RNA electrophoresis

Optimization of an RNA electrophoresis workflow for E-Gel EX agarose gels

Keywords
RNA, electrophoresis, gel agarose, gel electrophoresis, RNA analysis, RNA electrophoresis, E-Gel EX agarose gels, E-Gel Power Snap Plus Electrophoresis System

Abstract
Agarose gel electrophoresis is an established method for the separation of nucleic acids. However, using it for the analysis of RNA can be challenging. Accurate analysis requires optimization of conditions in which RNA can be denatured and analyzed for precise assessment of fragment size before degradation occurs. Invitrogen™ E-Gel™ EX agarose gels integrate an easy-to-use precast format, high sensitivity, and rapid nucleic acid analysis into an all-in-one system for a simplified RNA analysis workflow. Here we investigate RNA denaturation conditions and sample amounts for an optimal RNA analysis workflow for E-Gel EX agarose gels.

Key findings
- RNA samples and ladders exhibit optimal separation resolution and fewer artifacts when treated in 50% formamide and heated at 70°C for 10 minutes
- RNA ladders exhibit optimal resolution on E-Gel EX gels with 500 ng to 1 µg loaded per well, depending on the ladder type
- RNA samples have different optimal load ranges, based on the sizes of the fragments:
  - Smaller fragments are optimally resolved at loads of 20–70 ng per well
  - Larger fragments (>2,000 bp) are optimally resolved at loads of 70–200 ng per well

Introduction
Electrophoresis is one of the oldest methods in molecular biology for the analysis of nucleic acids [1]. It is a simple and efficient technique for analysis of fragment sizes and concentration, quality control, and fragment isolation. Electrophoretic analysis of DNA is generally straightforward under most conditions, as DNA is less prone to degradation and can provide relatively fast and reliable results. However, the use of agarose electrophoresis for the analysis of RNA often requires additional considerations.

RNA tends to form secondary structures through complementary base pairing. Therefore, accurate assessment of RNA fragment size by agarose gel electrophoresis requires a denaturation step to revert the molecule to a linear structure [2]. Multiple RNA denaturation agents have been described, including methyl mercury hydroxide, urea, guanidine thiocyanate, and the most common one, formaldehyde [3]. Additionally, strong denaturing reagents often inhibit RNases, helping maintain RNA integrity during separation and gel handling [4].

Traditional electrophoresis-based approaches for RNA analysis require extra time for buffer preparation, hand-pouring of gels, and prolonged run times. The additional need for denaturing reagents adds an extra layer of complexity, as it requires the full process to be performed under a fume hood to help prevent exposure to toxic chemicals.

E-Gel EX agarose gels are being increasingly adopted in RNA analysis workflows. They simplify the electrophoresis process by eliminating the need for labor-intensive gel and buffer preparation as well as the use of toxic denaturing reagents. This study provides guidelines on optimal RNA ladder and sample concentrations, preparation, and a protocol to help bring the time savings and ease of use of the Invitrogen™ E-Gel™ Power Snap and Power Snap Plus systems to RNA workflows.

Materials and methods

Sample preparation

RNA samples and standard markers were diluted in nuclease-free water to the desired working concentration in a maximum total volume of 10 µL. Samples were then brought to a final volume of 20 µL, the maximum well capacity of Invitrogen™ **E-Gel™ EX 1% agarose gels** or **E-Gel™ EX 2% agarose gels**, with either 100% formamide or nuclease-free water.

RNA denaturation

RNA was denatured by: (1) addition of formamide (up to 50% final concentration), (2) heating samples at 70°C for 10 minutes, or (3) using both formamide addition and heating. All heated samples were cooled on ice prior to loading the samples on the gel.

Gel run and documentation

E-Gel EX 1% or 2% agarose gels were placed in the **E-Gel Power Snap Electrophoresis System** or **E-Gel Power Snap Plus Electrophoresis System**. RNA samples and reference marker ladders were loaded into wells and allowed to rest for 1 minute prior to initiating the run. Gels were run using a pre-programmed protocol specific to the gel type.

