

Duplex dsRNA (double-stranded RNA) detection by Acridine Orange (AO) Staining¹

A major undesired byproduct of T7 RNAP IVT is the sense-antisense duplex RNA, which results from the hybridization of the desired mRNA (sense RNA) and a fully complementary antisense RNA. Acridine orange is a dye that binds RNA regardless of its secondary structure but fluoresces differently depending on the RNA secondary structure. Acridine orange emits green fluorescence when bound to dsRNA (antisense-sense duplex RNA) and red fluorescence when bound to ssRNA (single-stranded RNA).

Materials and Instruments

- 5% TBE-PAGE (Not provided):

Reagent	Volume
30% acrylamide	3.3 mL
10X TBE Buffer	2.0 mL
ddH ₂ O	14.7 mL
30% APS	50 μ L
TEMED	10 μ L

- 10X TBE Buffer (Not provided): 108g Tris-HCl, 7.44g EDTA, 55g Boric Acid, pH 8.3, dissolved in 1L water.

Experimental Procedure

Setup:

1. Prior to the experiment, remove the reagent from the -20°C freezer and allow it to thaw on ice.
2. Ensure that the entire process is conducted in an RNase-free environment to maintain the integrity of the IVT system.
3. Follow the recipe below to set up the IVT reaction.
4. T7 RNAP and the DNA template are the last items to be added.

IVT Reaction:

Reagent	Volume (μ L)
10X Buffer I	2
NTP (5mM/each)	4
Murine RNase Inhibitor (40U/ μ L) (Catalog #GMP-RNI-ME101)	0.5
Pyrophosphatase, Inorganic (0.1U/ μ L) (Catalog #GMP-PYR-YE101)	0.5
T7 RNAP	2



Linearized 512B DNA template (512 nt transcript)	2
ddH ₂ O	9

1. Initiate the IVT reaction at 37°C for 17 hours.
2. Digest the DNA template using DNase I (4U/μL) (KACTUS, GMP-DNI-EE001-11) at 37°C for 30 minutes.
3. Purify the RNA from the reaction mixture using either LiCl precipitation or column purification techniques.

LiCl Precipitation:

1. For LiCl precipitation, add LiCl solution (Provided) to the IVT reaction at a 2.5M final concentration and place it in a -20°C freezer for 30 minutes.
2. Centrifuge the mixture at 16000g for 15 minutes and discard the supernatant carefully.
3. Use 50-100μL 70% ethanol to wash the precipitant and centrifuge at 16000g for 5 minutes.
4. Repeat the previous step 2 times to clean the precipitant.
5. Dry the precipitant in the fume for about 15–30 minutes until the ethanol is completely gone.
6. Dissolve the precipitant in 50–150μL RNase-free water.
8. The purified RNA can be directly used for later experiments or frozen by liquid nitrogen and stored at -80 °C.

Column Purification:

1. Follow the instructions provided by the supplier.
2. Measure the purified RNA on Nanodrop to confirm its concentration.
3. The purified RNA can be directly used for later experiments or frozen by liquid nitrogen and stored at -80°C.

TBE-PAGE:

1. Mix 200-1000ng of purified RNA with 5x TBE loading buffer (Provided).
2. Incubate the mixture at 65°C for 3 minutes and then at 4°C for 1 minute.
3. Load the mixed sample onto a 5% TBE-PAGE and run it at a constant voltage of 180V for 35 minutes in 1x TBE buffer.
4. After running, the gel should be stained with 25μg/mL acridine orange (AO) (Powder Provided) for 10-15 minutes and visualized using UV light.

Acridine Orange (AO) Staining:

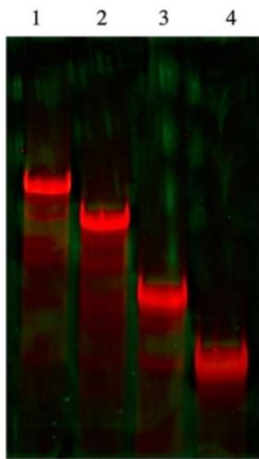
1. After running the gel, carefully remove it from the electrophoresis chamber and place it in a staining tray.
2. Add enough AO staining solution (25μg/mL) to completely cover the gel.
3. Incubate the gel in the staining solution for 5 minutes.
4. Discard the staining solution and add de-staining solution (water) to the tray to cover the gel.
5. Incubate the gel in the de-staining solution for 10 minutes.
6. Remove the gel from the de-staining solution and place it on the imaging platform of an iBright-1500 imaging system.
7. Visualize the gel by scanning it simultaneously at the GFP channel and Westerndot 655 channel.



Template sequence information (Ty promoter in yellow):

TAATACGACTCACTATAAGGGAGAAGCTCTTACACCTGATTCATTTCCATTGTTTTCTGCAGCAGCAATCCGGT
TTCTGTCTTCAATTGTCAACAGTTCCTCCTCCATGCACTTATCCAAGACGTCTCTAACTAGAAGCTTGTCCACCA
GAGTGGGCTGAAGGAGGTTTCAGCAGTTGGAGATATTCATCATGAGCGTTCTCAAACGATGGAGAGGGCAAGT
CCGTGAGCTCAGGGTTCATGTAGCGGGCGGCCAGAGGGGCTGCCGGTTCTCCGGAGGGCCTCCACGAATTCC
CGAGTCCAACCAAGGTGCCAGACTCCCTTCTCCAAGGTGCTCAGCAGCAGTTCAACTGCCTGCATGTTCCCG
GAGGTGGCGACTGTCCTCTGAATCTGCTCCTTACCTCTGCAGGCAGAAAGGTCAGGTAGTCCAGCACAGGC
TCCACCTGGATGTACATTTTACCCTGGCCCTGAAGCACGAGATGAGATAGCGGAAATTCTCGTCTGTGGAAT
ACCCATTGACATTCTCCC

Sample Results



512 nucleotide transcript run at 240v for 25 minutes, stained with AO staining solution and visualized on the Westerndot 655 channel.

References

1. Mu, X., Greenwald, E., Ahmad, S. & Hur, S. An origin of the immunogenicity of in vitro transcribed RNA. Nucleic Acids Res 46, 5239-5249 (2018).