CELLSCRIPT™
RNA for Translation in Cells

Min-Immune™ Gold dsRNA Removal Kit

Cat. No. **MGDR250125**

INTRODUCTION

The Min-Immune™ Gold dsRNA Removal Kit provides a novel enzymatic solution for the removal of double-stranded RNA (dsRNA) contamination present in RNA samples produced by *in vitro* transcription (IVT). Each 25-reaction kit will treat 1,500 µg of IVT RNA or mRNA.

The removal of dsRNA from mRNA preparations has been shown to be essential for reducing the innate immunogenic response to the mRNA in cells[1-4]. Alternative dsRNA removal methods, such as reverse-phase HPLC[5], hydroxyapatite chromatography[6] and cellulose chromatography[7], are associated with high capital costs as well as reduced final product yields. The Min-Immune Gold dsRNA Removal Kit provides an easy to use, scalable method for removing the dsRNA content of IVT or mRNA preps without a reduction of the single-stranded RNA yield.

MATERIALS

Materials Supplied

Important: Store at –20°C in a freezer without a defrost cycle. Do not store at –70°C.

Min-Immune™ Gold dsRNA Removal Kit Contents (25 reactions)	
Kit Component	Reagent Volume
Min-Immune™ Gold RNase III (20X) in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.1% Triton® X-100.	150 µl
Min-Immune™ Gold 10X RNase III Treatment Buffer	300 µl
ScriptGuard™ RNase Inhibitor, 40 U/µl in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM DTT, 0.1 mM EDTA and 0.1% Triton X-100.	75 µl
5 M Ammonium Acetate	2 x 1.6 ml
RNase-Free Water	2 x 1.7 ml

Materials Required, but not Supplied

- mRNA or IVT RNA for treatment.
- 70% ethanol

Optional Materials

- dsRNA-specific detection system including:
  - dsRNA-specific antibody (e.g., J2 antibody [Absolute Biotech-Exalpha])
  - Dot/slot blotting system for use with the antibody
  - An image analyzer for blot visualization and/or quantification
  - dsRNA standards

For more information, consult the appropriate safety data sheet (SDS) at www.cellscript.com.

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SPECIFICATIONS

Unit Definitions

One standard reaction from the Min-Immune Gold dsRNA Removal Kit treats 60 μg of RNA.

One unit of ScriptGuard RNase Inhibitor results in 50% inhibition of 5 ng of RNase A. RNase III is not inhibited by ScriptGuard RNase Inhibitor.

Functional Testing

The Min-Immune Gold dsRNA Removal Kit is functionally tested by treatment of pseudouridine-containing RNA, which is then purified by ammonium acetate precipitation. Treated and untreated RNAs are assayed for dsRNA content via immunoblotting with dsRNA-specific J2 antibody and a horseradish peroxidase coupled secondary antibody, then quantified via chemiluminescence. dsRNA-content in treated RNA must be reduced to <0.01% when loaded alongside known concentrations of a pseudouridine-containing in-house dsRNA luciferase standard containing a 1,675 bp central dsRNA region. **Note:** use of different dsRNA controls, for standard curve production, will lead to different final calculated dsRNA concentrations.

Contaminating Activity Assays

All components of the Min-Immune Gold dsRNA Removal Kit are free of detectable RNase and DNase activities except for the inherent activity of the Min-Immune Gold RNase III.

BEFORE YOU START: IMPORTANT TIPS

- **RNA Source**:

  Both IVT RNAs and mRNAs may be used as input RNA sources for dsRNA-content removal. The input RNA should be purified from any previous enzymatic reaction using your method of choice. See the Technical Appendix for options. **It is important that the RNA is resuspended in RNase-free Water and NOT in an EDTA-containing solution such as $T_{10}E_1$ buffer.**

- **Maintaining an RNase-Free Environment**:

  Highly stable RNases are ubiquitous, including on human skin.

