubated with two dsRNA-binding domains, one labeled with LgBiT and the other with SmBiT. When these binding domains interact with dsRNA, the LgBiT and SmBiT subunits are brought into proximity, reconstituting the NanoBiT® enzyme and producing luminescence in the presence of the Lumit® substrate. The resulting luminescence is directly proportional to the dsRNA concentration in the sample, enabling rapid and accurate quantification without wash steps.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® dsRNA Detection Assay	**1 each**	**W2041**

Each kit provides sufficient reagents to test one 96-well plate with one dsRNA standard curve. Includes:

- 15µl dsRNA Standard
- 15µl Lumit® dsRNA Sensor-SmBiT
- 15µl Lumit® dsRNA Sensor-LgBiT
- 8ml dsRNA Assay Buffer (5X)
- 160µl Lumit® Detection Substrate B

PRODUCT	SIZE	CAT.#
Lumit® dsRNA Detection Assay 5X	**1 each**	**W2042**

Includes 5 × Cat.# W2041 kits. Each kit contains:

- 15µl dsRNA Standard
- 15µl Lumit® dsRNA Sensor-SmBiT
- 15µl Lumit® dsRNA Sensor-LgBiT
- 8ml dsRNA Assay Buffer (5X)
- 160µl Lumit® Detection Substrate B

Storage Conditions: Upon arrival, immediately transfer components to a –30°C to –10°C freezer. To prevent repeated freeze-thaw cycles, **do not** store the components in a frost-free freezer. Briefly centrifuge tubes to collect contents at the bottom of the tube and mix by pipetting prior to use.

3. Before You Begin

Components do not contain preservatives; handle aseptically to avoid microbial and nuclease contamination. The assay buffer contains a carrier protein; do not vortex when preparing or mixing intermediate dilutions.

Materials to Be Supplied By the User

- nuclease-free water or Barnstead NANOpure® purified water
- white 96-well plate polypropylene plate (e.g., Thermo Fisher Scientific Cat.# 267350; Eppendorf Cat.# 0030601475; Greiner Cat.# 650207; or NBS surface [Corning® Cat.# 3600, Greiner Cat.# 655904])
  Note: Assay is **not** compatible with tissue culture-treated plates.
- lids for 96-well plate (Corning® Cat.# 3098) or adhesive plate sealer
- sterile polypropylene tubes for sample handling and dilutions
- sterile polypropylene dilution reservoirs with lid (Dilux™ Cat.# D-1002 or USA Scientific Cat.# 3823-3512) for standard curve dilution series
- reagent reservoir trays
- plate shaker for mixing multiwell plates
- plate reader capable of detecting glow-type luminescence from multiwell plates (e.g., GloMax® Discover System, Cat.# GM3000)