Results of the runs were documented using the built-in camera of the E-Gel Power Snap or Power Snap Plus system. Gel images and fragment sizing results were exported from the systems using an external USB flash drive.

Reagents and instruments used in the optimization studies are listed in Table 1.

Table 1. RNA markers, agarose gels, and instruments used in optimization of RNA analysis.

Reagents and instrumentation	Cat. No.
E-Gel EX Agarose Gels, 1%	G401001
E-Gel EX Agarose Gels, 2%	G401002
E-Gel Power Snap Electrophoresis System	G8300
E-Gel Power Snap Plus Electrophoresis System	G9301
RiboRuler High Range RNA Ladder	SM1821
RiboRuler Low Range RNA Ladder	SM1831
Millennium RNA Markers	AM7150
Century-Plus RNA Markers	AM7145
Formamide (formerly Acros Organics Cat. No. 181090010)	044066.AY

Results and discussion

Determining denaturation method

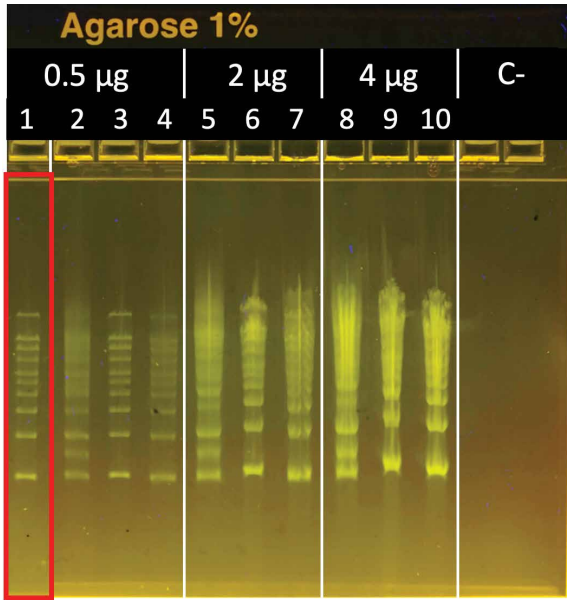
RNA tends to form secondary structures through complementary base pairing. This can prevent consistent and predictable migration of RNA samples and markers through an agarose gel and cause smearing. Therefore, it is important to denature RNA samples prior to electrophoresis.

Invitrogen™ **Millennium™ RNA Markers** (500–9,000 bases) and **Century™-Plus RNA Markers** (100–1,000 bases) were tested under different denaturing conditions on 1% and 2% E-Gel EX agarose gels, respectively. Markers were either not denatured, treated in 50% formamide or with heat alone, or treated with both formamide and heat (Figure 1).

In addition to testing different denaturation conditions, the amount of RNA marker loaded into each well was also varied from 0.5 µg to 4 µg. This was done to better identify the optimal quantity of RNA per well to maximize separation and resolution on E-Gel EX agarose gels.

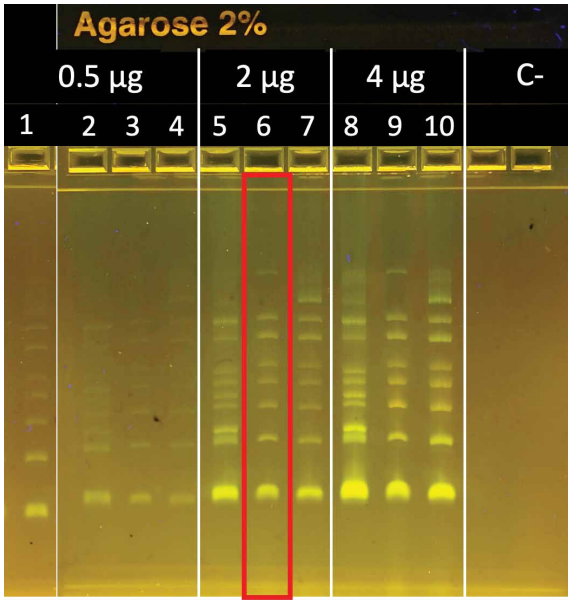
The analysis revealed the optimal RNA amounts per well to be 0.5 µg for the Millennium RNA Markers and 2 µg for the Century-Plus RNA Markers (Figure 1, red box). The results show that heating alone was not sufficient to obtain optimal clarity and fragment separation for either RNA marker type. However, the use of formamide as a single denaturing agent resulted in significantly improved resolution over the control sample, suggesting that additional treatment of samples with heat may not be necessary.

