**In Vitro Synthesis of 2’-Fluoro-Modified RNA Transcripts That Are Completely Resistant to RNase A Digestion Using the DuraScribe™ T7 Transcription Kit**

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**Introduction**

*In vitro* transcription of DNA into RNA has become an increasingly important technique for genomic research. EPICENTRE’s new DuraScribe™ T7 Transcription Kit produces about 50 µg of “DuraScript™ DNA” that is completely resistant to RNase A.

The DuraScribe T7 Transcription Kit utilizes a mutant form of T7 RNA Polymerase (DuraScribe™ T7 RNA Polymerase) that uses the same T7 transcription promoters as standard T7 RNA Polymerase. However, unlike standard T7 RNA Polymerase, DuraScribe T7 Polymerase efficiently incorporates 2’-modified-NTPs (e.g., 2’-fluoro-dNTP), as well as canonical NTPs (ATP, CTP, GTP, UTP), into full length RNA transcripts *in vitro*. DuraScript RNA is produced by completely replacing CTP and UTP with 2’-fluoro-dCTP (2’-F-dCTP) and 2’-fluoro-dUTP (2’-F-dUTP) in a DuraScribe *in vitro* transcription reaction (Figure 1). The presence of the 2’-fluoro-dC and 2’-fluoro-dU nucleotides in DuraScript RNA prevents its digestion by RNase A. However, DuraScript RNA is not resistant to other RNases that digest RNA at other nucleotides such as RNase T1 or RNase H. The sensitivity of DuraScript RNA to these RNases is beneficial in some applications.

Here we report the stability and yields of DuraScript RNA as well as its resistance to RNase A and related ribonucleases found in the lab environment.

**Materials and Methods**

DuraScribe T7 *in vitro* transcription reactions were performed as described in the DuraScribe kit literature. Unless otherwise noted, 20 µl DuraScribe reactions containing 1X DuraScribe™ T7 Transcription Buffer, 10 mM DTT, 1 µg of a linearized 3 Kb DNA Control Template (which produces a 1.4 Kb RNA transcript), 5 mM each ATP, GTP, 2’-F-dUTP and 2’-F-dCTP, and 2 µl DuraScribe T7 Enzyme Mix, were incubated at 37º C for 4 hours.

DuraScript RNA transcripts were precipitated from transcription reactions by addition of an equal volume (20 µl) of 5 M ammonium acetate. The tubes were held on ice for 15 minutes followed by high speed centrifugation for 15 minutes in a microcentrifuge. The pelleted DuraScribe RNA was resuspended in water and quantitated by UV spectrophotometry.

**Results**

**Yields of DuraScript RNA**

The DuraScribe T7 Enzyme Mix is formulated to utilize very high concentrations of nucleotides in order to produce the highest possible yields of DuraScript RNA. Duplicate DuraScribe transcription reactions were incubated for 2, 4, or 6 hours at 37ºC. The standard 2-hour DuraScribe transcription reaction consistently produced 40 - 60 µg of full-length DuraScript RNA from 1 µg of the control DNA template (Figure 2). Incubating the reaction for 6 hours improved the yield by approximately 10%.

The standard 20 µl DuraScribe reaction was scaled-up to increase the yield of DuraScript RNA. All reaction components, including the DNA template, were scaled up 5X and 25X (to 100 µl and 500 µl reaction volumes, respectively). The 5X reaction produced >200 µg and the 25X reaction generated >600 µg of DuraScript RNA.

Frequently, the DNA template is the limiting component in an *in vitro* transcription reaction. The yield of DuraScript RNA produced from limiting amounts of template was determined by performing DuraScribe reactions, in duplicate, using decreasing amounts of the linearized Control Template DNA. As shown in Figure 3, a DuraScribe reaction produces microgram amounts of DuraScript RNA from as little as 10 ng of DNA template.

**Figure 1.** DuraScribe™ T7 RNA Polymerase efficiently incorporates 2’-F-dCTP and 2’-F-dUTP into full length DuraScript™ RNA. The presence of the fluorine at the 2’-position of the 2’-F-dC and 2’-F-dU nucleotides prevents RNase A digestion. The result is DuraScript RNA that is completely resistant to RNase A and related ribonucleases.

**Figure 2.** Forty to sixty µg of a 1.4 Kb DuraScript™ RNA is produced in a standard 4-hour DuraScribe™ T7 Transcription reaction using the control DNA template.