  Creating an RNase-free work environment and maintaining RNase-free solutions is critical for successful work with RNA.

  It is strongly recommended to:

  – Use RNase-free tubes and pipette tips.

  – Always wear gloves when handling kit components or samples containing RNA and change gloves frequently, especially after touching potential sources of RNase contamination such as doorknobs, pens, pencils and human skin. Do not touch any kit component or tube containing RNA with an ungloved hand.

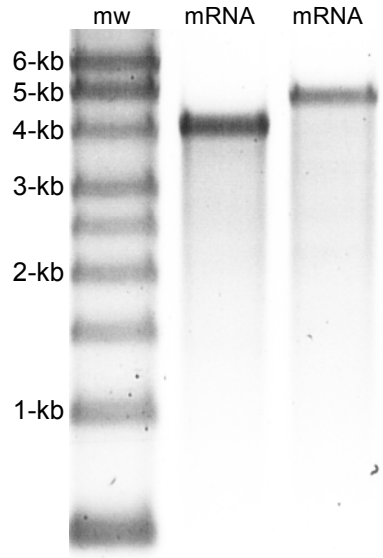
  – Keep all kit components tightly sealed when not in use. Keep all tubes containing RNA tightly sealed during the incubation steps.

- **Treatment of single-stranded RNAs (ssRNA) containing inherent double-stranded regions**:
  The Min-Immune Gold dsRNA Removal Kit was designed to remove the contaminating dsRNA-content, produced as a transcription reaction byproduct, from the desired ssRNA transcription reaction product. However, some RNAs naturally contain dsRNA regions or structures within them. Some of these are vital functional components of the RNA (e.g., cellular factor binding sites). The lengths of these dsRNA regions dictate their sensitivity to both Min-Immune Gold RNase III cleavage (see Example Data on the following pages) as well as to recognition by intracellular dsRNA-specific pattern recognition receptors. Because of this, we recommend that all new RNA sequences to be treated with the Min-Immune Gold dsRNA Removal Kit be characterized for Min-Immune Gold RNase III cleavage sensitivity by performing a 1X (or smaller) reaction treatment followed by analysis via denaturing agarose gel electrophoresis of treated versus non-treated samples prior to treatment of larger amounts of the same RNA. See Example Data on the following pages.

Example Data:

Figure 1

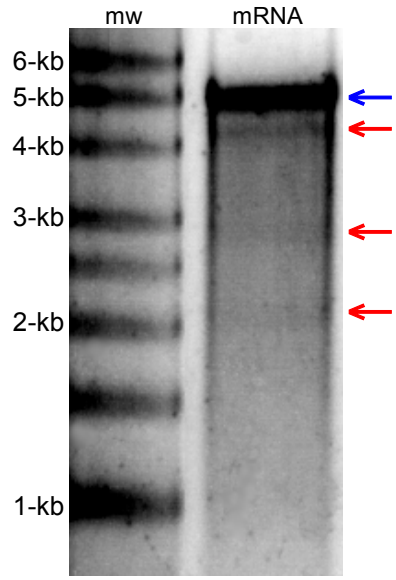
Formaldehyde-denaturing 1% agarose gel.
Two different treated Ψ-mRNA samples.
Standard image exposure (Syngene® G:Box).



Min-Immune Gold dsRNA Removal Kit treated RNAs, in this example: pseudouridine-containing mRNAs should appear as single full-length bands of ssRNA lacking any background banding pattern that was not present in the untreated RNA sample.

Figure 2

Formaldehyde-denaturing 1% agarose gel.
Treated Ψ-mRNA sample.
Image over exposure (Syngene G:Box).