Millennium RNA Markers denaturation



Well number	Formamide (final conc. 50%)	Heating (70°C, 5 min)
1	+	–
2, 5, 8	–	–
3, 6, 9	+	+
4, 7, 10	–	+

Century-Plus RNA Markers denaturation



Well number	Formamide (final conc. 50%)	Heating (70°C, 5 min)
1	+	–
2, 5, 8	–	–
3, 6, 9	+	+
4, 7, 10	–	+

Figure 1. Testing RNA denaturing conditions for E-Gel EX gel separation and analysis. Millennium and Century-Plus RNA Markers were analyzed untreated and following treatment with 50% formamide, heating, or both. Tested loading quantities were 0.5 µg, 2 µg, and 4 µg. Results indicate that 0.5 µg was the optimal loading quantity for the Millennium markers while 2 µg provided the best resolution for Century-Plus markers. For Millennium markers, formamide treatment alone was sufficient to achieve good resolution and separation. For Century-Plus markers, treatment with heat and formamide achieved fine resolution.

Determining optimal loading quantity

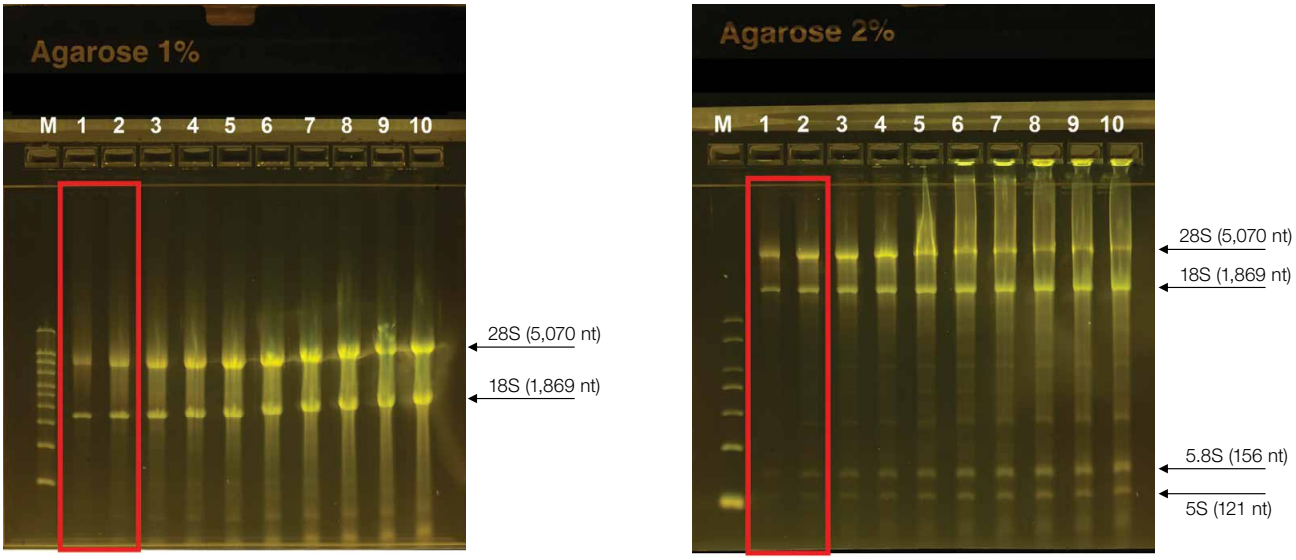
We also investigated the optimal RNA sample quantity that can be loaded per well on E-Gel EX agarose gels, using a universal human reference RNA (UHRR) as reference. Prior to loading, the samples were prepared in 50% formamide as described previously. Considering that the single-strand composition of RNA is known to reduce the binding efficiency of the detection stain used in E-Gel EX agarose gels, the minimum amount was chosen to be 0.25 µg, with a maximum of 4 µg.

