# RNA Transcription, Purification, Characterization, And NMR Sample Preparation

**……'How To' series, 2004**

January 13th

# Steps Involved

**Synthesis of RNA sequence**
(*in vitro* T7 Polymerase-based RNA transcription)

**Purification of Synthesized RNA**
(denaturing PAGE, Electro-elution)

**Characterization**
(PAGE, UV Melting, NMR)

**NMR Sample Preparation**
(Buffer choice, Lyophilization vs Speed-Vac, Annealing)

# T7 RNA Polymerase-based *in vitro* Transcription

To produce large quantities of RNAs for NMR

**Limitations:**
Requires 5'-end to be purine-rich!
and may produce 3'end heterogeneity!

T7 bacteriophage RNA polymerase is single polypeptide chain enzyme, extremely efficient and allow RNA synthesis in large quantity

Can be obtained commercially, but becomes expensive for large scale RNA synthesis, thus has to be expressed and purified *in-house*

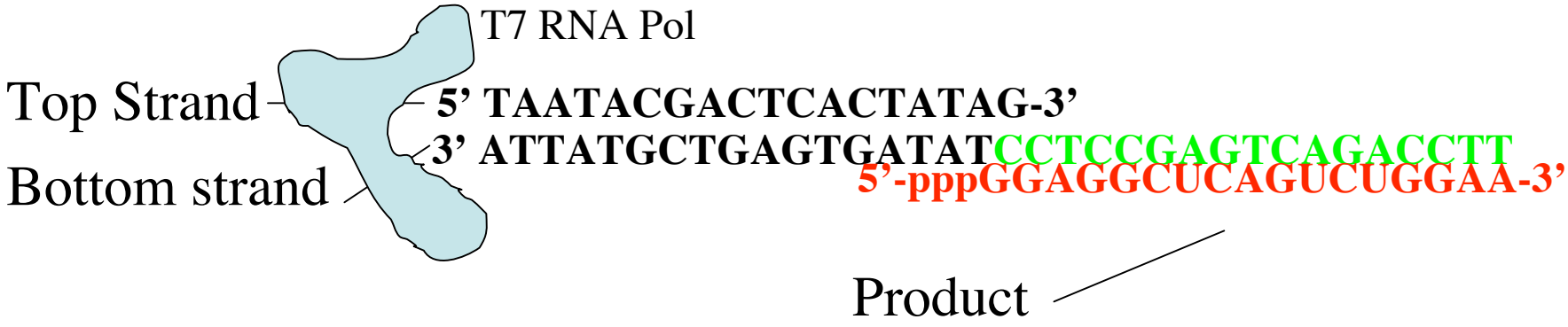
# T7 RNA Polymerase Information

Over-expression plasmid (pAR1219) developed by Studier et al.
It has gene encoding T7 RNA polymerase under control of lac UV5 Promoter. Plasmid also carries lac Iq gene encoding lac repressor. Production of T7 RNA polymerase is induced by adding IPTG.

T7 RNA polymerase is easily proteolysed by protease OmpT and E.Coli BL21 strain cells lacking this enzyme are recommended host for the plasmid.

# T7 RNA Polymerase-based *in vitro* Transcription of RNA Oligonucleotide of your interest

RNA sequence **5'-pppGGAGGCUCAGUCUGGAA-3'**



**Transcription Cocktail**

Template DNA duplex (0.01mM)
**rNTPs (4mM each)**
**$MgCl_2$ (5 times NTP conc., 20-30mM range)**
**5X Transcription buffer**
(contains Tris-HCl, pH 8.1, 20mM spermidine, Triton X-100, 100mM DTT, PEG)
**T7 Polymerase Enzyme (1mg/ml)**
**Sterile water**

**TOTAL REACTION VOLUME 10-20mL**

**Incubation for 2-4 Hrs.**

| Component | []i/mM | Vol.add/ml | []f/mM |
|---|---|---|---|
| **Top Strand** | 0.02 | 0.60 | 0.0006 |
| **Bottom Strand** | 0.02 | 0.60 | 0.0006 |
| **ATP** | 100.00 | 0.80 | 4.00 |
| **CTP** | 100.00 | 0.80 | 4.00 |
| **GTP** | 100.00 | 0.80 | 4.00 |
| **UTP** | 100.00 | 0.80 | 4.00 |
| **MgCl2** | 200.00 | 3.00 | 30.00 |
| **T7 RNApol** | 0.25 | 0.20 | 0.0025 |
| **5x Trans. Buffer** | 5x | 4.00 | 1x |
| **H2O** | | 8.40 | |
| | Final Volume | 20.00 | |
| | | | |
| **Edta** | 250.00 | 2.40 | 30.00 |
| **NaAcetate** | 3M | 2.00 | .10x rxn vol. |
| **EtOH chilled** | | 50.00 | 2.5x rxn vol. |

