

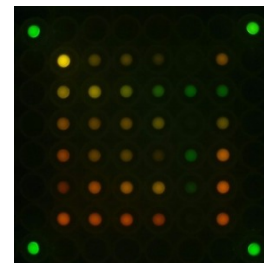
Jun 14, 2017

Step 3: Performing cell-free RNAPT7 reactions

 In 2 collections

DOI

<https://dx.doi.org/10.17504/protocols.io.ieecbbe>



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DOI: <https://dx.doi.org/10.17504/protocols.io.ieecbbe>

Protocol Citation: Anibal Arce Medina 2017. Step 3: Performing cell-free RNAPT7 reactions. **protocols.io**
<https://dx.doi.org/10.17504/protocols.io.ieecbbe>

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Protocol status: Working

Created: June 12, 2017

Last Modified: March 23, 2018

Protocol Integer ID: 6310

Keywords: free rnapt7 reactions this protocol, free rnapt7 reaction, free rnapt7 transcription, free rnapt7, transcription, including amino acid, amino acid, performing cell, reaction, coupled reaction, cell extract, detail in step

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).

Materials

STEP MATERIALS

☒ Sodium hexametaphosphate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #305553-25G**

☒ PEG-8000 **Promega Catalog #V30111**

☒ Wizard SV Gel and PCR Clean-Up System **Promega Catalog #A9281**

☒ Storage Mat III **Catalog #Costar / Corning 3080**

☒ 96 Well Clear V-Bottom Polypropylene Not Treated Microplate **Catalog #3357**

☒ Sodium hexametaphosphate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #305553-25G**

☒ PEG-8000 **Promega Catalog #V30111**

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Protocol materials

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Thaw all the necessary solutions on ice

- 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).

Important Considerations

- 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

Note

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



Sodium hexametaphosphate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #305553-25G**



PEG-8000 **Promega Catalog #V30111**



Wizard SV Gel and PCR Clean-Up System **Promega Catalog #A9281**

Mastermix preparation

- 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

- 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.



Storage Mat III **Catalog #Costar / Corning 3080**



96 Well Clear V-Bottom Polypropylene Not Treated Microplate **Catalog #3357**

Preparation of 5X reactions per 3 technical replicates

- 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**.



- 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.**

Note

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

Load the 96-well plate

- 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

- 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

- 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.