The analysis (Figure 2) demonstrated smearing and slowed migration when RNA was loaded at quantities of 0.75 µg per well and higher. The optimal range was determined to be 0.25–0.5 µg per well.

RNA fragment analysis

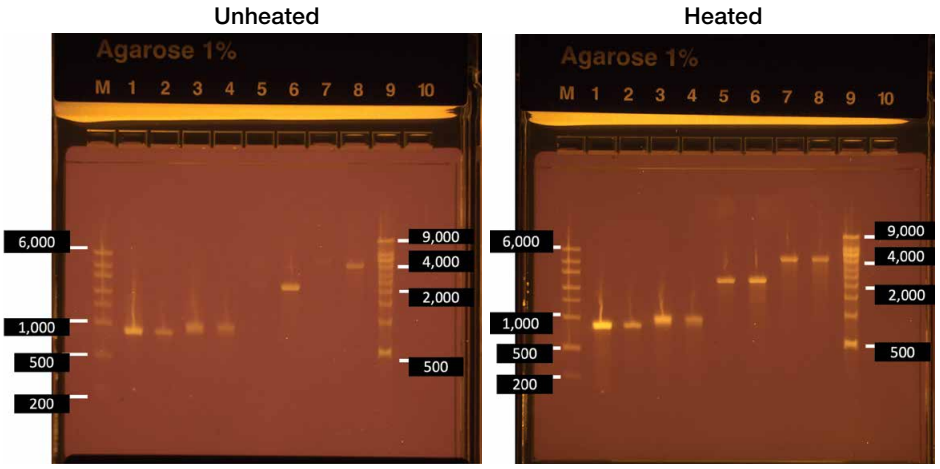
The optimal conditions identified previously were applied to an actual instance of fragment analysis (Figure 3). The results highlighted two major differences.

A load of 250 ng (0.25 µg) of RNA per well had significant smearing, so the amounts of sample loaded were reduced for subsequent experiments (data not shown). Specifically, 100 ng per well was used as a starting point. With only 50% formamide, the sample bands appeared faint and less defined. Therefore, heat treatment at 70°C for 10 min was added to sample preparation (Figure 3).



Well number	1	2	3	4	5	6	7	8	9	10
UHRR quantity, ng	250	500	750	1,000	1,500	2,000	2,500	3,000	3,500	4,000

Figure 2. Determining optimal RNA loading per well. UHRR samples were denatured in 50% formamide and loaded into either 1% or 2% E-Gel EX agarose gels in a range of 250–4,000 ng per well. Millennium and Century-Plus RNA markers were used on 1% and 2% agarose E-Gel EX gels, respectively, as reference standards. Results showed clear separation of bands with minimal smearing when loaded at 0.25 and 0.5 µg per well (red boxes).

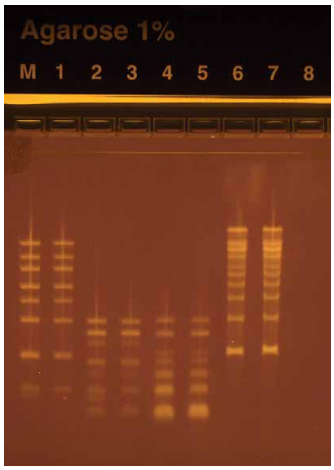


Well	Sample	Size (bases)	Quantity (ng/well)
M	RiboRuler HR RNA Ladder	Various	1,000
1	eGFP (unpurified)	868	100
2	eGFP (purified)		100
3	eGFP poly(A) (unpurified)	1,017	100
4	eGFP poly(A) (purified)		100
5	TV_Liu_eGFP (unpurified)	2,716	100
6	TV_Liu_eGFP (purified)		100
7	TV_NOS_eGFP (unpurified)	4,525	100
8	TV_NOS_eGFP (purified)		100
9	Millennium RNA Markers	Various	1,000
10	—		

Figure 3. Treatment with heat in addition to 50% formamide enables more complete RNA denaturation in diverse samples. Samples were denatured either with formamide alone (left) or in combination with heat at 70°C for 10 minutes followed by cooling on ice prior to loading (right).