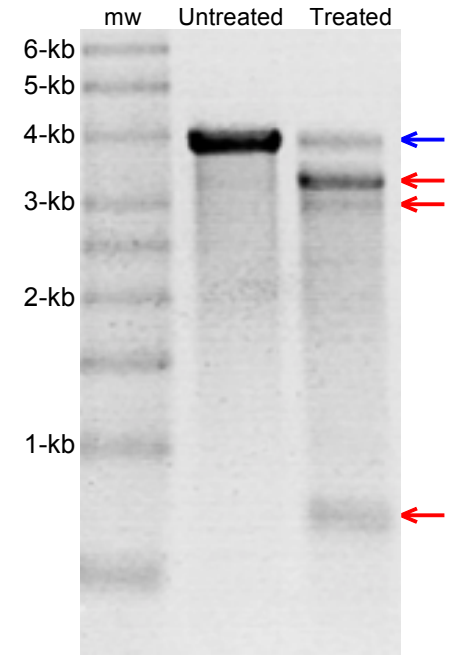


Some RNA samples can form transient dsRNA regions (molecular breathing) during the reaction which are substrates for Min-Immune Gold RNase III cleavage. These will appear as faint less than full length background bands.

- **Blue** arrow = full length mRNA.
- **Red** arrows = cleaved RNA fragments from transient Min-Immune Gold RNase III sensitive sites.

This does not affect the bulk functionality of the treated RNA.

Figure 3
Formaldehyde-denaturing 1% agarose gel.
Treated & Untreated Ψ-mRNA sample.
Standard image exposure (Syngene G:Box).

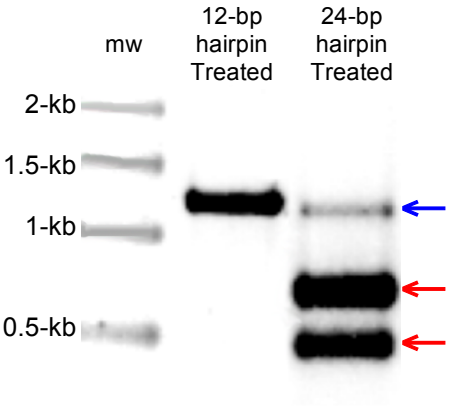


Some RNA samples contain inherent stable dsRNA regions due to the sequence of the RNA which are substrates for Min-Immune Gold RNase III cleavage. These will appear as strong less than full length background bands along with a diminished full length RNA band.

- **Blue** arrow = full length mRNA.
- **Red** arrows = cleaved RNA fragments from inherent Min-Immune Gold RNase III sensitive sites.

This will greatly affect the bulk functionality of the treated RNA. See the Troubleshooting section for options on how to handle such RNAs.

Figure 4
Formaldehyde-denaturing 1% agarose gel.
Treated U-IVT RNA samples.
Standard image exposure (Syngene G:Box).



A 1.25-kb RNA construct was designed to contain an inherent dsRNA region which included either a 12-bp or 24-bp hairpin in the secondary structure, and treated with Min-Immune Gold RNase III.

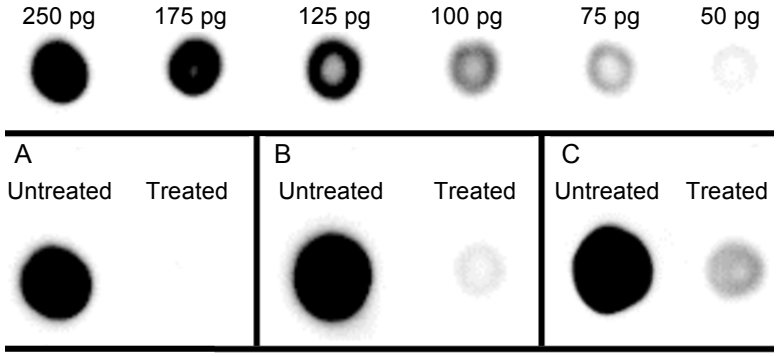
- **Blue** arrow = full length RNA.
- **Red** arrows = cleaved RNA fragments from Min-Immune Gold RNase III sensitive sites.

Duplexes of 12-bp dsRNA are not recognized by Min-Immune Gold RNase III while duplexes of 24-bp dsRNA are recognized.

