Antibody Validation			
Company and location	Antibody	Catalog #	RRID
Easybio, China	Goat Anti-Mouse IgG(H+L) HRP	BE0102	AB_2923205
Cell line			
Cell name	ATCC number	RRID	
HEK293T cells	ATCC CRL-3216	CVCL_0063	
Vero cells	ATCC CCL81	CVCL_0059	
Virus			
VACV-WR	Kind gift from Prof. Min Fang from the Institute of Microbiology, CAS		
VACV-VTT	Prof. Wenjie Tan from (National Institute for Viral Disease Control and Prevention, China CDC)		
MPXV clade IIb	Isolated from a mpox patient by Wuhan Institute of Biological Products Co., Ltd.		
Enzyme			
Company and location	Antibody	Catalog #	RRID
New England Biolabs, USA	BamHI	R0136M	-
Novoprotein, China	T7 High Yield RNA Transcription kit	E131	-
Kit			
Company and location	Antibody	Catalog #	RRID
Mirus Bio, USA	TransIT-mRNA	MIR 2250	-
Thermo Fisher, USA	Ribogreen RNA reagent	R11490	-
Dakewe Biotech Co, China	Precoated ELISPOT Kit	2210006	-

T7 RNA Transcription Enzyme Mix, GMP Grade

Cat. No.: GMP-E131 **animal-free, ampicillin-free**

01/ Product Description

As a biological macromolecule, mRNA can be synthesized on a large scale by in vitro transcription (IVT). T7 promoter is one of the promoters with the highest transcription efficiency. Therefore, T7 RNA Polymerase can be used for in vitro transcription to obtain more synthetic products. T7 RNA Transcription Enzyme Mix has been optimized by a series of transcription systems. One reaction can transcribe up to 150-200μg of RNA, and the synthesized RNA can be used downstream in many aspects such as mRNA vaccine preparation, RNA structure and function research, RNase protection, probe hybridization, RNAi, microinjection and in vitro translation application.

The original enzymes of T7 RNA Transcription Enzyme Mix produced in *E. coli*. Our manufacturing processes are strictly controlled to ensure the end products free from host protein or nucleic acid contaminations and other impurities following the Pharmaceutical Manufacturing Guidelines. We guarantee the manufacturing and quality control comply with GMP regulation for tracking each and every step of the manufacturing process, including raw material sourcing.

This product has passed the HALAL certification.

02/ Quality Criterion

Element	Standard
Appearance	Clear and transparent solution
Visible Particles	Meet the specification
pH	7.5-8.5
Performance	1μl enzyme mix can be used to transcribe no less than 150μg RNA
Endonuclease Residues	The degradation of substrate was ≤10%
Exonuclease Residues	The degradation of substrate was ≤10%
RNase Residues	The degradation of substrate was ≤10%
Bacterial Endotoxins	<10EU/mg
Exogenous DNA Residues	≤100pg/mg
Host-cell Protein Residues	≤50ppm
Mycoplasma	Negative
Heavy Metals	≤10ppm
Microbial Limit	Total aerobic microbial count≤1cfu/10ml, total yeasts and molds count≤1cfu/10ml

03/ Complying to Following Regulations

1. ISO 9001:2015, certified facility.
2. GMP Appendix – Cellular therapeutic product National Medical Products Administration.
3. The Pandect of Genetic Therapeutic Product for Human Chinese Pharmacopoeia Commission.
4. USP Chapter <1043>, Ancillary Materials for Cell, Gene, and Tissue-Engineered Products.
5. USP Chapter <92>, Growth Factors and Cytokines Used in Cell Therapy Manufacturing.
6. Ph. Eur. General Chapter 5.2.12, Raw Materials of Biological Origin for the Production of Cell-based and Gene Therapy Medicinal Products.

International Region
Website: www.novoprotein.com
E-mail: product@novoprotein.com

China
TEL: +86-400-600-0940; +86-21-50798060
Website: www.novoprotein.com.cn
E-mail: product@novoprotein.com.cn
ADD: BLDG 1, No.11 Jialilue Road, Zhangjiang Hi-Tech Park, Shanghai 201203, China

04/ Application

1. Single stranded RNA synthesis
2. RNA probe synthesis.
3. siRNA precursor synthesis
4. Precursor for RNA splicing preparation
5. Capped RNA synthesis.

05/ Storage Conditions

At -20±5℃.

06/ Product Packaging

SKU	Components	Volume
GMP-E131-01A	T7 RNA Transcription Enzyme mix, GMP Grade	50μl
GMP-E131-M001	T7 RNA Transcription Enzyme mix, GMP Grade	1ml
GMP-E131-M010	T7 RNA Transcription Enzyme mix, GMP Grade	10ml

07/ Precautions

1. Template efficiency and incubation time:

   This kit can generate 150-200μg of RNA with 1μg of template input, however, the yield of different templates will vary depending on the sequence, structure, length, purity of the template and the sequence and length of the specific RNA polymerase promoter. Contaminants that affect transcript yield include RNases or contaminants such as phenol, trace metals, and SDS.

2. Optimized reaction:

   The recommended reaction conditions are suitable for in vitro transcription of most templates, however, for some templates, the yield can be improved by increasing the reaction time (4 hours-overnight reaction) and increasing the amount of template.

3. The amount of templates:

   The table below summarizes our experience in regulating the amount of templates. Results may vary depending on the template used, and extending the reaction time to 4-6 hours increases the yield of RNA.

The amount of templates	The yield of RNA
1000ng (1μg)	130-160μg
500ng (0.5μg)	110-130μg
100ng (0.1μg)	30-50μg
50ng (0.05μg)	15-25μg
10ng (0.01μg)	10-20μg
1ng (0.001μg)	3-8μg

4. Maintain RNase-free environment:

   Use RNase-free tubes and pipettes;

   Gloves should be worn and changed frequently when handling kit components or samples containing RNA, especially after exposure to potential sources of RNase contamination such as doorknobs, pens, pencils, and human skin.

   All reagents should be sealed when not in use. During incubation, all tubes containing RNA were sealed.

5. Since the 10×Transcription Buffer contains spermidine, which may bind nucleic acid and, generate insoluble complex at low temperature, it is recommended not adding template DNA and enzyme until the last step.

08/ Template Preparation

Linearized plasmids with double-stranded T7 promoter, PCR products or synthetic DNA fragments can be used as templates for in vitro transcription of T7 High Yield RNA Transcription Kit, and the templates can be dissolved in TE buffer or RNase-free Water.

1. Plasmid template (recommended to add 1μg of linearized plasmid to each reaction as template)

Plasmids with T7 promoter can be used as transcription templates. The linearization and purity of plasmids will affect the yield of transcription and the integrity of RNA. Due to the lack of effective termination, circular plasmids will transcribe RNA products of different lengths. To obtain RNA of a certain length, the plasmid must be fully linearized, and the linearized plasmid must ensure that the duplex is blunt-ended or 5' -terminal protruding.

2. PCR product template (recommended to add 0.1μg~1μg to each reaction as template)

PCR products with T7 promoter can be used as templates for in vitro transcription. The T7 promoter was added to the 5' end of the upstream primer of the sense strand when PCR amplifying the template. The PCR product was purified and used as a template.

3. Synthesized DNA template (recommended to add 0.1μg~0.5μg to each reaction as template)

Synthetic DNA fragments with T7 promoters can also be used as templates for in vitro transcription.

09/Protocol

1. In Vitro Transcription

1) Mix the components well except T7 RNA Transcription Enzyme mix, GMP Grade, centrifuge briefly to collect the liquid at the bottom of the tube, and put it on ice for later use.

Note: Please don't add template DNA and enzyme until the last step.

