Step 3: Performing cell-free RNAPT7 reactions

Anibal Arce Medina

Abstract

This protocol explains how to prepare, load and run cell-free RNAPT7 transcription/translation coupled reactions. To be able to follow this protocol it is necessary to have prepared all the solutions needed including **amino acids and Energy solutions** and **cell extracts (** explained in detail in Step 1 and Step 2 of this collection).

Citation: Anibal Arce Medina Step 3: Performing cell-free RNAPT7 reactions. **protocols.io** dx.doi.org/10.17504/protocols.io.ieecbbe
Published: 14 Jun 2017

Guidelines

Protocol

Thaw all the necesary solutions on ice

Step 1.

Thaw all of the following solutions **on ice**: batch (or batches) of cell extract, 10X maltodextrin-based energy solution, stock amino acid solution (at ~17nM each amino acid) solution, 0.03 g/ml Sodium Hexametaphosphate (HMP) solution, 100mM Magnesium acetate solution, 3,5 M Potassium acetate solution, and plasmid DNA. This may take some time (>1 h for the cell extract).

ANNOTATIONS

Fernan Federici 13 Jun 2017

In this protocol, all reaction components (cell extract, amino acid solution, energy solution, Mg, and K salts, etc) are added separately to a MasterMix. This allows fine tuning and tweaking of specific components in the Tx-Tl reaction. This has advantages such as finding optimal conditions for each cell extracts, and so on.

Otherwise, it can be prepared a **stock reaction buffer** solution that contains all the reagents needed for the reaction **without cell extract and without DNA**. This reaction buffer can be frozen and stored at -80, thawed and mixed with cell extract and DNA right before starting reactions.

Important Considerations

Step 2.

- After thawing, the amino acid solution may have some precipitation. Vortex it well before use.
- We never vortex the cell extract.
- For optimal conditions, DNA should be prepared at least at 70mM and should be extra-cleaned using PCR clean-up or similar. For this propose, we normally use Promega's kit.

- HMP 0.03 g/mL solution should be warmed near boiling temperatures for 5 minutes before aliquoting. Then, aliquots can be kept at -20°C.

- The typical volume of a cell-free reaction is 5uL. In our hands, the best reactions had been obtained with the following compositions per 5uL:

Each 5 ul of cell-free reaction contains:

Energy Solutiion	0.5 ul
(Cell extract from BL21 DE3 STAR strain)	2.5 ul
Aminoacid solution	1 ul
40% PEG solution	0.25 ul
HMP solution	0.1 ul
3.5 M Potassium Acetate solution	0.08 ul
100mM Magnesium Acetate solution	0.074 ul
DNA (70 mM)	0.496 ul

REAGENTS

Sodium hexametaphosphate 305553-25G by Sigma Aldrich

PEG-8000 V30111 by Promega

Wizard SV Gel and PCR Clean-Up System A9281 by Promega

NOTES

Anibal Arce Medina 12 Jun 2017

Potassium-Glutamate and Magnesium-Glutamate can be used instead Potassium and Magnesium acetate obtaining similar results

Mastermix preparation

Step 3.

Prepare Mastermix accordingly to the composition of the cell-free reaction. Take in consideration that the Mastermix serves for the preparation of 5X reactions, which are used to load three wells (replicates) with 5 ul reaction. The Mastermix contains all the solutions shown in step 2, except DNA. Put all the reagents into a 1.5ml Eppendorf tube **on ice**. The Mastermix should be prepared in this order: Aminoacid solution, 0.03 g/ml HMP solution, 3.5 M Potassium acetate solution, Energy solution, 40 %PEG, and at least, the cell extract. Mix well the master mix after addition of each ingredient.

96-well plate and caps preparation

Step 4.

Place the 96-well Plate (V-shaped bottom clear polystyrene) on ice, and cut the storage mat into strips of three caps. Keep the cap strips handy, in order to seal the wells right after loading the 5ul reactions in order to prevent evaporation.

REAGENTS

- Storage Mat III Costar / Corning 3080 by Contributed by users
- 96 Well Clear V-Bottom Polypropylene Not Treated Microplate 3357 by Contributed by users

Preparation of 5X reactions per 3 technical replicates

Step 5.

Prepare a 5x mastermix, as shown in step 2, per 3 technical replicates.

For each, add 2.48 ul of DNA solution (or 2.48 ul of water for the negative controls) on an 1.5ml Eppendorf tube **on ice.**

Step 6.

Mix the 5X reaction tubes (DNA + mastermix) several times by pipetting up-and-down. Be sure not to generate bubbles. **Keep on ice before loading the 96-well plate.**

NOTES

Anibal Arce Medina 12 Jun 2017

It is important to mix well the cell-free reactions in order to avoid variability among the technical replicates.

Load the 96-well plate

Step 7.

Place 5ul of the 5X reaction tube (DNA + mastermix) into the bottom of the well without adding bubbles. The 96-well plate should be kept on ice all the time during the loading step to avoid premature reaction initiation. The 5X reaction volumes are used for loading three wells (5ul each), the rest of the volume is not loaded. Immediately after loading three samples, seal the wells with the cap-strips cut earlier, to avoid evaporation.

Incubation and signal measurement

Step 8.

Place the 96-well plate into the plate reader and kept at 24 °C without agitation for 24 hours. DeGFP fluorescence intensity is read using appropriate filters. Normally, deGFP signal can be detected during the first 3 hours of the experiment.

Representative results

Step 9.

The following are representative results obtained following this protocols.

Cell-free reactions were run in three independent cell extracts, ranging from 7 pM to 7 nM of DNA (proT7-deGFP-TT7).

A) Fluorescence intensity was measured in a plate reader. Max fluorescence increases with the increase of DNA concentration. Batch-to -batch variability is evidenced.

B) End-point reactions taken using open-source Rasperryscope.

C) Cell-free RNAPT7 end-point reactions of other fluorescent reporters. The picture was taken using the open-source Rasperryscope.