Figure 5

dsRNA immuno-dot blot assayed with dsRNA-specific J2 antibody detection using horseradish peroxidase and chemiluminescent detection.

1 μg of Untreated or Treated mRNA was blotted for each experimental sample.



Pseudouridine-containing dsRNA quantification standards

Untreated and treated pseudouridine-containing mRNA samples.

A) Example result of an mRNA prep where the dsRNA content is totally removed by treatment. This mRNA can be used for downstream applications.

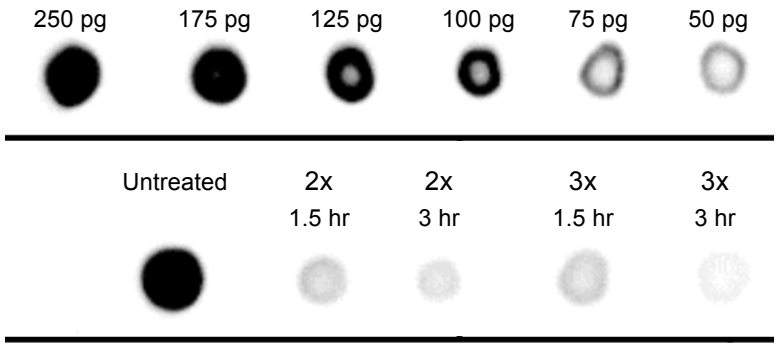
B) Example result of an mRNA prep where the mRNA contains an inherent dsRNA region which is not recognized by Min-Immune Gold RNase III but is recognized by the J2 antibody. This mRNA can be used for downstream applications. See Figure 6 below.

C) Example result of an mRNA prep where the dsRNA content is not totally removed by treatment. This mRNA would require retreatment prior to many downstream applications.

Figure 6

dsRNA immuno-dot blot assayed with dsRNA-specific J2 antibody detection using horseradish peroxidase and chemiluminescent detection.

1 μg of Untreated or treated mRNA was blotted for each experimental sample.



Pseudouridine-containing dsRNA quantification standards

Untreated and treated pseudouridine-containing mRNA samples.

Example result of an mRNA prep where the mRNA contains an inherent dsRNA region which is not recognized by Min-Immune Gold RNase III but is recognized by the J2 antibody. The dsRNA region could not be removed with two (2x) or three (3x) Min-Immune Gold RNase III treatments even with extended incubation times (1.5 & 3 hrs). This mRNA can be used for downstream applications.

PROCEDURE

A. Min-Immune Gold dsRNA Removal Treatment

1. Recommended: save an amount of untreated RNA to run on a denaturing agarose gel versus the treated RNA for post treatment RNA analysis.

   For the RNA to be treated, combine the following reagents at room temperature in the order indicated below:

Min-Immune Gold dsRNA Removal Treatment Reaction	
Component	Amount
RNase-Free Water	x µl
Min-Immune Gold 10X RNase III Treatment Buffer	12 µl
ScriptGuard RNase Inhibitor	3 µl
60 µg RNA (IVT or mRNA)	y µl
Min-Immune Gold RNase III (20X)	6 µl
Total Volume	120 µl

2. Incubate at 37°C for 60 minutes.

3. Proceed to RNA Purification.

B. RNA Purification

The Min-Immune Gold dsRNA Removal Kit includes 5 M Ammonium acetate for high-salt purification of the RNA. This method selectively precipitates ssRNA while leaving most of the protein and Min-Immune Gold RNase III digestion products in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size. Follow the protocol below when using the ammonium acetate method. For alternative RNA purification options, see the Technical Appendix.

1. Add one volume (100 µl) of 5 M Ammonium acetate to the reaction tube, mix well.

2. Incubate for 10-15 minutes on ice.

3. Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.

4. Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.