2) Add the following components:

1. Unmodified RNA transcription system

Components	Quantity
10×Transcription Buffer, GMP Grade	2μl
ATP/GTP/CTP/UTP (100mM)	1.5μl for each
Template DNA	100ng-1μg
T7 RNA Transcription Enzyme mix, GMP Grade	1μl
RNase Free Water Up to	20μl

2. Modified RNA transcription system

Components	Quantity
10×Transcription Buffer, GMP Grade	2μl
Modified ATP/GTP/CTP/UTP (100mM)	1.5μl for each
Template DNA	100ng-1μg
T7 RNA Transcription Enzyme mix, GMP Grade	1μl
RNase Free Water Up to	20μl

Modified NTP such as pUTP, 5-Me-CTP, N1-Me-pUTP, 5-OMe-UTP, etc.

3. Co-transcription system

Components	Quantity
10×Transcription Buffer, GMP Grade	2μl
Modified ATP/GTP/CTP/UTP (100mM)	1.5-2μl for each
CAP1 GAG (100mM)	1.5-2μl
Template DNA	100ng-1μg
T7 RNA Transcription Enzyme mix, GMP Grade	1μl
RNase Free Water Up to	20μl

This system takes the Novoprotein CAP1 GAG as an example. If other cap analog are used, please refer to its recommended reaction system.

3) Gently mix the components with a pipette, collect by centrifugation briefly, and incubate at 37°C for 3 h.

▲In order to avoid the influence of evaporation on the reaction system, it is recommended to carry out the reaction in a PCR machine. The reaction time can be appropriately adjusted according to the length of the product fragments. For example, if RNA less than 0.3 kb is synthesized, the reaction can be extended to 4 h or longer, and the 16 h overnight reaction will not affect the quality of the product.

▲The mixing time of the reaction buffer and the enzyme or template should be shortened as much as possible before the reaction starts. The high-salt environment can easily affect the enzyme activity, which can be adjusted by adding the enzyme and template at the end.

4) Add 2-4U of DNase I, GMP Grade (Cat. No.: GMP-E127) to the reaction system, incubate at 37°C for 15 min, and digest the transcribed DNA template (optional).

▲Compared with product RNA, the content of template DNA is very low. Generally, it does not need to be removed. It can also be digested with DNase I.

5) The synthesized RNA can be used for downstream experiments after electrophoresis analysis and purification.

▲The product concentration is extremely high, and it needs to be diluted with RNase-free Water before detection.

2. RNA Purification

2.1 Method 1: Phenol/chloroform purification method

Phenol/chloroform extraction removes proteins and most free nucleotides.

a. Add 160μl RNase-free Water to dilute the product to 180μl.

b. Add 20μl of 3M sodium acetate (pH 5.2) to the diluted product and mix well with a pipette.

c. Add 200μl of phenol/chloroform mixture (1:1) for extraction, centrifuge at 10,000 rpm for 5 min at room temperature, and transfer the upper layer solution (aqueous phase) to a new RNase-free EP tube.

d. Add the same volume of chloroform as water to extract twice, and collect the upper aqueous phase.

e. Add 2 volumes of absolute ethanol and mix well, incubate at -20°C for at least 30 minutes, and centrifuge at 15,000 rpm for 15 minutes at 4°C.

f. Discard the supernatant and add 500μl of pre-chilled 70% ethanol to wash the RNA pellet, centrifuge at 15,000 rpm at 4°C, and discard the supernatant.

g. Open the lid and dry for 2 min. Add 20-50μl RNase-free Water or other buffers to dissolve the RNA precipitate.

h. Store at -70°C.

2.2 Method 2: Column purification

Column purification can remove proteins and free nucleotides.

Add 80μl RNase-free Water to dilute the product to 100μl before purification, and then purify according to the column purification instructions.

▲ Due to the high RNA yield, in order to avoid exceeding the loading capacity of the binding column, please estimate the number of columns required.

2.3 Method 3: Magnetic beads purification

Magnetic beads purification can remove proteins and free nucleotides.

Purify according to the magnetic bead purification instructions.

2.4 Method 4: Lithium chloride purification

a. Add 30μl Lithium Chloride Precipitation Solution (7.5 M Lithium Chloride, 50 mM EDTA) and 30μl RNase Free Water to 20μl product RNA (Note: RNA is less than 300nt or concentration is less than 100ng/μl, effective precipitation cannot be obtained by this method. The best precipitation effect was obtained when RNA concentration was greater than 400ng/μl. When the concentration of the transcription product is low, at 100 400ng/μl, it does not need to be diluted with water and precipitates directly with 30μl Lithium Chloride Precipitation Solution.).

b. After mixing, put it at -20℃ for at least 30 min.

c. Centrifuge at 12,000 rpm for 15 min, remove the supernatant, and collect the pellet.

d. Wash three times with pre-chilled 70% ethanol.

e. Detection after reconstitution in RNase Free Water.

3. RNA Quantification

Ultraviolet absorption method: Free nucleotides will affect the accuracy of quantification. Please perform RNA purification before using this method.

Dye method: RNA quantification is performed with RiboGreen dye, free nucleotides will not affect the quantification, and RNA in purified or unpurified reaction products can be accurately quantified.

10/ FAQ

1. How to choose a restriction endonuclease when linearizing a plasmid template?

A plasmid with a promoter can be used as a transcription template. The linearization and purity of the plasmid will affect the yield of transcription and the integrity of the RNA. Since the circular plasmid has no effective termination, RNA products of different lengths will be transcribed. In order to obtain RNA of a specific length, the plasmid must be completely linearized, and the linearized plasmid must ensure that the double strand is blunt-ended or 5' -terminal protruding. Therefore, it is necessary to select a class II restriction endonuclease that can produce a blunt end or 5' -terminal protruding, and the recognition site of the enzyme is a rare site.

2. Is there a requirement for the purity of the transcription template?

Template DNA should be RNase A-Free and high purity, and the recommended OD260/280 is 1.8~2.0.

3. Does the transcription template have to be removed?

It is best to add DNase I to remove the template after transcription is complete.

4. Low transcript yield or transcription failure:

A control group and an experimental group are recommended. For low yields in the control group, please contact Novorotein for

technical support. If the experimental yield of the control group is normal but the yield of the experimental group is low, there may be a quality problem of the template itself that leads to the low yield. Please try the following solutions:

a) There are components that inhibit the reaction in the experimental template. It is recommended to repurify the template to determine the quantification and integrity of the template;

b) For the problem of the experimental template sequence, it is recommended to extend the reaction time at 37°C, increase the amount of template input, or try other promoters and RNA polymerases.

c) Due to the high concentration of 10×Transcription Buffer, the high-salt environment will lead to the inactivation of the polymerase. At the same time, the buffer contains arginine, which will form a precipitate with the template DNA. When preparing the reaction solution, it is necessary to adjust the order of component addition and calculate the system, add the components in the following order: water, buffer, NTP, template, enzyme.

5. Low yield of short fragment transcripts:

When the transcript is less than 0.3kb, prolonging the reaction time or increasing the amount of template can improve the RNA yield.

6. Product electrophoresis tailing phenomenon:

a) The experimental operation process is contaminated by RNase;

b) DNA template is contaminated with RNase;

c) It is recommended to repurify the template DNA, and pay attention to RNase contamination control in all experimental procedures.

7. The RNA product fragment is larger than expected:

The plasmid template is not completely linearized or the 3' end of the sense strand has an overhang structure. It is recommended to re-linearize the plasmid template to ensure that the plasmid is completely linearized and the linearized plasmid must be blunt-ended or 5' -terminal protruding;

RNA has incomplete denatured secondary structure, replace the denaturing gel to detect RNA products.

8. The RNA product fragment is smaller than expected:

a) The template sequence includes a termination sequence similar to T7 RNA polymerase, which leads to premature termination of transcription. It is recommended to try to replace the RNA polymerase;

b) Advanced structure is formed in the template, it is recommended to add SSB protein;

c) RNase contamination.

