Materials not included:
Nuclease-free water, incubator, PAGE/Urea gel, TBE running buffer, power supply, SYBR Gold, Typhoon scanner and UV light box.

Fluorescein Labeled RNA Probe:
The probe is an in vitro RNA transcript (300-mer), internally labeled with Fluorescein-12-UTP, supplied at 40 ng/µl. The sequence of the transcript can be found on the product page at www.neb.com.

Table 1. Reaction Set Up for RNase Contamination Assay

<table>
<thead>
<tr>
<th>Components</th>
<th>Each Rxn</th>
<th>10 Reactions with 10% Overage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>7 µl</td>
<td>77 µl</td>
</tr>
<tr>
<td>10X NEBuffer 4</td>
<td>1 µl</td>
<td>11 µl</td>
</tr>
<tr>
<td>RNA probe</td>
<td>1 µl</td>
<td>11 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>9 µl</td>
<td>99 µl</td>
</tr>
</tbody>
</table>

1X NEBuffer 4:
- 50 mM Potassium acetate
- 20 mM Tris-acetate
- 10 mM Magnesium acetate
- 1 mM Dithiothreitol (pH 7.9 at 25°C)

2X RNA Loading Dye:
- 95% Formamide
- 0.02% SDS
- 0.02% Bromophenol blue
- 0.01% Xylene cyanol
- 1 mM EDTA

Proteinase K:
4 mg/ml

Protocol:

1. Preparation of test samples
Each 10 µl standard reaction uses 1 µl of test sample. The test sample should be diluted in sample storage buffer if it is very concentrated (e.g., enzyme stock). It is recommended that a reaction with a sample diluent (e.g., enzyme storage buffer) as well as a water control should be included along with test samples. The recommended minimal reactions are three.

If a custom reaction buffer is used for sample testing, replace 10X NEBuffer 4 with 10X custom reaction buffer. Be sure to include appropriate controls. If the test sample is water, we recommend replacing the water volume with sample water (8 µl). Be sure to include a control reaction with RNase-free water.

Samples containing very high concentration of salt or organics may inhibit RNase activity and interfere with PAGE.

2. Assay set up
It is highly recommended that a master mix with enough overage is prepared. Table 1 shows an example of a master mix preparation. After combining the components, mix them thoroughly by vortexing or by flicking the tube several times and then pulse-spin briefly.

3. Incubate the reactions in a dry air incubator at 37°C for 1 – 16 hours (overnight)
Incubation time varies depending on the quality requirements of test samples. More specifically, it depends on the applications of the test sample. For example, if the NTPs will be used in an in vitro transcription at 37°C for 16 hours, the NTPs should be incubated for 16 hours. Because the assay is more sensitive when detergent is present, 2 hour incubation is sufficient for samples containing detergent.

4. Proteinase K treatment to release bound RNA probe (optional)
If the test sample can potentially bind to the RNA probe, e.g., MMLV reverse transcriptase, the test reaction should be treated with 1 µl of Proteinase K at room temperature for 5 – 10 minutes before gel analysis. This treatment will release the bound RNA probe and help with gel image analysis. You may choose to treat all of the reactions with proteinase K when you are unsure whether the test samples will bind to the RNA probe. Proteinase K treatment does not interfere with the assay. Figure 1A shows the advantage of PK treatment of reactions with fast dyes that bind to the RNA probe (reactions 1 and 2) and no difference for reactions with samples that do not bind to RNA probe (control and reaction 3).

5. Stop reaction by adding 10 µl of 2X RNA loading dye to each reaction
Add 10 µl of 2X RNA loading dye to each reaction. Mix thoroughly and pulse-spin briefly. If you are unable to proceed with gel analysis, store the samples at –20°C. Samples can be stored at –20°C for at least three days.

6. Gel electrophoresis
We recommend analyzing the reactions on a denaturing polyacrylamide gel (PAGE-Urea gel, 5–10%). Before loading, heat the samples at 65–70°C for 5–10 min, pulse-spin briefly. Load 10 µl of each sample onto gel. Run the gel until the bromophenol blue (the fast dye) is approximately 2–3 cm from the bottom of the gel. If the gel runs too far, the free label will run off the gel. For a standard mini-gel, the running time is about 30–45 min.

7. Visualization and analysis
a) On a fluorescein capable imaging system
The integrity of the fluorescein labeled RNA probe can be visualized by a fluorescein capable imaging system directly. After electrophoresis, remove the gel carefully and scan it on the Typhoon scanner or equivalent. Compare the RNA probe intensities of test samples to that of a valid water control. If the RNA probe is mostly intact, the sample passes the RNase contamination assay (e.g., reactions 2 and 3 in Figure 1A).

Figure 1A: Proteinase K treatment releases bound RNA probe. After incubation for 16 hours at 37°C, control and test reactions were analyzed on 6% PAGE-Urea gel. The gel was imaged on a Typhoon scanner. PK treatment releases bound RNA probe (reactions 1 and 2). The +PK panel shows reactions 2 and 3 with intact RNA probe, indicating that the samples are clean. Reaction 1 shows heavy degradation with free label at the bottom, indicating that the sample is contaminated with RNase activity.