5. Remove the 70% ethanol with a pipette without disturbing the RNA pellet.

6. Allow pellet to dry, then resuspend in RNase-Free Water, $T_{10}E_1$ or other buffer suitable for subsequent applications of the RNA.

7. While usually unnecessary, steps 1-6 may be repeated a second time for even cleaner RNA.

TROUBLESHOOTING

Symptom	Solution
Faint, minor, less than full length background bands are seen on the gel post-treatment	RNA samples were not fully denatured. • Be sure to be running a denaturing agarose gel. • Rerun the samples with an increased heat denaturing step prior to gel loading.
	RNA sample formed transient dsRNA regions (molecular breathing) during the reaction which were substrates for Min-Immune Gold RNase III cleavage. • See example Fig 2, page 4. • If very minor, no further treatment nor treatment changes are needed. • If minor, treat subsequent samples of this RNA more gently. Reduce incubation to 30 minutes or reduce the amount of Min-Immune Gold RNase III used by 50%.
Strong, major, less than full length background bands are seen on the gel post-treatment	RNA sample contained an inherent stable dsRNA region of sufficient length to be a substrate for Min-Immune Gold RNase III cleavage. • See example Figs 3 & 4, page 5. • The Min-Immune Gold dsRNA Removal Kit is not appropriate for treating this particular RNA sequence. If the dsRNA structure is crucial to RNA functionality, use an alternative method for dsRNA removal. If the dsRNA structure is not crucial to RNA functionality, mute the dsRNA structure to remove the duplex formation by altering the RNA sequence and retreat the new RNA.
dsRNA assessment indicates remaining dsRNA content	RNA contains a region of dsRNA. • See example Figs 5 & 6, page 6. • dsRNA region may be inherent to the RNA sequence and recognizable by the dsRNA-specific antibody but may not be recognizable by the Min-Immune Gold RNase III or intracellular dsRNA-specific pattern recognition receptors. • If very minor, no further treatment nor treatment changes are needed. • If minor, the RNA sample may be retreated with Min-Immune Gold RNase III. If the dsRNA assessment still shows dsRNA content after retreatment, then the dsRNA region is most likely inherent to the RNA sequence. Try the RNA in the downstream application. If the dsRNA assessment now shows virtually no dsRNA, the next time that specific RNA is to be made, more strongly treat the RNA by extending the incubation time to 2 or 3 hours or increasing the amount of Min-Immune Gold RNase III used in the reaction.
	Abnormally high amounts of dsRNA were present in the RNA sample. • Retreat the RNA sample. • The next time that specific RNA is to be made, more strongly treat the RNA by extending the incubation time to 2 or 3 hours or increasing the amount of Min-Immune Gold RNase III used in the reaction. • Consider using an altered transcription protocol that would produce less dsRNA during the IVT reaction.
	Min-Immune Gold dsRNA Removal reaction was inefficient or failed. • Retreat the RNA sample. If the problem persists contact CELLSCRIPT Technical service.

TECHNICAL APPENDIX

A. RNA Purification

Purify the RNA by your preferred method. The method chosen should remove residual proteins and Min-Immune Gold RNase III digestion products from the ssRNA. Several options are listed below. RNA can be stored at –20°C or –70°C. If the RNA is to be stored indefinitely, store the RNA as an ethanol pellet.

I) **RNAClean XP beads** (Beckman Coulter): Removes all proteins and Min-Immune Gold RNase III digestion products from the ssRNA.