| | **TIME** | **8:15** | **9:15** | **10:15** | **11:15** | **0:15** | **13:15** | **14:15** | **Totals** |
|---|---|---|---|---|---|---|---|---|---|
| | dilution uL (in 1mL) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | |
| **Cell #1** | Vol. (uL) | 1100 | 900 | 940 | 990 | 980 | 950 | 960 | 6820 |
| | abs. | 0.03 | 0.06 | 0.034 | 0.049 | 0.018 | .01 | .003 | ! |
| | OD amount | 3.3 | 5.4 | 3.196 | 4.851 | 1.764 | 0.95 | 0.288 | 19.749 |
| **Cell #2** | Vol. (uL) | 650 | 670 | 700 | 780 | 970 | 940 | 1160 | 5870 |
| | abs. | 0.021 | 0.046 | 0.04 | 0.038 | 0.005 | .005 | .006 | ! |
| | OD amount | 1.365 | 3.082 | 2.8 | 2.964 | 0.485 | 0.47 | 0.696 | 11.862 |

## Effective Variables in Transcription Reaction

1. **DNA Template final concentration 200nM-1uM**
   *(best results, generally, around 500nM)*

2. **rNTP concentration (2-4mM)**
   *(can be lowered for specific NTPs)*

3. **Magnesium Concentration** *(most important!)*

4. **T7 RNA Polymerase concentration** (0.5-2%)

5. **Incubation time**

## Once Transcription incubation is over…

1. Quenching the reaction by EDTA 1.5 times total Mg conc.
2. PPTN. By Ethanol/3M 5.3pH NaoAc. O/N at -20C
3. Spinning at 12g for 15min
4. Wash with 70% EtOH (chilled)
5. Spin again.
6. Save the pellet. Let it dry at RT.
7. Add 6X BPP/XCC Dye in 7M Urea.
8. Heat in 7M Urea to dissolve

Load onto 20% PAGE Denaturing. O/N
600-1000V Constant (better if you pre-run for 1hr or so)

## Next Day….

1. Cut the band using UV shadowing
2. Electro-elute using 1000MW cut off membrane. (0.5x TBE)
3. Check OD at 260nm of the collected sample to quantitate RNA.
4. (USE Extinction coefficient calculated from web site)
5. BioPolymer Calculator **paris.chem.yale.edu/extinct.html**
5. PPT again using EtOH/NaoAC

**Dialysis**

6. Dissolve and dialyze against
    - Water/EDTA(10mM) 24Hrs (1L volume)
    - Water/1mM EDTA (24Hrs)
    - Water only (24Hrs.)

Collect all liquid from membrane and again take OD to measure RNA quantity- Store at –20C.