4. dsRNA Detection Assay Protocol

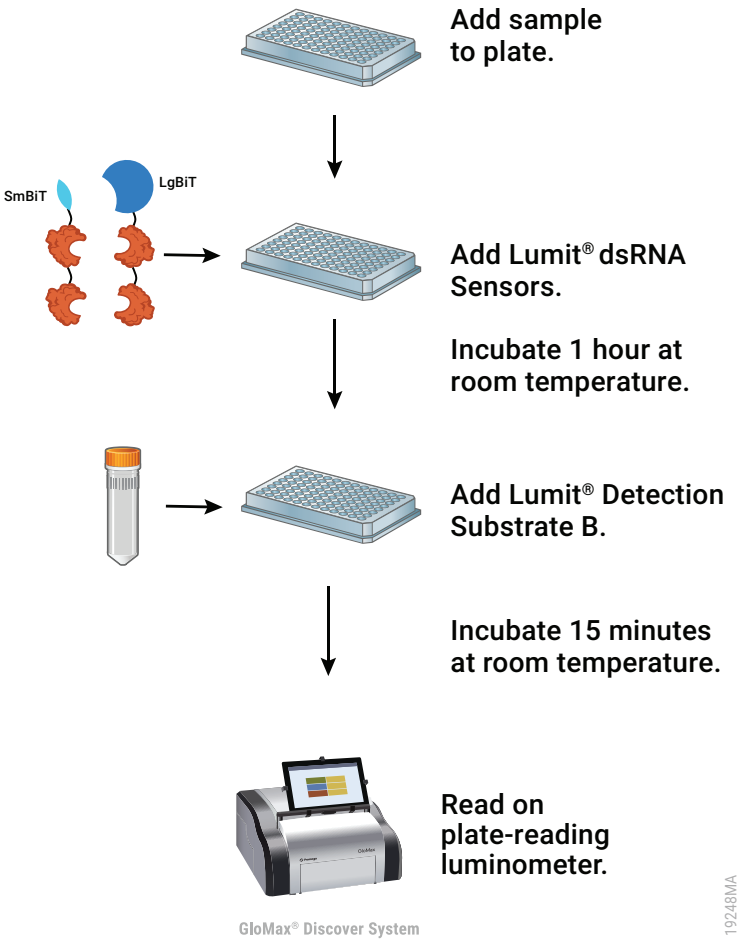

Figure 1. Lumit® dsRNA Detection Assay schematic protocol.

4.A. Preparing dsRNA Standard Curve

Prepare the dsRNA Standard, Lumit® dsRNA Sensors and Lumit® Detection Substrate B on the day of use. A standard curve must accompany test samples for each assay plate. The protocol below describes preparation of reagents sufficient for one 96-well plate.

Note: Prior to use, briefly centrifuge tubes to collect contents at the bottom of the tube. Mix by pipetting.

Assay Buffer

1. Thaw the dsRNA Assay Buffer (5X) in a room temperature water bath or on the benchtop and mix thoroughly prior to use.
2. Prepare 35ml of Assay Buffer by diluting with nuclease-free water, as shown in the table:

Component	Final Concentration	Volume Added
dsRNA Assay Buffer (5X)	20%	7ml
nuclease-free water	80%	28ml

3. Thaw tube of dsRNA Standard (100µg/ml) and gently mix by pipette.
4. Using sterile polypropylene tubes, prepare serial dilutions to achieve a 2.5ng/ml solution in Assay Buffer as follows (see Figure 2):
   a. Add 10µl of 100µg/ml dsRNA Standard to 90µl of Assay Buffer (1:10). Mix thoroughly by pipetting.
   b. Transfer 10µl of 10µg/ml dsRNA Standard to 990µl of Assay Buffer (1:100). Mix thoroughly by pipetting.
   c. Transfer 25µl of 100ng/ml dsRNA Standard to 975µl of Assay Buffer (1:40) to create 2.5ng/ml dsRNA.

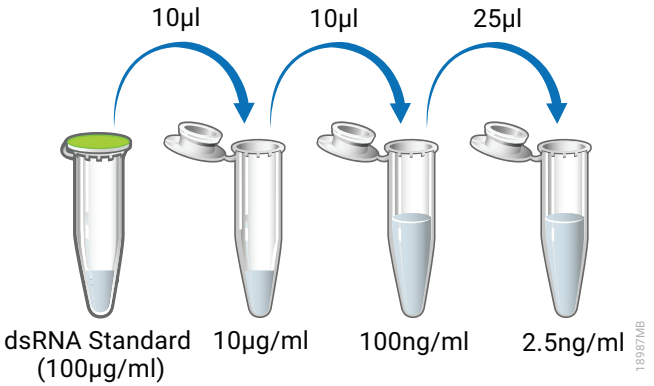

Figure 2. Dilution scheme for dsRNA Standard.

4.A. Preparing dsRNA Standard Curve (continued)

5. To a polypropylene dilution reservoir, add 500µl/well of Assay Buffer to wells 1–7.
6. Add 1ml of diluted dsRNA Standard (2.5ng/ml) to well 8.
7. Prepare samples for an eight-point standard curve by performing serial twofold dilutions (500µl/well) from wells 8–2.
8. Stop after mixing well 2. Well 1 is the negative control (0ng/ml dsRNA).

Note: To develop a standard curve with your own dsRNA, perform a preliminary experiment to determine the actual linear response concentration range. Choose a concentration range that maintains the linear response of the assay. The final linear concentration range may be impacted by the presence of modified nucleosides, the absolute determined concentration of sample material, purity and sensitivity of your plate luminometer. See Figure 4 for a representation of linear range and luminescence response outside the linear range.