11/ Related Product

Cat. No.	Product Name	Cat. No.	Product Name
GMP-M062	Vaccinia Capping Enzyme, GMP Grade	GMP-E125	RNase Inhibitor, GMP Grade
GMP-M072	mRNA Cap 2´-O-Methyltransferase, GMP Grade	GMP-E127	DNase I, GMP Grade
GMP-M012	*E. coli* Poly(A) Polymerase, GMP Grade	GMP-M036	Pyrophosphatase, Inorganic (yeast), GMP Grade
GMP-E121	T7 RNA Polymerase, GMP Grade	GMP-S023-026	NTPs, GMP Grade

*Trans*IT®-mRNA Transfection Kit

Protocol for MIR 2225, 2250, 2255, 2256

Quick Reference Protocol, MSDS and Certificate of Analysis available at mirusbio.com/2225

INTRODUCTION

*Trans*IT®-mRNA Transfection Kit is designed to transfect RNA into a broad range of cell types with minimal cellular toxicity. RNA delivery avoids transcriptional regulation effects by directly delivering the RNA to the cytoplasm for expression. The *Trans*IT-mRNA Transfection Kit can be used to deliver a variety of RNA molecules including mRNAs and viral RNAs (2–10 kb). It can be used for multiple applications such as short-term protein expression, viral production and replication studies.

*Trans*IT-mRNA Transfection Kit contains two components, namely: *Trans*IT-mRNA Reagent and the mRNA Boost Reagent. This kit is compatible with serum; transfection efficiency is optimal when transfections are performed in the presence of serum, with no medium change required.

SPECIFICATIONS

Storage	Store both *Trans*IT-mRNA Reagent and mRNA Boost Reagent at 4°C. ***Before each use***, warm to room temperature and vortex gently.
Product Guarantee	1 year from the date of purchase, when properly stored and handled.

Warm *Trans*IT-mRNA and mRNA Boost Reagent to room temperature and vortex gently before each use.

MATERIALS

Materials Supplied

*Trans*IT-mRNA Transfection Kit is supplied in ***one*** of the following formats.

Product No.	Volume of *Trans*IT-mRNA Reagent	Volume of mRNA Boost Reagent
MIR 2225	1 × 0.4 ml	1 × 0.4 ml
MIR 2250	1 × 1.0 ml	1 × 1.0 ml
MIR 2255	5 × 1.0 ml	5 × 1.0 ml
MIR 2256	10 × 1.0 ml	10 × 1.0 ml

Materials required, but not supplied

- Cultured cells
- Appropriate cell culture medium
- Purified RNA
- Serum-free medium (e.g. Opti-MEM® I Reduced-Serum Medium)
- Sterile tube for transfection complex preparation
- Micropipets
- Reporter assay as required

For Research Use Only.

BEFORE YOU START:

Important Tips for Optimal RNA Transfection

Optimize reaction conditions for each cell type to ensure successful transfections. The suggestions below yield high efficiency RNA transfection using the *Trans*IT-mRNA Transfection Kit. **Table 1** on page 3 presents recommended starting conditions depending on culture vessel size.

- **Cell density (% confluence) at transfection.** Determine the optimal cell density for each cell type to maximize transfection efficiency. Passage the cells 18–24 hours before transfection to ensure that the cells are actively dividing and reach the appropriate cell density (generally ≥80% confluence) at the time of transfection.
- **RNA features.** The structure of the transfected RNA substrate can have a profound effect on transfection results. For example, an *in vitro* transcribed RNA substrate that is designed to express a protein product should be capped and polyadenylated, simulating a wild type mammalian messenger RNA (mRNA). The presence of the 5′ cap structure and 3′ polyA tail will both stabilize the mRNA after transfection and improve translation efficiency. Many *in vitro* transcription kits include cap analogs that can be incorporated into the 5′ end of the *in vitro* transcript. The transcript can also be polyadenylated using polyA polymerase or by the presence of a run of dA bases in the DNA transcription template. In some cases, the *in vitro* transcript can be produced with an internal ribosome entry site (IRES) in the 5′ untranslated region of the mRNA. The presence of the IRES can increase transcription in the absence of a 5′ cap.
- **RNA purity.** RNA used for transfection should be highly purified and sterile. We recommend mMESSAGE mMACHINE® T7 Ultra kit (Ambion) for *in vitro* transcription followed by purification of RNA transcripts using RNeasy® spin columns (Qiagen®). However, other methods of purification can also produce RNA of sufficient quality for highly efficient transfection. Avoid RNase contamination to maintain the integrity of the RNA molecules. RNA integrity can be verified using agarose gel electrophoresis.
- **RNA quantity.** As a starting point, use 1 µg of RNA per well of a 12-well plate. The optimal RNA amount can be determined by titrating from 1–3 µg of RNA per well of a 12-well plate. For certain applications, the optimal amount may be outside the recommended range of this protocol.
- ***Trans*IT-mRNA Reagent.** As a starting point, use 2 µl of *Trans*IT-mRNA Reagent per µg of RNA. The optimal *Trans*IT-mRNA Reagent volume can be determined by titrating the reagent from 1–3 µl per well of a 12-well plate. Please refer to Table 1 on Page 3 for recommended starting conditions.
- **mRNA Boost Reagent.** As a starting point, use 2 µl of mRNA Boost Reagent per µg of RNA. The optimal mRNA Boost volume should be determined by titrating the reagent from 1–3 µl per well of a 12-well plate. Refer to Table 1 on Page 3 for recommended starting conditions.
- **Complex formation conditions.** Prepare *Trans*IT-mRNA Reagent:mRNA Boost:RNA complexes in serum-free growth medium. Mirus recommends Opti-MEM I Reduced-Serum Medium. Use RNA stocks that range in concentration from 1–3 µg/µl.
- **Cell culture conditions:** Culture cells in the appropriate medium, with or without serum. There is no need to perform a medium change to remove the transfection complexes. The *Trans*IT-mRNA Transfection Kit yields improved efficiencies when transfections are performed in complete growth medium (instead of serum-free medium) without a post-transfection medium change.

Depending on the goal of the experiment, it may not be necessary to cap and polyadenylate the RNA transcript. For example, some viral transcripts do not need to be capped and polyadenylated in order to start replicating and/or produce infectious virus after transfection.

Do not use serum or antibiotics in the medium during transfection complex formation.

- **Presence of antibiotics:** Antibiotics will inhibit transfection complex formation and therefore should be excluded from the complex formation step. Transfection complexes can be added to cells grown in complete culture medium containing low levels of antibiotics (0.1–1X final concentration of penicillin/streptomycin mixture).
- **Post-transfection incubation time.** The best post-transfection incubation time can be determined empirically by testing a range of incubation times from 4–48 hours. Optimal incubation times will depend on the characteristics of the RNA being transfected. For transfection of an mRNA encoding a protein, the recommended starting incubation range is from 12–18 hours. This recommendation was determined using a capped, polyadenylated *in vitro* transcript expressing firefly luciferase and may be shorter if the transfected RNA is not capped or polyadenylated. However, depending on the goal of the transfection experiment, longer incubation times may also be required. For instance, viral rescue by RNA transfection may require more time to generate infectious virus.

Table 1. Recommended starting conditions for RNA transfections with the *Trans*IT-mRNA Transfection Kit.

