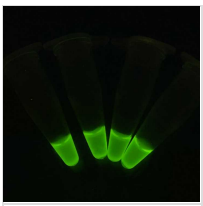

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rNTPs Stock Preparation / IVT Standard Reaction

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Laboratorio de Tecnologias Libres

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ABSTRACT

This protocol describes the preparation of rNTPs stock solutions for their use on IVT and other molecular reactions.

The rNTPs preparation is adapted from a protocol by Berglund Lab (Goers, 2006), it contains instructions to prepare [M]**100 millimolar (mM)** stock solutions of each ribonucleotide with a final pH of 7-8 each. It is important to consider that each rNTP has a slightly different molar mass, and this value can also vary according to the degree of hydration of the reagent and provider. The molar mass value you should use to determine the resuspension volume must be the one indicated on the flask that contains each rNTP.

In our case, we have:
ATP (583,36 g/mol)
GTP (567,10 g/mol)
UTP (550,09 g/mol)
CTP (527,12 g/mol)

The second part of this protocol describes the preparation of an **in vitro transcription (IVT)** reaction of fluorescent RNA aptamers, like Spinach or Broccoli. In our lab, we use it to test, validate and standardize our buffers, enzymes, ribonucleotides and other reagents. Spinach and Broccoli aptamers bind to DFHBI-1T and this interaction “mimics a GFP fluorophore”, as the reaction product emits a green fluorescence that can be observed in a UV transilluminator.

The homemade IVT-aptamer reaction was based on procedures used by the *Adamala-Engelhart Laboratory* (Heili et al., 2018 & Aufdembrink et al., 2020), with our own modifications.

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MATERIALS TEXT

For rNTPs stock

- NTPs
- NaOH [M]**1 Molarity (M)** Solution
- 1:1 HCl Solution
- Nuclease-Free Water
- pH Strips

For IVT Standard Reaction

- 5X In Vitro Transcription Buffer
- rNTPs, 100 mM each
- T7 Polymerase (2 mg/mL)
- DMSO
- Nuclease-Free Water
- DFHBI-1T 40 mM

rNTPs Preparation

1 Determine the mass of rNTPs that is going to be used. In this case, **100 mg** of each rNTP are recommended.

2 Calculate the necessary volume to obtain a [M] **100 millimolar (mM)** solution of each rNTP.

We did this by estimating how many mmoles are in 100mg and used that value to estimate the volume for each 100mM solution. In our case, we calculated:
- ATP (583,36 g/mol) --> 1714 uL
- GTP (567,10 g/mol) --> 1763 uL
- UTP (550,09 g/mol) --> 1818 uL
- CTP (527,12 g/mol) --> 1898 uL

If you are using 100 mg of each rNTP, this result should be around **1600-2100 µL**

3 Weight **100 mg** of each rNTP separately in an 1,5 mL Eppendorf tube. Deposit them on ice.

We recommend doing this step slowly, as we experienced a lot of static between the reagents and the tube when we were doing this.

4 Add **1300 µL** of nuclease-free water to each tube. Vortex and deposit them on ice.

Since you need to adjust the pH of the solution, we recommend adding less water at first than the amount you will actually need so you can have a broad range of volume available for the next step.

5 Measure the pH of the solution using a pH strip.

6 ⚠

Adjust the pH of the solution using NaOH [M] **1 Molarity (M)** and HCl 1:1 to range between 7-8

rNTPs solutions are originally very acidic (pH 2-5), so you will probably need to add only base. The HCl solution is only required if you overshoot the pH by adding too much NaOH. We recommend adding the first **200 µL** of NaOH 1M and from there continue in volumes between **5-20 µL** until you get to the pH 7-8 range. **Measure the pH each time with a pH strip and have a record of how much volume you are adding to each solution.** Be

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careful to not go over the final volume.

7 **If needed**, add nuclease-free water up to the final volume of each solution. Vortex each tube.

8 Aliquot each tube separately, label them and store them at **-20 °C** until use.

IVT Standard Reaction 3h

9 **5X IVT buffer**

Our 1X IVT buffer is composed of Tris Acetate **pH 8.1** **30 millimolar (mM)** , Magnesium glutamate **24 millimolar (mM)** , Potassium glutamate **90 millimolar (mM)** , Spermidine **2 millimolar (mM)** and DTT **1 millimolar (mM)**

A	B	C	D
Stock solutions	**Volume for a 50mL solution**	**5X Concentration**	**1X concentration**
Tris-Ac pH 8.1 500mM	3 ml	150 mM	30 mM
Mg-Glutamate 500mM	2.4 ml	120 mM	24 mM
K-Glutamate 100mM	4.5 ml	450 mM	90 mM
Spermidine 1M	100 uL	10 mM	2 mM
DTT 1M	50 uL	5 mM	1 mM

Considerations should be made that these concentrations can be further optimized.

We aliquote this 5x transcription buffer and store it at **-20 °C**

10

DFHBI-1T 40 mM

In case Broccoli or Spinach aptamer are used to benchmark the quality of the IVT reaction, it is necessary to prepare the following:

For a **40 millimolar (mM)** solution, mix **5 mg** of DFHBI-1T **Sigma Aldrich Catalog #SML2697-25MG** with **390 µL** of DMSO.

Vortex, aliquot in 1,5 mL Eppendorf tubes and save at **-20 °C** covered with aluminium foil.

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11 Test Reaction Protocol

The amounts shown here are for a single **20 µL** reaction.

11.1 Remember to thaw, vortex and quickly spin tubes down before opening for dispensing. It is preferable to keep all the tubes on ice when not in use.

When stored, all the reagents should be at **-20 °C** .

A	B	C
	1x (uL)	**Final concentration**
IVT Buffer 5x	4	1X
ATP 100 mM	1	10 mM
CTP 100mM	1	10 mM
UTP 100mM	1	10 mM
GTP 100mM	1	10 mM
DFHBI-1T 40 mM	1	2 mM (if needed)
T7 Pol Mix (2 mg/mL)	2	0,1 mg/mL
H20	7	
DNA	2	Spinach or Broccoli aptamer
	20	

Although we have not formally tested this, we followed the general recommendations of using linear dsDNA rather than plasmids as a substrate for IVT. We normally use linear dsDNA from a cleaned up-PCR reaction at a concentration ranging from 70-200 ng/uL stock.

3h

11.2 Incubate the reaction at **37 °C** **for** **03:00:00** for downstream applications, it can also be incubated overnight, however little increase in yield is seen in our hands.

Although not necessary. We observed an increase in the reaction yields by supplementing the reaction with **1 µL** of

Pyrophosphatase, Inorganic (E.coli) - 10 un

Biolabs Catalog #M0361S

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