4.B. Preparing Test Samples

Dilute samples containing >2.5ng/ml of dsRNA to ensure they are within the linear range of the assay. If the dsRNA concentration is unknown, we recommend several dilutions (e.g., undiluted, 1:10, 1:100) to ensure one dilution is within the linear range of the assay. When analyzing the data, if multiple samples fall within the linear range, consider using their average calculated concentration.

Note: Samples with dsRNA concentrations that are too high will result in reduced luminescence values (see Figure 4).

To extend the supply of the dsRNA Assay Buffer (5X) provided with the kit, TE buffer (10mM Tris-HCl/1mM EDTA, pH 8.0) can be used for initial sample dilutions down to 100µg/ml.

In our experience, dsRNA concentrations range from 0.05%–1% for in vitro transcription products with simple purification (e.g., ion-exchange column). Therefore, we recommend diluting the sample to a concentration of 200ng/ml total RNA, which is likely to result in dsRNA readings within the linear range of the assay (0.04–2.5ng/ml).

Note for testing IVT samples: Several dilutions with total RNA concentrations higher and lower than 200ng/ml are recommended to ensure the readings fall within the linear range of the assay.

1. Prepare appropriate serial dilutions of test samples in polypropylene tubes using Assay Buffer.

4.C. Adding Samples to Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0									
B	0.04ng/ml	0.04ng/ml	0.04ng/ml									
C	0.08ng/ml	0.08ng/ml	0.08ng/ml									
D	0.16ng/ml	0.16ng/ml	0.16ng/ml									
E	0.31ng/ml	0.31ng/ml	0.31ng/ml									
F	0.63ng/ml	0.63ng/ml	0.63ng/ml									
G	1.25ng/ml	1.25ng/ml	1.25ng/ml									
H	2.5ng/ml	2.5ng/ml	2.5ng/ml									

Figure 3. Recommended plate layout. Use columns 1–3 for the dsRNA standard curve for each plate. Use the remaining wells for test samples.

1. Using an electronic multichannel pipette, add 50µl/well of dsRNA standard curve samples (prepared in Section 4.A) to a white 96-well assay plate.
2. Add 50µl/well of test samples to the same plate.
3. Place lid on plate and keep on bench top while preparing the dsRNA sensor reagent in Section 4.D.

4.D. Preparing and Adding dsRNA Sensor Reagent

Prepare the following reagent and use within 60 minutes.

1. Remove the Lumit® dsRNA Sensors from the freezer and briefly centrifuge. Gently mix each sensor by pipette.
2. Add 10µl of Lumit® dsRNA Sensor-SmBiT to 918µl of Assay Buffer. Mix thoroughly by pipetting.
3. Add 10µl of Lumit® dsRNA Sensor-LgBiT to 64µl of Assay Buffer. Mix thoroughly by pipetting.
4. Prepare dsRNA sensor reagent by adding 20µl of each sensor working dilution to 7ml of Assay Buffer. Mix thoroughly using a 5ml pipette.
5. Add dsRNA sensor reagent to a reagent reservoir tray. Using a multichannel pipette, add 50µl/well of dsRNA sensor reagent to sample wells. Add lid and place plate on plate shaker.
6. Gently shake at approximately 300rpm for 1 minute.
7. Incubate plate for 60 minutes at room temperature.

4.E. Preparing and Adding Lumit® Detection Substrate

Prepare this substrate near the end of the 60-minute incubation (Section 4.D, Step 7). The diluted substrate is stable for 30 minutes once prepared. Protect from light until use.

1. Remove Lumit® Detection Substrate B from freezer and mix by pipetting.
2. Add 160µl of Substrate B to 3.04ml of Assay Buffer and mix by inverting the tube.
3. Add the substrate to a reagent reservoir tray. Using a multichannel pipette, add 25µl/well to all samples.
4. Gently shake the plate for 1 minute at 300rpm on a plate shaker. Protect the plate from light during incubation.
5. Incubate plate for 15 minutes at room temperature, then record luminescence.

5. Data Analysis

1. Determine background by obtaining the average luminescence relative light units (RLU) from the 0ng/ml dsRNA standard.
2. Subtract this background RLU from all samples (standard curve and test samples).
3. Use curve fitting software such as GraphPad Prism to create a dsRNA standard curve using these subtracted values: Plot both y (RLU, subtracted) and x ([dsRNA]) axes in log format.
4. Interpolate test sample concentrations using linear regression curve fitting.
5. Multiply the results by the sample dilution factor to determine the concentration of dsRNA in the undiluted sample.