Culture vessel	96-well plate	48-well plate	24-well plate	12-well plate	6-well plate	10-cm dish	T75 flask
Surface area	0.35 cm^2	1 cm^2	1.9 cm^2	3.8 cm^2	9.6 cm^2	59 cm^2	75 cm^2
Complete growth medium	92 µl	263 µl	0.5 ml	1 ml	2.5 ml	15.5 ml	19.7 ml
Serum-free medium	9 µl	26 µl	50 µl	100 µl	250 µl	1.5 ml	1.9 ml
RNA (1µg/µl stock)	0.09 µl	0.25 µl	0.5 µl	1 µl	2.5 µl	15.5 µl	19.7 µl
*Trans*IT-mRNA Reagent	0.18 µl	0.5 µl	1 µl	2 µl	5 µl	31 µl	39.4 µl
mRNA Boost Reagent	0.18 µl	0.5 µl	1 µl	2 µl	5 µl	31 µl	39.4 µl

Surface areas are based on Greiner tissue culture plates and Falcon 10-cm dishes and T75 flasks. All volumes given are per well (or per dish) for a given culture vessel.

If small volumes of *Trans*IT-mRNA and mRNA Boost need to be pipetted, dilute the reagents in serum-free medium before each use to avoid pipetting errors. ***Do not*** store diluted reagents.

RNA TRANSFECTION PROTOCOL

The following procedure describes how to perform RNA transfections in 12-well plates. The surface areas of other culture vessels are different and transfections must be scaled accordingly. Appropriately increase or decrease the amounts of serum free medium, *Trans*IT-mRNA Reagent, mRNA Boost Reagent, RNA and complete culture medium based on the surface area of the cell culture vessel (please refer to **Table 1** on Page 3).

Transient RNA transfection protocol per well of a 12-well plate

A. Plate cells

1. Approximately 18–24 hours before transfection, plate cells in 1.0 ml complete growth medium per well in a 12-well plate. Ideally cells should be ≥80% confluent prior to transfection.

   For adherent cells: Plate cells at a density of 0.8–3.0 × 10^5 cells/ml.

   For suspension cells: Plate cells at a density of 2.5–5.0 × 10^5 cells/ml.
2. Incubate the cell cultures overnight.

Divide cultured cells 18–24 hours before transfection such that the cells reach optimal cell density at the time of transfection.

B. Prepare *Trans*IT-mRNA Reagent:mRNA Boost:RNA complexes (Immediately before transfection)

1. Warm *Trans*IT-mRNA and mRNA Boost reagents to room temperature and vortex gently before using.
2. Place 100 µl of Opti-MEM I Reduced-Serum Medium in a sterile tube.
3. Add 1 µg (1 µl of a 1 µg/µl stock) RNA. Pipet gently to mix completely.
4. Add 2 µl mRNA Boost Reagent to the diluted RNA mixture. Pipet gently to mix completely.
5. Add 2 µl *Trans*IT-mRNA Reagent to the diluted RNA mixture. Pipet gently to mix completely.
6. Incubate at room temperature for 2–5 minutes to allow sufficient time for complexes to form. *Do not incubate the complexes for more than 5 minutes.*

C. Distribute the complexes to cells in complete growth medium

1. Add the complexes (prepared in Step B) drop-wise to different areas of the wells. It is not necessary to replace the complete growth medium with fresh medium.
2. Gently rock the culture vessel back-and-forth and from side-to-side to evenly distribute the *Trans*IT-mRNA Reagent:mRNA Boost:RNA complexes.
3. Incubate for 4–48 hours depending on the nature of the transfected RNA and the goal of the experiment.
4. Harvest cells and assay as required.

Warm *Trans*IT-mRNA and mRNA Boost reagents to room temperature and vortex gently before each use.

Do not let the complexes incubate longer than 5 minutes as this may decrease transfection efficiency.

*Trans*IT-mRNA is a low-toxicity reagent. There is no need to change culture medium after transfection, unless required by your cell type or culture conditions. If required, perform a medium change at least 4 hours post-transfection.

For protein translation assays, the recommended starting incubation range is from 12–18 hours.

For non-capped and/or non polyadenylated transcripts, incubation times may be shorter.

TROUBLESHOOTING GUIDE

Problem	Solution
LOW RNA TRANSFECTION EFFICIENCY	
*Trans*IT-mRNA or mRNA Boost Reagent was not mixed properly.	Warm *Trans*IT-mRNA and mRNA Boost Reagents to room temperature and vortex gently before each use.
Suboptimal *Trans*IT-mRNA Reagent:mRNA Boost Reagent ratio	Determine the optimal reagent levels to use for RNA transfection by testing a range of *Trans*IT-mRNA Reagent levels (1, 2, and 3 µl) with a range of mRNA Boost Reagent amounts (1, 2, and 3 µl). Following this guideline, nine different transfection complexes will be formed and transfected in order to identify the best transfection conditions for the particular RNA and cell type.
Suboptimal complex formation time	We recommend incubating the transfection complexes for 2–5 minutes. Longer incubation times may result in lower transfection efficiency.
Suboptimal amount of *Trans*IT-mRNA Reagent	Determine optimal amount of *Trans*IT-mRNA Reagent for each cell type. Titrate the *Trans*IT-mRNA Reagent from 1–3 µl per well of a 12-well plate, e.g. test 1, 2 and 3 µl of *Trans*IT-mRNA Reagent per well of a 12-well plate.
Suboptimal amount of mRNA Boost Reagent	Determine optimal amount of mRNA Boost Reagent for each cell type. Titrate the mRNA Boost Reagent from 1–3 µl per well of a 12-well plate, e.g. test 1, 2, and 3 µl of *Trans*IT-mRNA Reagent per well of a 12-well plate.
Suboptimal amount of RNA	Determine the RNA concentration accurately. Use RNA preps that have an $A_{260/280}$ absorbance ratio of 1.8–2.0. The optimal RNA concentration generally ranges between 1–3 µg/well of a 12-well plate. Start with 1.0 µg/well of a 12-well plate. Consider testing more or less RNA while scaling the amount of reagent accordingly.
Low-quality RNA	Use highly purified, sterile and contaminant-free RNA for transfection. We recommend using purifying the RNA using a column procedure such as Qiagen's RNeasy spin columns. Avoid RNase contamination as degradation of the RNA substrate will significantly diminish expression of the transfected RNA.
Inhibitor present during transfection	Serum and antibiotics inhibit transfection complex formation. Prepare *Trans*IT mRNA Reagent:mRNA Boost:RNA complexes in serum-free growth medium. We recommend Opti-MEM I Reduced-Serum Medium. Once transfection complexes are formed, they can be added directly to cells cultured in complete growth medium containing serum and 0.1–1X antibiotics. Polyanions such as dextran sulfate or heparin can inhibit transfection. Use culture medium that does not contain these polyanions. If necessary, the transfection medium can be replaced with polyanion containing medium 24 hours post transfection.
Transfection incubation time	Determine the best transfection incubation time for each cell type and experiment. Test a range of incubation times (e.g. 12–72 hours). The optimal incubation time is generally 24–48 hours.
Proper controls were not included	To verify efficient transfection, use *Trans*IT-mRNA Reagent to deliver a reporter transcript such as a luciferase, beta-galactosidase or green fluorescent protein (GFP) encoding RNA that is capped and polyadenylated. To assess delivery efficiency of RNA, use Mirus' *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits to label the RNA substrate for intracellular tracking (please refer to Related Products on Page 7).