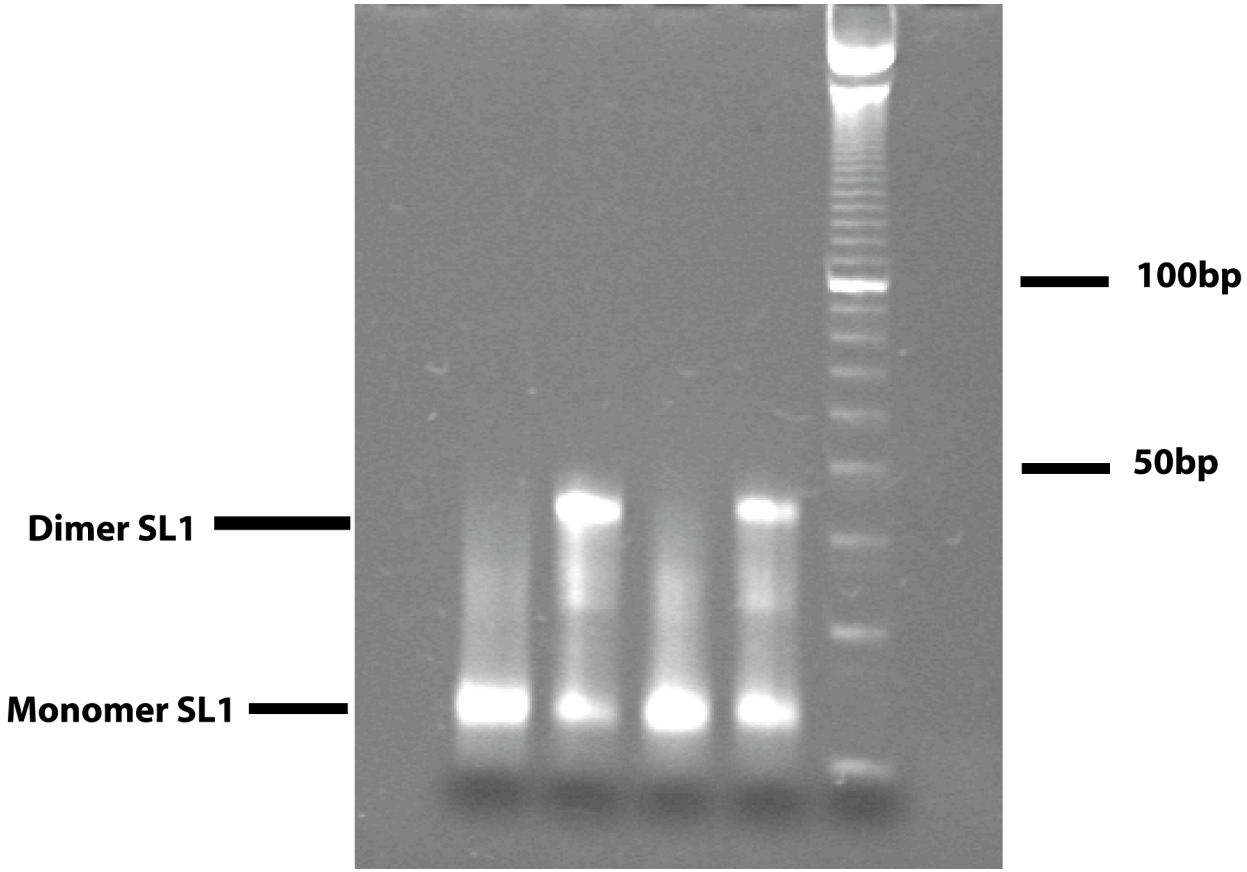
## Characterization:

Take 10-20uL containing ~0.05ODs and run denaturing 10-20% PAGE with known markers to check the integrity (single band) and size (MW) of your RNA.

**Trying to make Hairpin...it could also make duplex!**

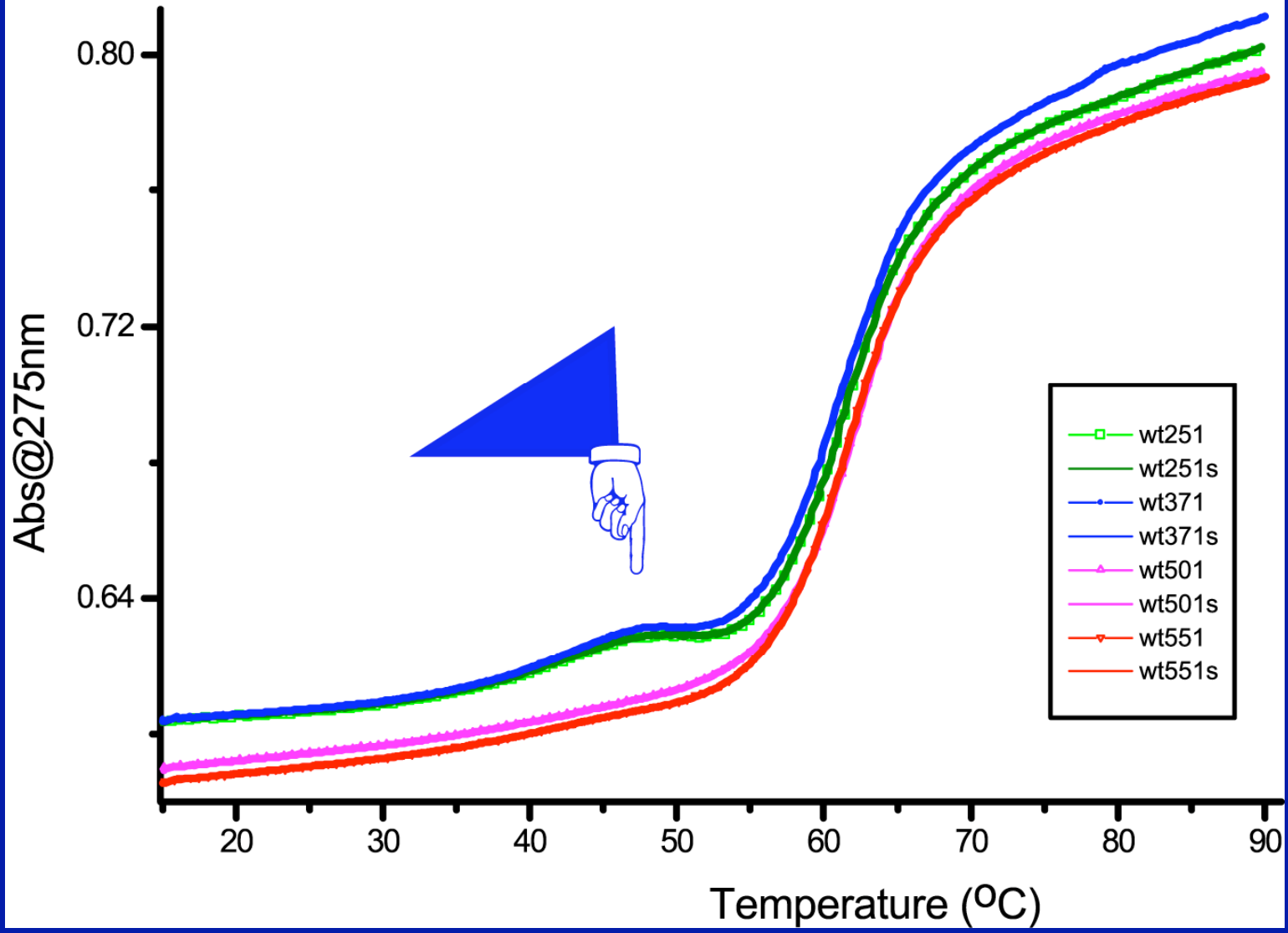
Check with Native PAGE in TBE or TBM. Run at 4C
Also, UV-melting can give information about the melting temperature, Buffer effects, etc.

