Use of ShortCut® RNase III for dsRNA removal from IVT samples

Ane Martin Anduaga, Ph.D., Joe Whipple, Ph.D., New England Biolabs, Inc.

INTRODUCTION

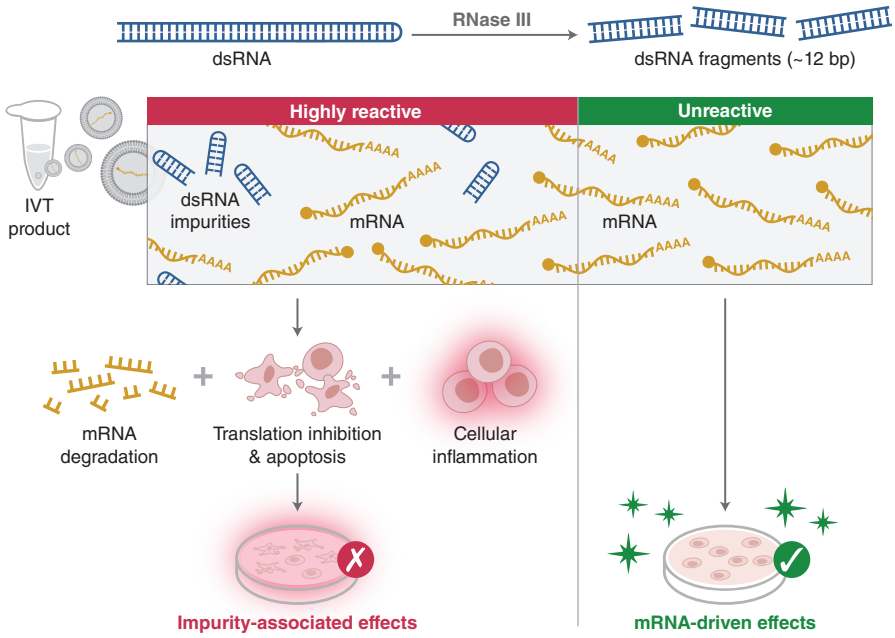
As RNA therapeutic development becomes more widespread, challenges arise for scientists to produce high-quality RNA that is free of impurities such as double-stranded RNA (dsRNA) that might trigger unwanted immune responses.

In vitro transcription (IVT) conditions can be optimized (e.g., template purification, altering NTP ratios, Mg^{2+} concentration, or using modified nucleosides) to minimize the generation of these byproducts during synthesis (1). However, if dsRNA content remains higher than desired, additional steps are required for purifying the IVT samples. Some purification strategies include cellulose/ethanol treatments (2), ion-pair reverse-phase HPLC (RP-HPLC) (3), or dsRNA-specific scavenger resin (4). Each of these strategies offer varying degrees of dsRNA removal effectiveness and pose their own challenges, such as using organic/flammable solvents and/or affecting production cost, mRNA recovery, and safety. Alternatively, dsRNA from IVT mRNA can be removed enzymatically using RNase III (5).

ShortCut® RNase III (NEB #M0245) was originally developed to process long dsRNA into a heterogeneous pool of short (18–25 bp) interfering RNAs (siRNA) suitable for RNA interference in mammalian cells. The original protocol provided is not compatible with a safe dsRNA removal strategy for IVT reactions. However, slight modifications in the reaction conditions reduce the risk of ssRNA degradation while removing the unwanted dsRNA from the sample. This application note provides a protocol for assessing the suitability of RNase III treatment using ShortCut RNase III (NEB #M0245) and shows the risk of using the original protocol or non-optimal conditions.

MATERIALS

- ShortCut® RNase III (NEB #M0245)
- 25 mM $MgCl_2$ (NEB #B9021)
- RNase Inhibitor Murine (NEB #M0314)
- Thermolabile Proteinase K (NEB #P8111)
- Monarch® Spin RNA Cleanup Kit (NEB #T2050)