**Figure 3.** Limiting amounts of DNA templates are readily transcribed in a standard 20 µl DuraScribe™ reaction.
Incorporation of Labeled Nucleotides by DuraScribe T7 Polymerase

Direct incorporation of biotin-, digoxigenin-, and fluorescent-nucleotides into DuraScript RNA by DuraScribe T7 Polymerase was examined. Since DuraScribe T7 Polymerase will incorporate 2’-deoxynucleotides, and to retain the RNase A resistance of the labeled DuraScript RNA, biotin-dUTP, digoxigenin-dUTP, and fluorescent labeled-dUTP were chosen as the labeled nucleotides. By replacing a portion of the 2’-fluoro-dUTP with the respective labeled-dUTP in the reaction, full length, non-radioactively labeled DuraScript RNA was produced. Transcripts were also labeled post-transcriptionally with a Cyanine dye after incorporating amino-hexyl-ATP into the DuraScript RNA during the DuraScribe transcription reaction. Labeling protocols are provided with the DuraScribe T7 Transcription Kit.

Resistance of DuraScript RNA to RNase A and Other Ribonucleases

DuraScript RNA and standard RNA transcripts, produced by in vitro transcription using standard T7 RNA Polymerase, were each incubated with 1 µg/ml (final concentration) of purified RNase A (Catalog # R5250, Sigma) for 30 minutes at 37ºC. An aliquot of each reaction was analyzed by gel electrophoresis to ascertain the extent of degradation by the RNase A. The standard RNA transcript was completely degraded while the DuraScript RNA remained intact (Figure 4) thus demonstrating the resistance of DuraScript RNA to even extreme levels of RNase A. It should be noted that some preparations of RNase A from nuclelease-rich sources such as bovine pancreas, contain contaminating RNases that may digest DuraScript RNA. Therefore, only the highest purity RNase A should be added to DuraScript RNA preparations.

Ribonucleases present on human hands and spread throughout the lab are a major problem for those working with RNA. Because of these “fingertip” nucleases, researchers are required to wear gloves, use DEPC-treated water, bake or autoclave glassware and tubes, add RNase inhibitors to reactions and even dedicate equipment especially for RNA. Therefore, the stability of DuraScript RNA in the presence of “fingertip” nucleases was tested. DuraScribe and standard in vitro transcription reactions were performed in duplicate as described previously except that either RNase-free sterile water was added to one set of transcription reactions and water that had been contaminated by the hands of a test subject was added to the other set of reactions. The RNA products of each reaction were then analyzed by gel electrophoresis to ascertain the extent of degradation by “fingertip” nucleases present in the contaminated water. Figure 5 shows that DuraScript RNA was completely resistant to common fingertip nuclease degradation while standard RNA transcripts are degraded.

Applications exist where selective sensitivity of RNA to ribonucleases is desired (e.g., RNase protection assays). We first evaluated the sensitivity of DuraScript RNA to RNase H. A DuraScript RNA transcript was annealed to two complementary DNA oligonucleotides. The DuraScript RNA/DNA hybrid was incubated with 0.1 Unit of E. coli RNase H for 30 minutes at 37ºC. Agarose gel analysis of the resulting digestion products produced the predicted pattern of fragments from complete and specific RNase H digestion of the DuraScript RNA/DNA hybrid. Therefore, DuraScript RNA behaves as standard RNA in an RNA/DNA hybrid and is subject to RNase H degradation.

Ribonuclease T1 digests RNA specifically at G residues. Because DuraScript RNA contains the canonical ribo-G nucleotides, it was predicted that DuraScript RNA would be susceptible to RNase T1 digestion. Agarose gel analysis of 200 ng DuraScript RNA that had been incubated 15 minutes at 37ºC with 2 Units of RNase T1 showed extensive degradation of the DuraScript RNA thus confirming that DuraScript RNA is readily digested by RNase T1.

Conclusion

EPICENTRE’s AmpliScribe™ High Yield Transcription Kits produce up to 150 µg of canonical RNA from a single reaction. The DuraScribe T7 Transcription Kit provides a useful new tool to efficiently prepare 2’-fluoro-modified RNAs that are resistant to RNase A. A 20 µl DuraScribe transcription reaction produces 40 – 60 µg of full-length 1.4 Kb DuraScript RNA using a linearized control template with a standard T7 polymerase promoter. The presences of the fluorine at the 2’-position of all C and U nucleotides in DuraScript RNA blocks digestion by RNase A present on human skin (“fingertip” nucleases). However, DuraScript RNA is still digested by ribonucleases that cleave at other than C or U nucleotides (e.g., RNase T1) or that cleave RNA by different reaction mechanisms (e.g., RNase H). The combination of DuraScript’s resistance to RNase A and its susceptibility to RNase H and RNase T1 and other specific ribonucleases is advantageous for many applications.

References


www.epicentre.com/durascribe.asp

DuraScribe™ T7 Transcription Kit

**Contents:**

- DuraScribe™ T7 Enzyme Mix, DuraScribe™ T7 10X Reaction Buffer, 2’-F-dCTP, 2’-F-dUTP, ATP, GTP, DTT, Control DNA Template, and Water.

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