TROUBLESHOOTING GUIDE continued

Problem	Solution
HIGH CELLULAR TOXICITY	
Cell density not optimal at time of transfection	Determine the best cell density for each cell type to maximize transfection efficiency. Use this density to ensure reproducibility. For most cell types, ≥80% confluence at the time of transfection is recommended.
	Divide the culture at least 18–24 hours before transfection to ensure that the cells are actively dividing and reach the appropriate cell density at time of transfection.
Transfection complexes and cells not mixed thoroughly after complex addition	Add *Trans*IT-mRNA:mRNA Boost Reagent:RNA transfection complexes drop-wise to different areas of the wells with plated cells. Gently rock the dish back-and-forth and from side-to-side to distribute the complexes evenly. Do not swirl or rotate the dish, as this may cause uneven distribution.
Transfection complexes were added to cells cultured in serum-free medium	Allow *Trans*IT-mRNA Reagent:mRNA Boost:RNA complexes to form in serum-free medium, then add these complexes to cells cultured in complete growth medium. The presence of serum in the growth medium improves transfection efficiency and reduces cytotoxicity. No culture medium change is required.
Low-quality RNA	Use highly purified, sterile and contaminant-free RNA for transfection.
	We recommend using purifying the RNA using a column procedure such as Qiagen's RNeasy spin columns.
	Avoid RNase contamination as degradation of the RNA substrate will significantly diminish expression of the transfected RNA.
Translated RNA is toxic to cells	If the target RNA is translated, include a control with non-coding RNA and *Trans*IT-mRNA Transfection Kit to compare the cytotoxic effects of the RNA being translated.
Cell morphology has changed	Mycoplasma contamination can alter cell morphology and affect transfection efficiency. Check your cells for Mycoplasma contamination. Use a fresh frozen stock of cells or use appropriate antibiotics to eliminate Mycoplasma.
	A high or low cell passage number can make cells more sensitive and refractory to transfection. Maintain a similar passage number between experiments to ensure reproducibility.

RELATED PRODUCTS

- Ingenio™ Electroporation Solution and Kits
- *Label* IT Plasmid Delivery Controls
- *Label* IT Tracker™ Intracellular Nucleic Acid Localization Kits
- *Trans*IT Cell Line Specific Transfection Reagents and Kits
- *Trans*IT-X2® Dynamic Delivery System
- *Trans*IT-2020 Transfection Reagent
- *Trans*IT-LT1 Transfection Reagent
- *Trans*IT-PRO™ Transfection Kit

For details on our products, visit www.mirusbio.com

Reagent Agent®

Reagent Agent® is an online tool designed to help determine the best solution for nucleic acid delivery based on in-house data, customer feedback and citations.

Learn more at:
www.mirusbio.com/ra

Contact Mirus Bio for additional information.

Mirus Bio LLC
5602 Research Park Blvd, Ste 210
Madison, WI 53719
Toll-free: 888.530.0801
Direct: 608.441.2852
Fax: 608.441.2849

Quant-iT™ RiboGreen™ RNA Reagent and Kit

Catalog Numbers R11490, R11491, T11493

Pub. No. MAN0002073 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Quant-iT™ RiboGreen™ RNA Reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating RNA in solution. Detecting and quantitating small amounts of RNA is important in many applications including measuring yields of *in vitro* transcribed RNA and measuring RNA concentrations before performing Northern blot analysis, S1 nuclease assays, RNase protection assays, cDNA library preparation, reverse transcription PCR, and differential display PCR.

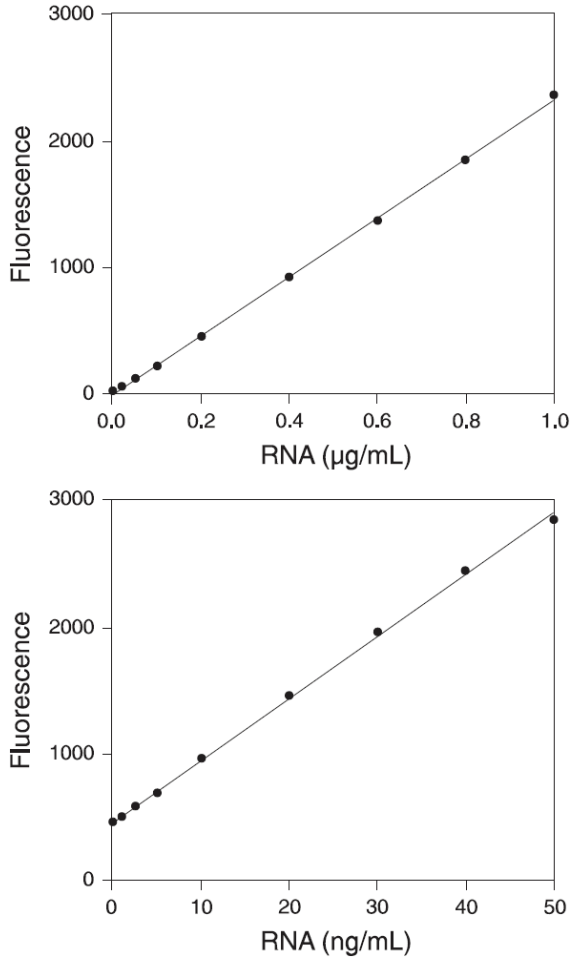

Figure 1 Dynamic range and sensitivity of the Quant-iT™ RiboGreen™ RNA Assay.
For the high-range assay (top panel), the Quant-iT™ RiboGreen™ RNA Reagent was diluted 200-fold into 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE) and 100 µL of the reagent solution was added to microplate wells containing 100 µL of ribosomal RNA in TE. For the low-range assay (bottom panel), the Quant-iT™ RiboGreen™ RNA Reagent was diluted 2,000-fold into TE and 100 µL of the reagent solution was added to microplate wells containing 100 µL of ribosomal RNA in TE. Samples were excited at 485 ± 10 nm and the fluorescence emission intensity was measured at 530 ± 12.5 nm using a fluorescence microplate reader. Fluorescence emission intensity was then plotted versus RNA concentration.

The Quant-iT™ RiboGreen™ RNA Reagent enables quantitation of as little as 1 ng/mL RNA (200 pg RNA in a 200 µL assay volume) with a fluorescence microplate reader using fluorescein excitation and emission wavelengths. The linear range of the Quant-iT™ RiboGreen™ RNA

Reagent extends over three orders of magnitude in RNA concentration (1 ng/mL to 1 µg/mL) using two dye concentrations (Figure 1). The high-range assay allows quantitation of 20 ng/mL to 1 µg/mL RNA, and the low-range assay allows quantitation of 1 ng/mL to 50 ng/mL RNA. This linearity is maintained in the presence of several compounds commonly found to contaminate nucleic acid preparations, including nucleotides, salts, urea, ethanol, chloroform, detergents, proteins, and agarose. Although the Quant-iT™ RiboGreen™ RNA Reagent also binds to DNA, pretreatment of mixed samples with DNase can be used to generate an RNA-selective assay (see "Eliminate DNA from samples" on page 5).

Contents and storage

Component	Quant-iT™ RiboGreen™ RNA Reagent[1]	Quant-iT™ RiboGreen™ RNA Assay Kit	Concentration	Storage[2]
	Cat. No. R11491	Cat. No. R11490		
Quant-iT™ RiboGreen™ RNA Reagent (Component A)	1 mL	1 mL	Solution in DMSO	2°C to 8°C[3] Desiccate Protect from light
20X TE Buffer, RNase-free (Component B)	Not applicable	25 mL	200 mM Tris-HCl, 20 mM EDTA, pH 7.5 in DEPC-treated water	≤30°C
Ribosomal RNA standard, 16S and 23S rRNA from *E. coli* (Component C)	Not applicable	5 × 200 µL	100 µg/mL in TE buffer	2°C to 8°C[4] Avoid freeze-thaw cycles
Number of labelings: 2,000 to 20,000 with an assay volume of 200 µL in a 96-well microplate format. The Quant-iT™ RiboGreen™ RNA Assay can be adapted for use in cuvettes or 384-well microplates.				
Approximate fluorescence excitation/emission maxima: 500/525 nm, bound to nucleic acid.				

[1] Stand-alone reagent does not include Components B and C.
[2] When stored as directed, products are stable for at least 6 months.
[3] For long-term storage, the Quant-iT™ RiboGreen™ RNA Reagent can be stored at ≤–20°C
[4] For long-term storage, store the rRNA standards at ≤–20°C or –70°C.