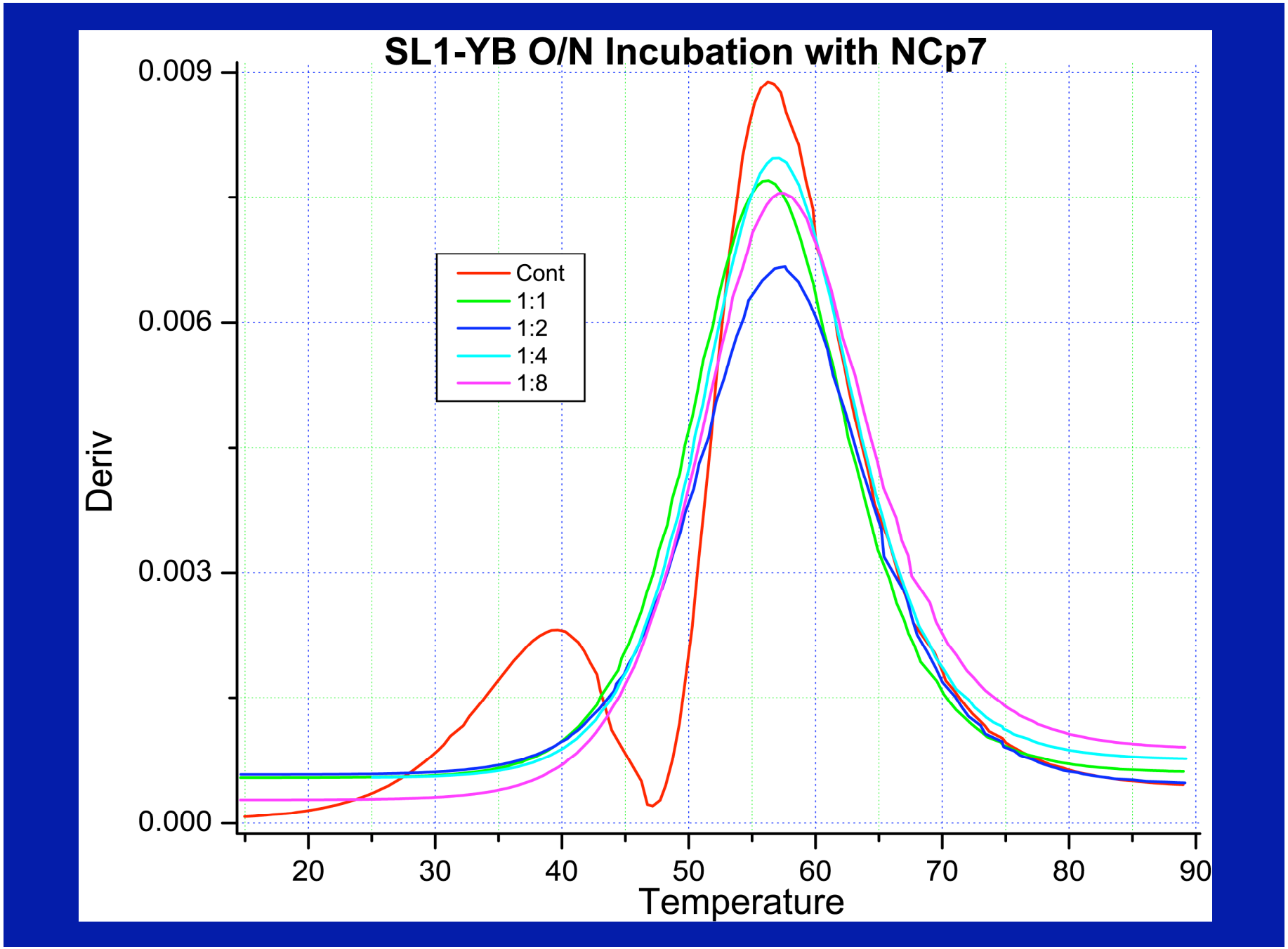
Finally, NMR (1D Imino and TOCSY ) to further check the RNA Integrity (conformation and sequence)