RNase III treatment using $MgCl_2$ preserves ssRNA integrity

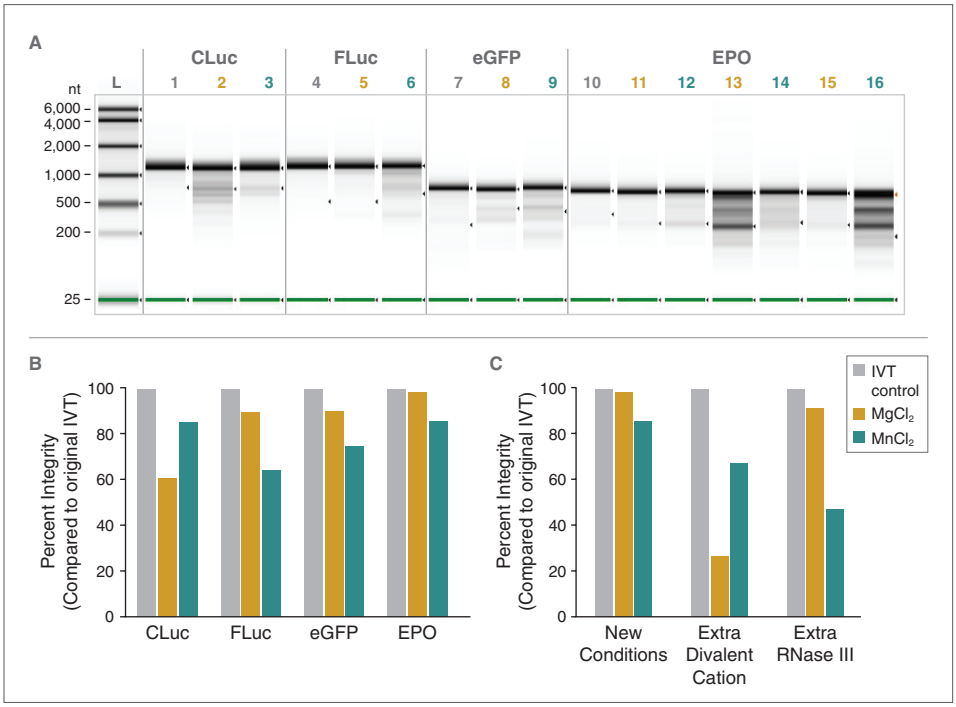
RNase III treatment was performed on IVT reactions from four different substrates of varying structural complexity: CLuc, FLuc, eGFP, and EPO. When sub-optimal RNase III and metal ion conditions were used (0.5 U of RNase III and either 0.5 mM $MgCl_2$ or 0.25 mM $MnCl_2$), the integrity of the ssRNA varied significantly across substrates (Figure 1A–B). The most structured substrate, CLuc, displays several ssRNA degradation products regardless of the metal ion employed. In contrast, FLuc and eGFP samples retained ~90% ssRNA integrity, and EPO maintained over 98% ssRNA integrity, but only when using $MgCl_2$. Interestingly, when the EPO sample, which is a great candidate for RNase III removal of dsRNA, was processed under conditions recommended in the original ShortCut RNase III protocol for siRNA generation (20 U RNase III and 20 mM of $MgCl_2$ or $MnCl_2$), its ssRNA integrity is substantially decreased (Figure 1A, C).

RNase III treatment significantly reduces dsRNA content

After assessing the ssRNA integrity post RNase III treatment, we quantified the dsRNA reduction using J2-based dot-blots. The RNase III treatment significantly reduced dsRNA content across all samples, with the greatest reductions observed when $MgCl_2$ was used as the divalent cation cofactor (Figure 2).

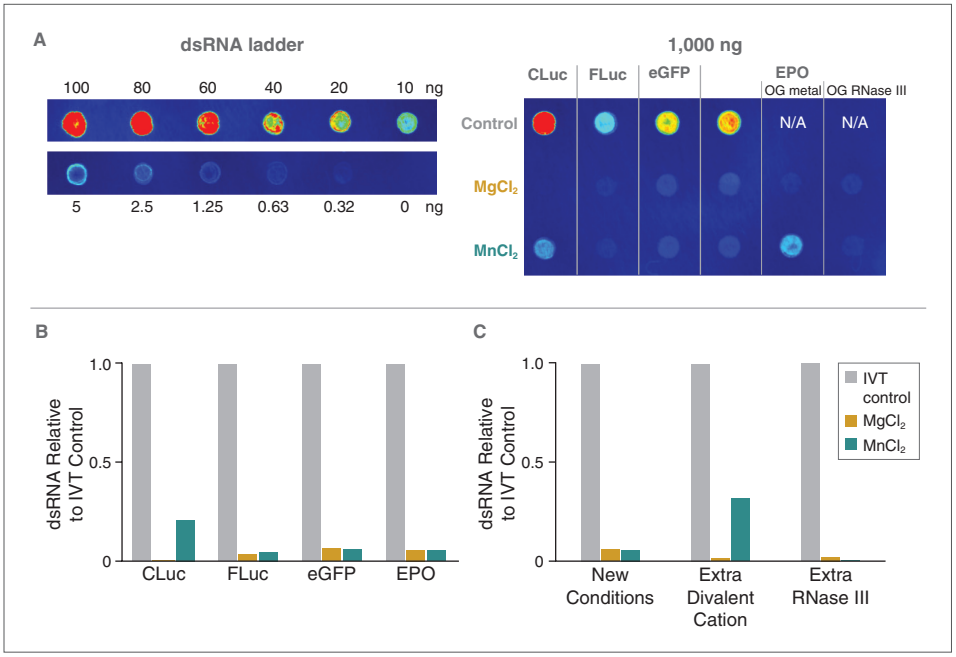
Given that the ssRNA integrity is significantly better when using $MgCl_2$, and the efficiency of dsRNA removal is comparable or improved with $MgCl_2$, we strongly recommend using $MgCl_2$ for RNase III-mediated dsRNA removal (Table 1).