The added heat treatment resulted in a remarkable improvement to band definition, as well as boosting signal strength in both samples and RNA markers used (Figure 3). Additional investigation of RNA markers under the same denaturing conditions (50% formamide and heat) eliminated phantom bands that appeared with Invitrogen™ RiboRuler™ High Range and RiboRuler™ Low Range RNA Ladders (Figure 4). There was no difference in the number of bands observed, between the heated and unheated samples of the Millennium and Century-Plus RNA markers. The data are consistent with initial observations presented in Figure 1, and highlight the advantages of combining 50% formamide and heat treatment for complete RNA denaturation to improve signal resolution.

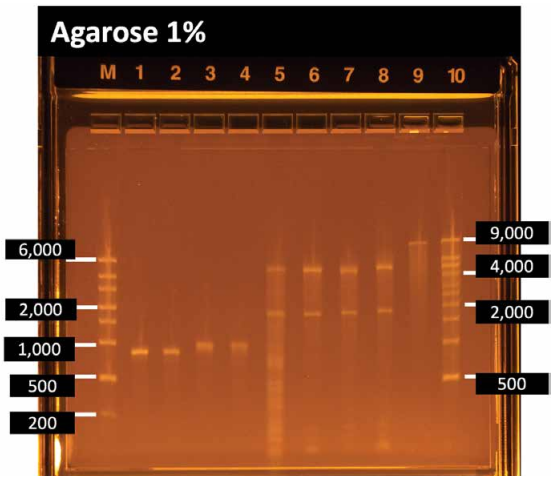
Finally, RNA electrophoresis was performed on a diverse collection of samples using the identified optimal conditions (Figure 5). Smearing was observed in total RNA extracts and the longest (9,000 bases) fragment tested, which could be due to impurities or degradation. On both gels, polyadenylated (poly(A)) samples exhibited a notable shift in size, making them easily distinguishable from non-poly(A) variants. Additionally, there was no significant difference between purified and unpurified RNA samples on both gel types, potentially further simplifying RNA analysis workflows.



Well	Sample
M	RiboRuler High Range RNA Ladder
1	
2	RiboRuler Low Range RNA Ladder
3	
4	Century-Plus RNA Markers
5	
6	Millennium RNA Markers
7	

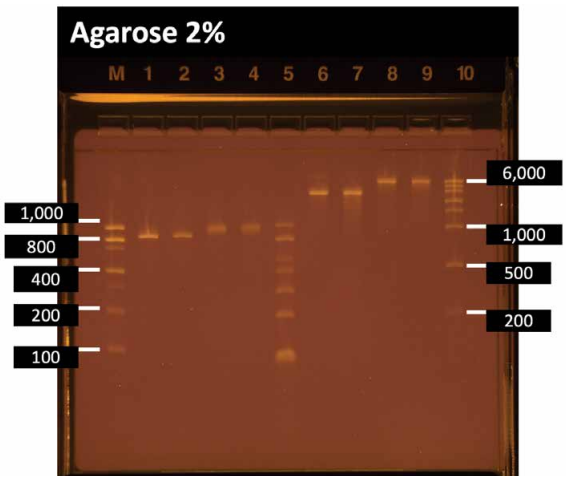
Figure 4. Treatment of RNA standard markers with both formamide and heat. After the combined treatment, RiboRuler Low Range and RiboRuler High Range RNA Markers did not show phantom bands.

Unheated



Well	Sample	Size (bases)	Quantity (ng/well)
M	RiboRuler High Range RNA Ladder	Various	1,000
1	eGFP (unpurified)	868	20
2	eGFP (purified)		50
3	eGFP poly(A) (unpurified)	1,017	20
4	eGFP poly(A) (purified)		50
5	Total RNA (placenta)	5,070 (28S), 1,869 (18S)	200
6	Total RNA (HeLa)		70
7	Total RNA (rat thymus)		70
8	Total RNA (Raji)		100
9	Millennium RNA Markers	9,000	120
10	Millennium RNA Markers	Various	800