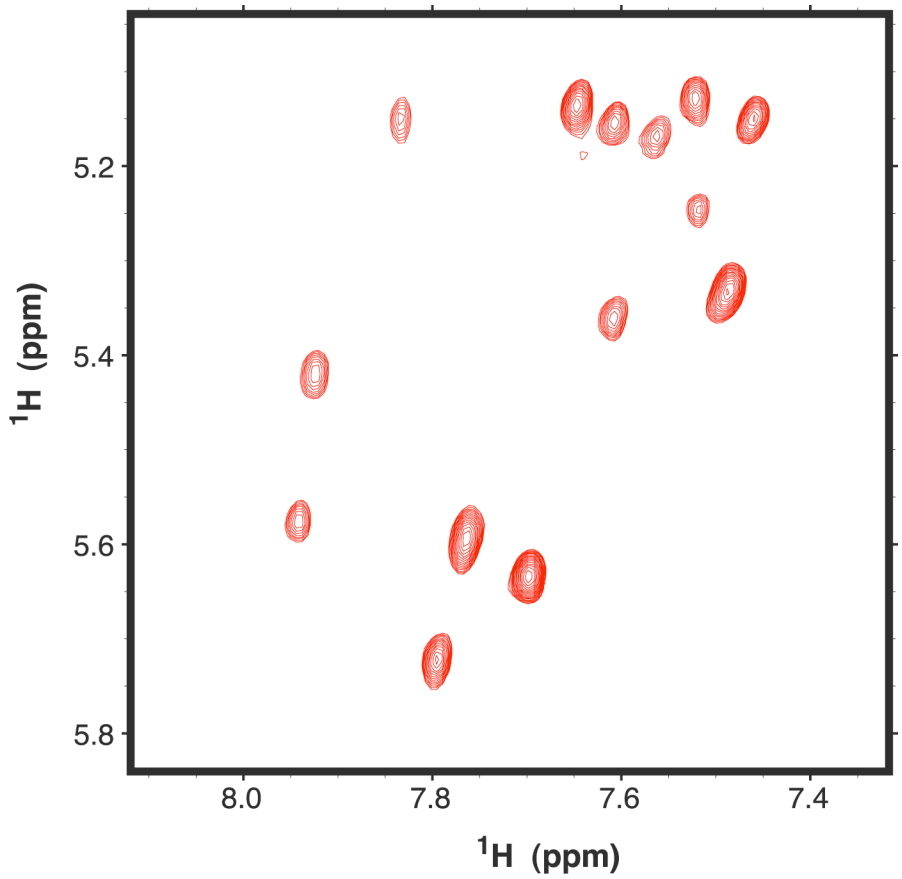



**Characterization of SL1 RNA Dimers and Monomers in 10% PAGE in TBE at RT**

# SL1-wt RNA melting in 0.1mM Mg-containing Buffer Annealed at RT, 37°C, 50°C and 55°C




SL1-YB O/N Incubation with NCp7
Deriv
0.009
0.006
0.003
0.000
20
30
40
50
60
70
80
90
Temperature
Cont
1:1
1:2
1:4
1:8




TOCSY NMR of RNA showing U and C H6-H5 signals

# Relevent References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Milligan and Uhlenbeck, Methods in Enzymology, Vol 180, 1988, Page 51-62

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RNA:Protein complexes for X-ray and NMR.Page:37-72.