FIGURE 1: **Integrity of ssRNA after RNase III treatment**



(A) Electropherogram traces of 15 ng of sample run in a High Sensitivity RNA ScreenTape® (Agilent®, 5067-5579) on a 4200 TapeStation® System (Agilent, G2991BA). Lanes 1–3 correspond to CLuc; 4–6 to FLuc; 7–9 to eGFP; and 10–16 to EPO. Lanes are color coded to represent the treatment conditions: grey for control and yellow or green for RNase III supplemented with $MgCl_2$ or $MnCl_2$, respectively. EPO was subjected to two additional reactions with the original ShortCut RNase III protocol concentrations of the corresponding metal (bands 13–14) or the original concentration of RNase III (bands 15–16). The ladder was run electronically. (B) Relative integrity of the band (% of sample corresponding to wanted band versus degradation bands) compared to the untreated control. (C) Integrity of EPO samples treated with the modified or original concentrations of the different metals or enzyme.

FIGURE 2: **Quantification of dsRNA using J2 dot blots**



(A) J2 blot showing dsRNA ladder (left) and 1 µg of control and RNase III-treated IVT reactions supplemented with $MgCl_2$ or $MnCl_2$ (right). (B) Quantification of the dsRNA content in each sample relative to the untreated control. (C) dsRNA quantification for EPO samples treated with optimized RNase III and metal concentrations vs. original protocol amounts of divalent cation or RNase III (OG).

Titration of RNase III and $MgCl_2$ concentration in RNase III reactions

By titrating RNase III and $MgCl_2$ concentrations, we determined that 0.1 U of RNase III and 0.25 mM $MgCl_2$ per 70 µg of IVT in a 100 µl reaction is the best starting point for most substrates.

Briefly, a mastermix containing 1x ShortCut Reaction buffer, 0.7 µg/µl IVT, and 1 U/µl RNase Inhibitor, Murine (NEB #M0314S) was prepared and divided into 12 aliquots. Each aliquot was supplemented with varying concentrations of $MgCl_2$ (0.5, 0.25, 0.1, or 0.05 mM) and RNAse III (0.5, 0.25, or 0.1 U). Reactions were incubated at 37°C for 20 min and inactivated using Thermolabile Proteinase K (NEB #P8111).

These titrations were carried out on the four substrates mentioned above: CLuc, FLuc, eGFP, and EPO. For eGFP, $MgCl_2$ concentration had a greater impact on dsRNA removal efficiency than the RNase III units, with concentrations above 0.25mM achieving maximal dsRNA reduction (Figure 3A). TapeStation analysis revealed that both $MgCl_2$ and RNase III influence substrate integrity, with $MgCl_2$ showing a stronger effect (Figure 3B–D).

Collectively, these results suggest that using 0.1 U of RNase III and 0.25 mM $MgCl_2$ provide the optimal balance between substrate integrity and dsRNA removal (Table 2). Similar results were obtained in all assessed substrates (data not shown) except for CLuc, the most structured substrate, which exhibited persistent ssRNA substrate degradation under conditions that achieved significant dsRNA reduction (Figure 4).