Heated



Well	Sample	Size (bases)	Quantity (ng/well)
M	RiboRuler Low Range RNA Ladder	Various	1,200
1	eGFP (unpurified)	868	30
2	eGFP (purified)		70
3	eGFP poly(A) (unpurified)	1,017	30
4	eGFP poly(A) (purified)		80
5	Century-Plus RNA Markers	Various	1,200
6	TV_Liu_eGFP (unpurified)	2,716	200
7	TV_Liu_eGFP (purified)		70
8	TV_NOS_eGFP (unpurified)	4,525	70
9	TV_NOS_eGFP (purified)		70
10	RiboRuler High Range RNA Ladder	Various	1,000

Figure 5. Analysis of a diverse range of RNA samples using optimized conditions for sample preparation and run. No differences were observed between purified and unpurified fragments. Poly(A) RNA can be distinguished from non-poly(A) RNA in both concentrations of agarose gels.

Conclusions

The E-Gel Power Snap Plus Electrophoresis System and E-Gel EX agarose gels offer a fast and efficient platform for rapid nucleic acid analysis. E-Gel precast agarose gels are provided in a ready-to-use format and do not require any additional preparation outside of addition of samples, ladders, and tracking dye. Further, these gels are offered in a wide variety of agarose concentrations, well formats, and throughput capacities for ultimate flexibility. Hence, the E-Gel Power Snap system offers an exceptional platform for the efficient separation of RNA fragments.

Sample preparation of RNA ladders and RNA samples representing commonly analyzed fragment sizes was optimized to leverage the benefits of the E-Gel Power Snap Electrophoresis System for RNA fragment analysis. Agarose electrophoresis of native RNA samples can produce unreliable results due to the formation of secondary structures by complementary base-pair bonding. Multiple methodologies have been reported to denature RNA and enhance detection and accuracy in RNA electrophoresis.

Here we established an RNA analysis workflow using E-Gel EX agarose gels with the E-Gel Power Snap Plus Electrophoresis System. We demonstrated that denaturation of the RNA in 50% formamide is sufficient in most cases to obtain optimal results. However, the addition of heat treatment can further reduce phantom bands and improve band definition in selected RNA samples. Finally, it was shown that maximal resolution could be obtained with an optimal loading of 20–80 ng per well for smaller bands (<1,000 bases), and 70–200 ng per well for larger bands (>1,000 bases). Collectively, this study helps provide foundational data to enable effective electrophoretic separation of RNA on the E-Gel Power Snap Plus system. The complete protocol for analysis of RNA samples using E-Gel EX agarose gels can be found in the E-Gel Power Snap and E-Gel Power Snap Plus system user guides.

References

1. Stellwagen NC (2009) Electrophoresis of DNA in agarose gels, polyacrylamide gels and in free solution. *Electrophoresis*. 30 Suppl 1(Suppl 1):S188-S195. doi:10.1002/elps.200900052
2. Rio DC (2015) Denaturation and electrophoresis of RNA with formaldehyde. *Cold Spring Harb Protoc*. 2015(2):219-222. Published 2015 Feb 2. doi:10.1101/pdb.prot080994
3. Masek T, Vopalensky V, Suchomelova P, Pospisek M (2005) Denaturing RNA electrophoresis in TAE agarose gels. *Anal Biochem*. 336(1):46-50. doi:10.1016/j.ab.2004.09.010
4. Mansour FH, Pestov DG (2013) Separation of long RNA by agarose-formaldehyde gel electrophoresis. *Anal Biochem*. 441(1):18-20. doi:10.1016/j.ab.2013.06.008

Ordering information

Product	Cat. No.
E-Gel EX Agarose Gels, 1%	G401001
E-Gel EX Agarose Gels, 2%	G401002
E-Gel Power Snap Electrophoresis System	G8300
E-Gel Power Snap Plus Electrophoresis System	G9301
RiboRuler High Range RNA Ladder	SM1821
RiboRuler Low Range RNA Ladder	SM1831
Millennium RNA Markers	AM7150
Century-Plus RNA Markers	AM7145
Formamide (formerly Acros Organics Cat. No. 181090010)	044066.AY

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