Required materials not supplied

- Nuclease-free pipettors and tips
- Nuclease-free water
- Microplates for Fluorescence-based Assays, 96-well (Cat. No. M33089)

Prepare the assay buffer

Prepare the 1X TE working solution by diluting the concentrated buffer (Component B) 20-fold with nuclease-free water. Prepare nuclease-free water by treating distilled, deionized water with 0.1% diethyl pyrocarbonate (DEPC), incubating for several hours at 37°C, and autoclaving for at least 15 minutes at 15 lbs/sq. inch to sterilize the water and eliminate DEPC.

IMPORTANT! TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) is used to prepare the Quant-iT™ RiboGreen™ RNA Reagent and for diluting RNA standards and samples. Make sure the TE solution is free of contaminating nucleases and nucleic acids. The 20X TE buffer included in the Quant-iT™ RiboGreen™ RNA Assay Kit is nuclease-free and nucleic acid–free.

Prepare the reagent

Two different dye concentrations are required to achieve the full linear dynamic range of the Quant-iT™ RiboGreen™ RNA Assay. Before preparing the working solution of the Quant-iT™ RiboGreen™ RNA Reagent, decide whether you wish to perform the **high-range** assay (20 ng/mL to 1 µg/mL RNA), **low-range** assay (1 ng/mL to 50 ng/mL RNA), or both.

On the day of the experiment, allow the Quant-iT™ RiboGreen™ RNA Reagent to warm to room temperature before opening the vial, then prepare an aqueous working solution of the Quant-iT™ RiboGreen™ RNA Reagent by diluting the concentrated DMSO stock solution (Component A) into TE, 200-fold for the **high-range** assay or 2,000-fold for the **low-range** assay. For microplate assays of a total 200 µL assay volume, you need 100 µL of the Quant-iT™ RiboGreen™ RNA Reagent working solution per sample.

For example, to prepare enough working solution to assay 100 samples in 200 µL volumes, add 50 µL Quant-iT™ RiboGreen™ RNA Reagent to 9.95 mL TE for the **high-range** assay or add 5 µL Quant-iT™ RiboGreen™ RNA Reagent to 9.995 mL TE for the **low-range** assay.

Note: Allow the Quant-iT™ RiboGreen™ RNA Reagent to warm to room temperature before opening the vial. Cold DMSO solutions absorb moisture from warmer, room temperature air, resulting in loss of efficacy for the reagent. Always store the DMSO stock solution in the

presence of desiccant when not in use. We recommend preparing the working solution in sterile, disposable polypropylene plasticware rather than glassware, as the reagent may adsorb to glass surfaces. Protect the working solution from light, as the Quant-iT™ RiboGreen™ RNA Reagent is susceptible to photodegradation. **For best results, use the working solution within a few hours of preparation.**

Prepare the RNA standard curve

1. Prepare a 2 µg/mL solution of RNA in TE using nuclease-free plasticware. Determine the RNA concentration on the basis of absorbance at 260 nm (A_{260}) in a cuvette with a 1 cm pathlength; an A_{260} of 0.05 corresponds to 2 µg/mL RNA.

   The ribosomal RNA standard (Component C), provided at 100 µg/mL in the Quant-iT™ RiboGreen™ RNA Assay Kit, is diluted 50-fold in TE to make the 2 µg/mL working solution. For example, 4 µL of the RNA standard mixed with 196 µL of TE is sufficient for the standard curve described in step 2.

   Note: For a standard curve, we commonly use 16S and 23S ribosomal RNA, although any purified RNA preparation may be used. It is sometimes preferable to prepare the standard curve with RNA similar to the type being assayed. However, most single-stranded RNA molecules yield approximately equivalent signals.

   Note: The RNA solution used to prepare the standard curve should be treated the same way as the experimental samples and should contain similar levels of contaminants. See "Effects of common contaminants" on page 4 for a list of contaminants tested in the Quant-iT™ RiboGreen™ assay.

2. For the **high-range** standard curve, dilute the 2 µg/mL RNA solution into microplate wells as shown in Table 1. For the **low-range** standard curve, dilute the 2 µg/mL RNA solution 20-fold into TE to make a 100 ng/mL RNA stock solution, then prepare the dilution series shown in Table 2.

   Table 1 Protocol for preparing a high-range standard curve.

Volume of TE buffer	Volume of 2 µg/mL RNA stock	Volume of 200-fold diluted Quant-iT™ RiboGreen™ RNA Reagent	Final RNA concentration in the assay
0 µL	100 µL	100 µL	1 µg/mL
50 µL	50 µL	100 µL	500 ng/mL
90 µL	10 µL	100 µL	100 ng/mL
98 µL	2 µL	100 µL	20 ng/mL
100 µL	0 µL	100 µL	blank

   Table 2 Protocol for preparing a low-range standard curve.

Volume of TE buffer	Volume of 100 ng/mL RNA stock	Volume of 2,000-fold diluted Quant-iT™ RiboGreen™ RNA Reagent	Final RNA concentration in the assay
0 µL	100 µL	100 µL	50 ng/mL
50 µL	50 µL	100 µL	25 ng/mL
90 µL	10 µL	100 µL	5 ng/mL
98 µL	2 µL	100 µL	1 ng/mL
100 µL	0 µL	100 µL	blank

3. Add 100 µL of the appropriate aqueous working solution of Quant-iT™ RiboGreen™ RNA Reagent (prepared in "Prepare the reagent" on page 2) to each microplate well. Use the high-range working solution for performing the high-range assay, and use the low-range working solution for performing the low-range assay. Mix well and incubate for 2–5 minutes at room temperature, protected from light.

4. Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm).

   Note: To ensure that the sample readings remain in the detection range, set the instrument's gain so that the sample containing the highest RNA concentration yields a fluorescence intensity near the microplate reader's maximum. For optimal detection sensitivity, the instrument gain can be increased for the low-range assay relative to the high-range assay. To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.

5. Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus RNA concentration (see Figure 1).

Analyze samples

1. Dilute the experimental RNA solution in TE to a final volume of 100 µL in microplate wells.

   Note: You can alter the amount of sample diluted, provided that the final volume remains 100 µL. High dilutions of the experimental sample may serve to diminish the interfering effect of certain contaminants. However, extremely small sample volumes should be avoided because they are difficult to pipet accurately. In addition, the level of assay contaminants should be kept as uniform as possible throughout an experiment, to minimize sample-to-sample signal variation. For example, if a series of RNA samples contain widely differing salt concentrations, then they cannot be compared to a single standard curve. To avoid this problem, simply adjust the concentration of contaminants to be the same in all samples, if possible (see "Effects of common contaminants" on page 4).

2. Add 100 µL of the aqueous working solution of the Quant-iT™ RiboGreen™ RNA Reagent (prepared in "Prepare the reagent" on page 2) to each sample. Incubate for 2–5 minutes at room temperature, protected from light.

3. Measure the fluorescence of the samples using the same instrument parameters used to generate the standard curve (see step 4). To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.

4. Subtract the fluorescence value of the reagent blank from that of each of the samples. Determine the RNA concentration of the sample from the standard curve generated in "Prepare the RNA standard curve" on page 3.

5. The assay can be repeated using a different dilution of the sample to confirm the quantitation results.

Effects of common contaminants

The Quant-iT™ RiboGreen™ Assay remains linear in the presence of several compounds that commonly contaminate nucleic acid preparations, although the signal intensity may be affected (Table 3). For the highest accuracy, the standards should be prepared under the same conditions as the experimental samples and contain similar levels of contaminants.

Table 3 Effects of common contaminants on the signal intensity of the assay.