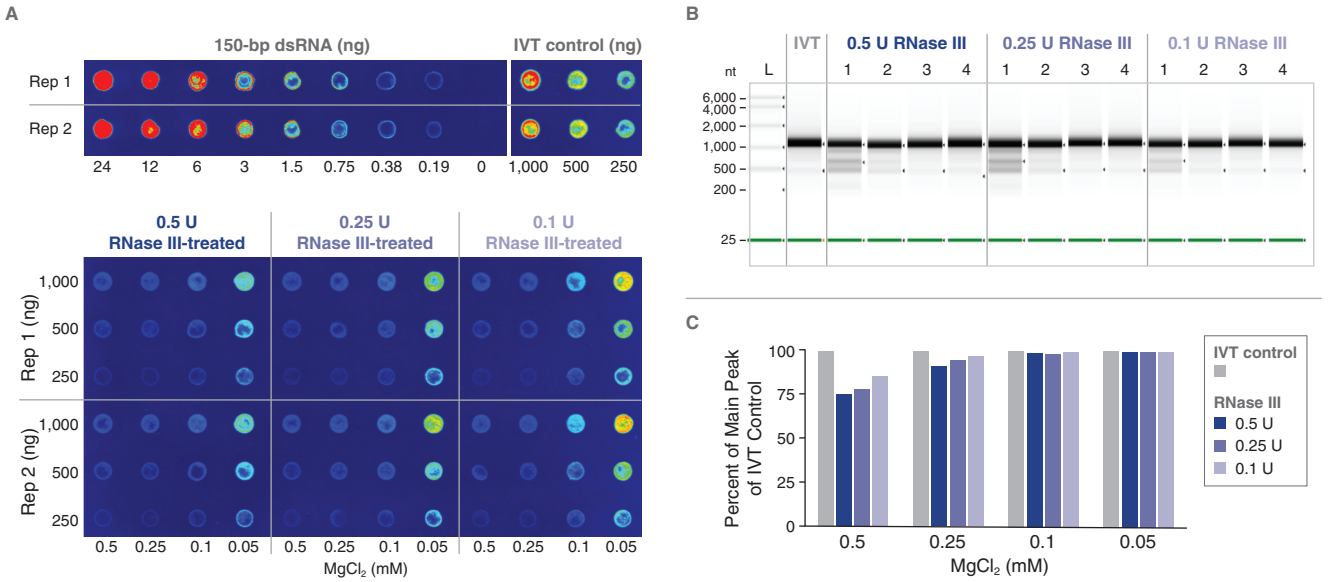
TABLE 1: **Summary of band integrity and dsRNA content of each substrate after RNase III treatment compared to untreated control**

	CLuc	FLuc	eGFP	EPO	EPO EXTRA M ETAL	EPO EXTRA RNASE III
% Band Integrity $MgCl_2$**	61.40	89.96	90.63	98.82	27.22	91.70
% dsRNA $MgCl_2$ compared to water	0.26	4.20	7.23	6.58	2.13	2.68
% Band Integrity $MnCl_2$ **	85.70	64.83	75.03	86.08	67.81	47.93
% dsRNA $MnCl_2$ compared to water	21.66	5.58	6.61	6.31	32.25	2.21

TABLE 2: **Integrity and dsRNA removal efficiency for eGFP samples**

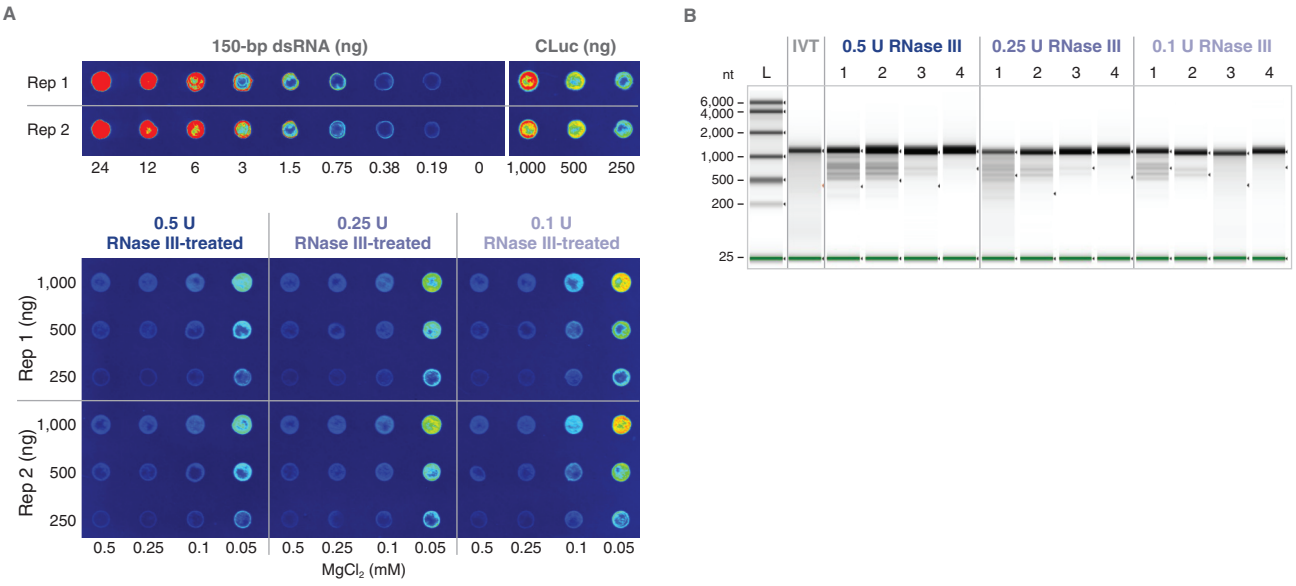
RNase III (U)	$MgCl_2$(mM)	ssRNA INTEGRITY	dsRNA REMOVAL
0.5	0.5	-	+++
0.5	0.25	++	+++
0.5	0.1	+++	+
0.5	0.05	+++	-
0.25	0.5	-	+++
0.25	0.25	++	+++
0.25	0.1	+++	-
0.25	0.05	+++	-
0.1	0.5	-	+++
0.1	**0.25**	**+++**	**+++**
0.1	0.1	+++	-
0.1	0.05	+++	--