1) Allow beads to warm to room temperature and vortex the beads prior to use.
2) Add 1.8 X volume of RNA XP beads to the reaction. For example, 180 µl of beads into a 100 µl reaction.
3) Pipet the samples up and down 15 times until thoroughly mixed. Let sit at room temperature for 5 minutes.
4) After the 5-minute incubation, place on magnetic plate for 5 minutes. Without disturbing the pellet, carefully aspirate the solution off the samples and discard it.
5) With samples still magnetized, dispense 200 µl of 70% ethanol into each tube and incubate at room temperature for 30 seconds. Carefully aspirate and discard the supernatant. Repeat for a total of 3 washes. After the final wash, carefully remove any residual ethanol using a pipette.
6) Remove from the magnet. Air-dry the bead pellets at room temperature for 10 minutes.
7) Add RNase-Free Water to each tube, and pipet the samples up and down ≥10 times being careful to thoroughly resuspend the beads. This is your final sample volume.
8) Incubate at room temperature for 10 minutes then magnetize for 5 minutes at room temperature, waiting for the solution to clear.
9) Once the solution has cleared, transfer the sample to a new clean tube. The RNA is now ready for downstream applications.

II) **RNA-Binding Purification Column**: Several options are available commercially from multiple vendors. Follow the manufacturer's recommended protocol. The final resuspension of RNA should be in RNase-Free Water, TE or other suitable buffer.

III) **Lithium Chloride Precipitation**: Selectively precipitates ssRNA, while leaving most of the protein and unincorporated NTPs in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Add one volume of 5 M lithium chloride, mix well.
2) Incubate for 15 minutes on ice.
3) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
4) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
5) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
6) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.
7) While usually unnecessary, steps 1-6 may be repeated a second time for even cleaner RNA.

Continued on next page.

A. RNA Purification

IV) **Organic Extraction / Ammonium Acetate Precipitation**: Removes all proteins and selectively precipitates ssRNA, leaving most of the Min-Immune Gold RNase III digestion products in the supernatant. Note: for this method, the RNA to be purified must be >100 bases in size.

1) Add one volume of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
2) Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
3) Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
4) Add one volume of 5 M ammonium acetate, mix well then incubate for 15 minutes on ice.
5) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
6) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
7) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
8) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.

V) **Organic Extraction / Chromatography / Ethanol Precipitation**: Removes all proteins and Min-Immune Gold RNase III digestion products from the ssRNA.

1) Add one volume of TE-saturated phenol/chloroform. Vortex vigorously for 10 seconds.
2) Spin in a microcentrifuge at >10,000 x g for 5 minutes to separate the phases.
3) Remove the aqueous (upper) phase with a pipette and transfer to a clean tube.
4) Remove unincorporated NTPs by spin column chromatography.[8] For commercially-available columns, follow the manufacturer's instructions for this step. Recover the RNA in ~100 µl.
5) Add one-tenth volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol to the tube, mix well.
6) Incubate for 15 minutes on ice.
7) Pellet the RNA by centrifugation at >10,000 x g for 15 minutes at 4°C.
8) Remove the supernatant with a pipette and gently rinse the pellet with 70% ethanol.
9) Remove the 70% ethanol with a pipette without disturbing the RNA pellet.
10) Allow pellet to dry, then resuspend in RNase-Free Water, TE or other suitable buffer.

RELATED PRODUCTS

- INCOGNITO™ T7-FlashScribe™ Ψ-RNA Transcription Kit
- INCOGNITO™ T7-FlashScribe™ N1meΨ-RNA Transcription Kit
- INCOGNITO™ T7 mScript™ Complete Ψ-mRNA Production System
- INCOGNITO™ T7 mScript™ Ψ-mRNA Production System
- INCOGNITO™ T7 mScript™ Complete N1meΨ-mRNA Production System
- INCOGNITO™ T7 mScript™ N1meΨ-mRNA Production System
- INCOGNITO™ T7 Ψ-RNA Transcription Kit
- INCOGNITO™ T7 5mC- & Ψ-RNA Transcription Kit
- INCOGNITO™ T7 ARCA 5mC- & Ψ-RNA Transcription Kit
- MessageMAX™ T7 ARCA-Capped Message Transcription Kit V2
- T7-FlashScribe™ Transcription Kit V2
- T7 mScript™ Complete Standard mRNA Production System
- T7 mScript™ Standard mRNA Production System V2
- T7-Scribe™ Standard RNA IVT Kit

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