Compound	Maximum acceptable concentration	% Signal change[1]
Salts		
Ammonium acetate	20 mM	4% decrease
Sodium acetate	20 mM	11% decrease
Sodium chloride	20 mM	15% decrease
Zinc chloride	1 mM	9% decrease
Magnesium chloride	0.5 mM	9% decrease
Calcium chloride	0.1 mM	2% increase
Cesium chloride	10 mM	8% decrease
Guanidinium thiocyanate	10 mM	9% decrease
Urea	3 M	13% decrease
Organic solvents		
Phenol	0.5%	5% decrease
Ethanol	20%	10% decrease
Chloroform	2%	15% increase
Detergents		
Sodium dodecyl sulfate	0.05%	10% decrease
Triton™ X-100	0.5%	8% decrease
Proteins		
Bovine serum albumin	0.2%	11% decrease
IgG	0.02%	4% decrease

Compound	Maximum acceptable concentration	% Signal change[1]
Other compounds		
Formamide	10%	12% decrease
Sucrose	>500 mM	4% decrease
Boric acid	100 mM	15% decrease
Polyethylene glycol	10%	10% decrease
Agarose	0.2%	3% increase

[1] The compounds were incubated at the indicated concentrations with the Quant-iT™ RiboGreen™ RNA Reagent in the presence of 1.0 mg/mL ribosomal RNA. All samples were assayed in a final volume of 200 µL in 96-well microplates using a fluorescence microplate reader. Samples were excited at 485 ± 10 nm and fluorescence intensity was measured at 530 ± 12.5 nm.

Eliminate DNA from samples

The Quant-iT™ RiboGreen™ RNA Reagent also binds to DNA. Fluorescence in samples that is due to the Quant-iT™ RiboGreen™ RNA Reagent binding to DNA can be eliminated by pre-treating the sample with RNase-free DNase, ensuring that the entire sample fluorescence is due to dye bound to RNA.

1. Prepare 10X DNase digestion buffer: nuclease-free 200 mM Tris-HCl, pH 7.5, containing 100 mM $MgCl_2$ and 20 mM $CaCl_2$.
2. Add 0.11 volume of 10X DNase digestion buffer to each DNA-containing sample (for example, to a 9 µL sample, add 1 µL 10X buffer).
3. Add ~5 units of RNase-free DNase I per µg of DNA estimated to be in the sample.
4. Incubate the sample at 37°C for 90 minutes.
5. Dilute the sample at least 10-fold into TE to diminish effects of the digestion buffer salts on the Quant-iT™ RiboGreen™ assay procedure.
6. Perform the Quant-iT™ RiboGreen™ assay as described (see “Analyze samples” on page 4).

Related products

Table 4 Bulk Reagents and Kits

Product	Quantity	Cat. No.
Quant-iT™ PicoGreen™ dsDNA Assay Kit	1 mL assay kit 10 x 100 µL	P7589 P11496
Quant-iT™ PicoGreen™ dsDNA Reagent	1 mL reagent 10 x 100 µL	P7581 P11495
TE Buffer (20X), RNase-free	100 mL	T11493
Quant-iT™ RiboGreen™ RNA Assay Kit	1 mL assay kit	R11490
Quant-iT™ RiboGreen™ RNA Reagent	1 mL reagent	R11491
Quant-iT™ RediPlate™ 96 RiboGreen™ RNA Quantitation Kit	1 plate	R32700
Quant-iT™ OliGreen™ ssDNA Assay Kit	1 mL assay kit	O11492
Quant-iT™ OliGreen™ ssDNA Assay Reagent	1 mL reagent	O7582

Table 5 Microplate Reader Assays

Product	Dynamic Range	Quantity	Cat. No.
Quant-iT™ 1X dsDNA Assay Kit, High Sensitivity	200 pg–100 ng	1,000 reactions	Q33232
Quant-iT™ 1X dsDNA Assay Kit, Broad-Range	4 ng–2 µg	1,000 reactions	Q33267
Quant-iT™ DNA Assay Kit, High Sensitivity	200 pg–100 ng	1,000 reactions	Q33120
Quant-iT™ DNA Assay Kit, Broad-Range	4 ng–1 µg	1,000 reactions	Q33130
Quant-iT™ RNA Assay Kit	5–100 ng	1,000 reactions	Q33140
Quant-iT™ RNA Reagent	5–100 ng	1,000 reactions	Q32884
Quant-iT™ RNA Assay Kit, Broad Range	20 ng–1 µg	1,000 reactions	Q10213
Quant-iT™ RNA XR Assay Kit	200 ng–10 µg	1,000 reactions	Q33225
Quant-iT™ microRNA Assay Kit	1–100 ng	1,000 reactions	Q32882
Quant-iT™ Protein Assay Kit	250 ng–5 µg	1,000 reactions	Q33210
Microplates for Fluorescence-based Assays, 96-well	—	10 plates	M33089

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: Pub. No. MAN0002073

Revision	Date	Description
A.0	15 March 2022	The format and content were updated. The version numbering was reset to A.0 in conformance with internal document control.
1.00	10 June 2008	New document for the Quant-iT™ RiboGreen™ RNA Assay Kit and Quant-iT™ RiboGreen™ RNA Reagent.

达科为 DAKEWE

达优

Mouse IFN-γ

Precoated ELISPOT Kit(strips)

Cat#:2210006/2210007

ELISPOT 试剂盒

说明书

本试剂盒仅供科研使用，请勿用于诊断
使用前请仔细阅读说明书并检查试剂盒组分

REV：C/2

达优

达科为 DAKEWE

Mouse IFN-γ

Precoated ELISPOT Kit(strips)

ELISPOT 试剂盒

Cat#:2210006
Cat#:2210007

| 产品简介

达优系列 ELISPOT 预包被试剂盒采用原装进口高亲和力高效价抗体对，经预包被 PVDF 板、低温干燥、真空密封包装等工艺流程制备。成品 PVDF 板上预包被的抗体分布均匀、效价稳定，2℃~8℃可存放 12 个月。

达优系列 ELISPOT 预包被试剂盒使实验检测时间从 3 天缩短为 2 天，大幅度减少无菌操作的实验步骤，减轻实验者的劳动强度和减少污染的几率。使得实验者能够轻松、高效地完成复杂的 ELISPOT 检测实验。

| 知识背景

IFN-γ亦称 II 型干扰素，主要由 T 淋巴细胞和 NK 细胞分泌，是细胞因子超家族中 IFN 家族的重要成员。IFN-γ具有广泛的生物学功能，如抗病毒、抗肿瘤、免疫调节、调控细胞增殖和分化等。

试剂盒提供的试剂、规格

产品名称	货号	试剂组分名称	规格	数量
Mouse IFN-γ Precoated ELISPOT Kit (strips)	2210006	Biotinylated Antibody	100 μL	5 支
		Streptavidin-HRP	500 μL	1 支
		Dilution Buffer R (10×)	18 mL	1 瓶
		Washing Buffer (50×)	75 mL	1 瓶
		AEC Dilution	50 mL	1 瓶
		AEC Solution Ⅰ (20×)	3 mL	1 瓶
		AEC Solution Ⅱ (20×)	3 mL	1 瓶
		AEC Solution Ⅲ (200×)	500 μL	1 支
		阳性刺激物	/	/
		预包被 PVDF 板	/	5 块
	2210007	Biotinylated Antibody	100 μL	5 支
		Streptavidin-HRP	500 μL	1 支
		Dilution Buffer R (10×)	18 mL	1 瓶
		Washing Buffer (50×)	75 mL	1 瓶
		AEC Dilution	50 mL	1 瓶
		AEC Solution Ⅰ (20×)	3 mL	1 瓶
		AEC Solution Ⅱ (20×)	3 mL	1 瓶
		AEC Solution Ⅲ (200×)	500 μL	1 支
		阳性刺激物	50 T	5 瓶
		预包被 PVDF 板	/	5 块