FIGURE 3: **Effect of $MgCl_2$ and RNase III titration on eGFP integrity and dsRNA removal**



(A) J2 blot showing dsRNA ladder (left) and control eGFP (top, right; 1000, 500, and 250 ng) as well as RNase III-treated eGFP samples under the reported amounts of $MgCl_2$ and RNase III (bottom).
(B) Electropherogram traces of 15 ng of sample run in a High Sensitivity RNA ScreenTape. First lane corresponds to the untreated eGFP IVT control; while the rest are samples treated with 0.5, 0.25, 0.1 U RNase III and decreasing $MgCl_2$ concentrations (1–4 correspond to 0.5, 0.25, 0.1, or 0.05 mM, respectively).

FIGURE 4: **Assessment of dsRNA and CLuc integrity on $MgCl_2$ and RNase III titration**



(A) J2 blot showing dsRNA ladder (top, left) and control CLuc (top, right; 1000, 500, and 250 ng) as well as RNase III-treated CLuc samples under the reported amounts of $MgCl_2$ and RNase III (bottom).
(B) Electropherogram traces of 15 ng of sample run in a High Sensitivity RNA ScreenTape. First lane corresponds to the untreated CLuc IVT control; while the rest are samples treated with 0.5, 0.25, 0.1 U RNase III and decreasing $MgCl_2$ concentrations (1–4 correspond to 0.5, 0.25, 0.1, or 0.05 mM, respectively).

CONCLUSION

It is essential to empirically verify that the substrate can tolerate RNase III treatment and does not contain transient dsRNA regions that may trigger ssRNA degradation.

Although the suggested concentrations of 0.25 mM $MgCl_2$ and 0.1U ShortCut RNase III work well for most assessed substrates, we recommend titrating these parameters for each new substrate to ensure safety and efficacy. We strongly advise running the treated samples on a gel to visually confirm sample integrity and quantifying dsRNA removal after every treatment.

SUGGESTED PROTOCOL FOR IVT SAMPLES

1. Dilute the ShortCut RNase III enzyme 1:10 in 1X ShortCut Reaction Buffer to a working concentration of 0.2 U/µl.
2. Set up the following reaction:

COMPONENT	VOLUME (µl)	CONCENTRATION IN SAMPLE
Diluted ShortCut RNase III[1]	0.5	1 mU/µl (or 0.1 U total)
ShortCut Reaction Buffer (10X)	10	1x
IVT sample	up to 70 µg	0.7 µg/µl
25mM $MgCl_2$ (NEB #B9021S)[1]	1	0.25 mM
RNase Inhibitor Murine (NEB #M0314S)	2.5	1 U/µl
Nuclease-free Water	to 100	—
Total Volume	**100**	

[1] $MgCl_2$ and ShortCut RNase III concentrations should be experimentally assessed on a substrate-by-substrate basis, but this is a good starting point

3. Incubate at 37°C for 20 min.
4. Stop the reaction by adding 1µl of Thermolabile Proteinase K (NEB #P8111) and incubation at 37°C for 15 min.
5. RNA purification using Monarch® Spin RNA Cleanup Kit (NEB #T2050).
6. Quality control check-ups:
   a. Run in gel/Tapestation®/Bioanalyzer® to verify the integrity of your IVT sample.
   b. Perform dsRNA quantification to confirm the removal of dsRNA.

References

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