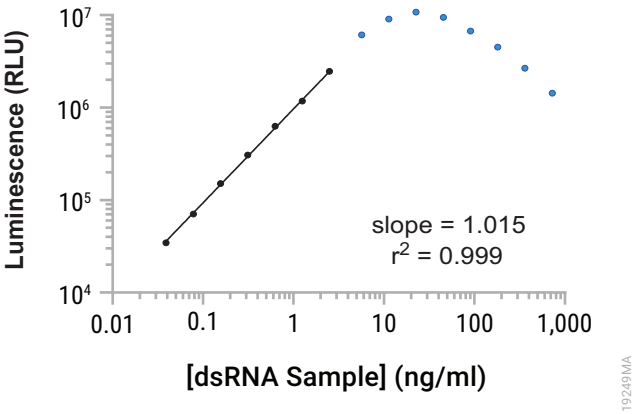

Figure 4. Standard curve response range of the dsRNA Standard, demonstrating linear (black circles) and nonlinear (blue circles) response concentrations. A nonlinear response showing signal inhibition is due to the loss of BiT complementation proximity. Luminescence was plotted after subtracting background, using a GloMax® Discover and a 0.5 second integration time. This figure is for illustration only. Always perform a standard curve on each experimental plate. Absolute RLU values will vary due to luminometer sensitivity and settings.

6. 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**

Symptom	Causes and Comments
Poor standard curve linearity	Careful pipetting and thorough mixing of all intermediate dilutions (standard, standard curve dilutions, SmBiT and LgBiT sensors) are critical for obtaining a linear response.
	Background subtracted data should be used to generate a standard curve, preferably plotting both y (RLU, subtracted) and x ([dsRNA]) axes in log format.
	Excess concentrations of dsRNA will demonstrate loss of linearity; if using a user-supplied dsRNA standard, adjustments to the standard curve may be necessary.
Increasing sample dilution results in higher luminescence	If the luminescence signal **increases** with an increasingly diluted sample, that sample contains dsRNA in excess of the linear range of the assay and must be further diluted. See Figure 4 (blue circles) for visualization.
Poor sensitivity	Careful pipetting and thorough mixing of all intermediate dilutions (standard, standard curve dilutions, SmBiT and LgBiT sensors) are critical for maintaining assay sensitivity.
	Low-sensitivity luminometers may not detect some of the more dilute standard curve point(s). Adjust gain to highest setting, if possible.
	Kit compatibility with dsRNA samples containing modified nucleotides has not been tested extensively. Reduced signal may occur when using samples containing modified nucleotides.
Poor replicate values	Some white plates are prone to poor replicate reproducibility; use recommended white plates.

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7. References

1. Chen, Y.G. and Hur, S. (2022) Cellular origins of dsRNA, their recognition and consequences. *Nat. Rev. Mol. Cell Biol.* **23**, 286–301.
2. Bonin, M. *et al.* (2000) Determination of preferential binding sites for anti-dsRNA antibodies on double-stranded RNA by scanning force microscopy. *RNA,* **6** 563–70.
3. Dixon, A.S. *et al.* (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol.* **11**, 400–8

8. Related Products

Supplemental dsRNA Reagents

Product	Size	Cat.#
Lumit® dsRNA Assay Buffer	8ml	W2010
Lumit® dsRNA Standard	15µl	W2040

RNA Production

Product	Size	Cat.#
RiboMAX™ Large Scale RNA Production Systems-SP6	—	P1280
RiboMAX™ Large Scale RNA Production Systems-T7	—	P1300
T7 RiboMAX™ Express Large Scale RNA Production System	—	P1320
T7 RiboMAX™ Express RNAi System	50 × 20µl	P1700
QuantiFluor® RNA System	1ml	E3310
RQ1 RNase-Free DNase	1,000 units	M6101
ProNex® Size-Selective Purification System*	10ml	NG2001

*Additional kit formats are available.

[a]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.

[b]U.S. Pat. Nos. 9,797,889, 9,797,890, 10,107,800 and 10,648,971 and other patents and patents pending.

[c]Patent Pending.