试剂盒的存放

未开封试剂盒在 2℃~8℃可稳定保存 12 个月；开封后尽量一次性用完，若要保存需密封分装保存。

需自备的材料设备

+ RPMI-1640 基础培养基（需要添加双抗）
+ 无血清培养基（完全培养基，即用型），推荐使用达优 ELISPOT 无血清培养基。
+ 货号 2210006 试剂盒无阳性刺激物，推荐使用达优阳性刺激物 PHA 或 PMA+Ionomycin。
+ 超净工作台
+ CO_2 细胞培养箱
+ 微量移液器及配套 Tip 头
+ 8 通道微量移液器
+ 0.5 mL，1.5 mL EP 管
+ 酶联斑点分析仪

检测操作

第一天：接种细胞，加入刺激物，培养（严格注意无菌操作）

试剂配制

1. **阳性刺激物：**现配现用；按标签重悬即为工作液。

2. **无血清培养基：**如果没有 ELISPOT 无血清培养基，可用含 5%~10%胎牛血清的 RPMI-1640 培养基代替。

操作步骤

1. **预包被板的活化：**200 μL/well 加入 RPMI-1640 培养基或者无血清培养基，室温静置 5~10 分钟后将其扣出。

2. **加入细胞悬液：**将调整好浓度的细胞悬液加入各实验孔，100 μL/well。

正对照孔：细胞浓度可采用 1×10^5 cells/well；

负对照孔：细胞浓度由实验者根据实验自行调整，跟实验孔保持一致；

背景负对照：加入不含细胞的无血清培养基（或重悬细胞所用的培养基）；

实验孔：细胞浓度由实验者根据实验自行调整。

3. **加入刺激物：**10 μL/well，具体如下：

正对照孔：加入工作浓度的阳性刺激物；

负对照孔：不加阳性刺激物；

背景负对照孔：不加阳性刺激物；

实验孔：加入实验者自己的刺激物（用无血清培养基或者 RPMI 1640 配制成 10×终浓度）。

4. **孵育：**所有样品和刺激物加完后，盖好板盖。放入 37℃，5%CO_2 培养箱静置培养 16~24 小时。孵育期间避免晃动，为防止蒸发，可根据实验室条件增加防蒸发措施。例如使用锡纸把板条包裹后进行孵育，包裹时需底部平整。

第二天：培养后操作（不再需要无菌操作）

- **试剂配制**

1. **注意：**各组分 1×工作液及显色液现配现用。

2. **Washing Buffer（50×）：**用去离子水稀释（1：50），制成 1×Washing Buffer 工作液备用。

3. **Dilution Buffer R（10×）：**用 1×PBS 稀释（1：9），制成 1×Dilution Buffer R 工作液备用。

4. **生物素标记的抗体（Biotinylated Antibody）：**用 1×Dilution Buffer R 工作液稀释（1：100），制成 1×Biotinylated Antibody 工作液。

5. **酶联亲和素（Streptavidin-HRP）：**用 1×Dilution Buffer R 工作液稀释（1：100），制成 1×Streptavidin-HRP 工作液。

6. **AEC 显色液：**在洁净的容器内将 AEC Dilution、AEC Solution Ⅰ (20×)、AEC Solution Ⅱ (20×)、AEC Solution Ⅲ (200×)按照 180：10：10：1 的比例混匀，即为工作液。可参见下表。AEC 显色液工作液室温下的半衰期约 30 分钟，使用时现用现配。

总体积	AEC Dilution	AEC Solution I (20×)	AEC Solution II (20×)	AEC Solution III (200×)
1 mL	0.9 mL	50 μL	50 μL	5 μL
2 mL	1.8 mL	100 μL	100 μL	10 μL
3 mL	2.7 mL	150 μL	150 μL	15 μL
4 mL	3.6 mL	200 μL	200 μL	20 μL
5 mL	4.5 mL	250 μL	250 μL	25 μL
8 mL	7.2 mL	400 μL	400 μL	40 μL
10 mL	9 mL	500 μL	500 μL	50 μL

操作步骤

1. **裂解细胞：**倾倒孔内细胞及培养基。加入冰冷的去离子水，200 μL/well，4℃冰箱放置 10 分钟低渗裂解细胞。

2. **洗板：**甩出孔内液体，加入 1×Washing Buffer 工作液，260 μL/well，停留 1 分钟后弃去孔内液体，重复六次，每一次在吸水纸上扣干。

3. **检测抗体孵育：**将 1×Biotinylated Antibody 工作液加入各实验孔，100 μL/well。37℃孵育 1 小时。

4. **洗板：**重复步骤 2。

5. **酶联亲和素孵育：**将 1×Streptavidin-HRP 工作液加入各实验孔，100 μL/well 37℃孵育 1 小时。

6. **洗板：**甩出孔内液体，加入 1×Washing Buffer 工作液，260 μL/well，停留 1 分钟后弃去孔内液体，重复五次，每一次在吸水

纸上扣干，洗涤完成后揭开板底座，用去离子水/自来水洗涤膜底面及底座，用吸水纸小心吸干底座及膜底残留的水迹，合上底座，加入1×Washing Buffer 工作液，260 μL/well，停留 1 分钟后弃去孔内液体，彻底扣干孔内液体。

7. **显色：**将现配的 AEC 显色液加入各实验孔，100 μL/well。室温避光静置 5~30 分钟，根据斑点生成情况选择终止显色时间。若室温低于 20℃，建议在 37℃孵箱做显色，每隔 5~10 分钟检查一次。

8. **终止显色：**倾倒孔内液体，揭开板底座，用去离子水/自来水洗涤各实验孔正反面及底座 3~5 遍，终止显色。将板放置在室温阴凉处，待其自然晾干后合上底座。

9. **ELISPOT 板斑点计数，**并记录斑点的各种参数，做统计分析。

洗涤指导

用排枪每孔加入 260 μL 洗涤缓冲液。加入洗涤缓冲液之后浸泡 1 分钟，然后扣出洗涤缓冲液。

注意：所有从孔中移出液体的步骤均需用力甩出或者扣出，勿用枪头去吸，以免碰刮、损伤到膜。洗涤步骤的最后一次操作需要在吸水纸上拍干。吸水纸最好采用进口棉纸，强度高，不掉屑，吸水量大，使用前需要高压灭菌。不充分的洗涤将加重膜背景，对斑点计数造成干扰。

| 注意事项及提示性说明

1. **Washing Buffer(50×)** 4℃存放后出现结晶析出为正常现象，用前半小时置于 37℃轻摇混匀可消除结晶，对实验结果无影响。

2. **Dilution Buffer R(10×)**有少量沉淀为正常的蛋白饱和析出，用前静置沉降，取用上清即可，不影响实验结果。

3. **AEC 底物的使用：** AEC 底物现用现配，颠倒混匀，并在半个小时内使用；避免使用聚苯乙烯（PS）材料的容器盛放；若正常使用试剂发生析出，加样过程中，间隔性混匀试剂，轻微析出不影响实验结果，同时注意避光。

4. 试剂盒恢复室温所使用的样品来源于人体，所有的样品都应考虑到潜在的感染危险。对所有样品及其成分的处理、使用、储存和放置都应遵守国家的有关规定。

5. 试剂中含有生物防腐剂，可能导致皮肤过敏。使用时应避免接触皮肤，操作时佩戴合适的手套。

6. 显色底物溶液对眼睛、呼吸系统和皮肤有刺激。呼吸吸入、皮肤接触或口腔摄入有害。使用时应避免吸入蒸汽和喷射物，避免皮肤和眼睛接触，应使用合适的防护服、眼镜和采取皮肤保护设施。