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Methods in Molecular Biology

Oocyte Expression with Injection of Purified T7 RNA Polymerase

Xavier Altafaj[1], Nathalie Joux[1], Michel Ronjat[1] & Michel De Waard[1$]

[1]Inserm U607, Canaux Calciques, Fonctions et Pathologies, CEA, Département Réponse et Dynamique Cellulaire, 17 Rue des Martyrs, 38054 Grenoble Cedex 09, France.

$ Send correspondence to Dr. Michel De Waard : **Tel. : (33) 4 38 78 68 13**

Fax : (33) 4 38 78 50 41

E-mail : mdewaard@cea.fr

Abstract

The *Xenopus* oocyte is a widely used system for protein expression. So far, investigators had the choice between two different techniques, i) injection into the cytoplasm of in vitro transcribed cRNA, or ii) injection into the nucleus of cDNA. Here, we report on a third expression technique that is based on the combined injection of cDNA and purified T7 RNA polymerase directly into the cytoplasm of oocytes.

Key Words

***Xenopus* oocyte, expression technique, T7 RNA polymerase, exogenous protein, injection.**

1. Introduction

The genomic era has generated an ever increasing need for protein structure and functional analyses. The *Xenopus laevis* oocyte cell machinery is remarkably well adapted for exogenous protein expression. Through specific technical developments, the ever-increasing use of this expression system has allowed the implementation of many new applications. *Xenopus* oocytes have been used to study protein post-translational modifications, channel ion fluxes with various biophysical approaches ***(1)***, changes in membrane capacitance ***(2)***, reconstituted transmitter release ***(3)***, channel gating currents, multi-protein subunit assembly ***(4)***, receptor pharmacology ***(5)***, sub-cellular metabolic activity ***(6)***, etc... Their relatively large sizes (~1 mm) allow investigators to perform biochemical analyses on single cells. For many applications, it takes one cell or less to detect expressed proteins by Western blotting or a metabolic labelling approach. The ease with which an oocyte can be injected allows for experiments that are difficult to achieve otherwise: injection of peptides ***(7)*** or recombinant proteins ***(8)***, and on-line recordings of the functional effects of non-permeable metabolic analogues. Original reports show that exogenous membranes, containing exogenous channels and receptors, can be inserted into the plasma membrane of oocytes and characterized. Examples include the microtransplantation of secretion vesicles from chromaffin cells and of membrane preparations from Alzheimer's brain ***(9)*** or Torpedo electroplaque membranes ***(10)***. Many of these applications have required methodological developments that are often specific for the oocyte system. For biochemical or biophysical studies, receptors and channels expressed in the plasma membrane can be studied in isolation following plasma membrane isolation ***(11-14)***. Similarly, nuclear functions can be studied after isolation of individual oocyte nuclei ***(15,16)*** or, conversely, the function of oocytes can be studied after their nuclei have been removed ***(17)***. New electrophysiological recording techniques have been developed specifically for the oocyte system, such as the cut-open oocyte voltage-clamp ***(18)*** or the transoocyte voltage clamp ***(19)*** techniques. Similarly, improved oocyte preparations have been proposed for patch-clamp experiments ***(20)***. Along with these techniques, the great size of these cells has required the

development of new extracellular ***(21)*** or intracellular ***(22)*** perfusion systems. With regard to structure-function analyses, unique *in vivo* incorporation of unnatural amino acids is possible into proteins expressed in *Xenopus* oocytes ***(23)***. Oocytes have also been used for expression cloning experiments ***(24)***. These are a few examples of the technological advances that have been made specifically for the oocyte system.

The use of the *Xenopus* oocyte system as an expression reporter was initially boosted with the discovery that exogenous haemoglobin messenger RNA is efficiently translated in frog oocytes ***(25)***. Two techniques have been widely used to induce the expression of proteins of exogenous origin: i) cDNA injection in the nucleus ***(26)*** or ii) injection of cRNA into the cytoplasm ***(27)***. Though nuclear injection is greatly facilitated by the relatively important size of the nucleus (up to 1/3 third of the total volume of the oocyte), it is still unreliable for two reasons. First, depending on the dexterity of each individual for injecting into the nucleus, only a fraction of the oocytes will express the gene of interest (one third, rarely more) ***(26)***. Second, the efficacy of transcription will heavily depend on the type of eukaryotic promoter being used. With different promoters, if one seeks to co-express at least two different genes, there is a non-negligible risk that the expression level of each desired gene turns out to be very different. Injection of cRNA in the cytoplasm of oocytes appears more reliable as a method for expression since 100% of the cells express the gene of interest and the expression level is easily controlled by defining the cRNA concentration and the injected volume. The main drawback of the technique however is the time-consuming experimentation linked to cRNA production, storage and manipulation. The aim of the present chapter is thus to present an alternative method that circumvents some of the drawbacks of both cytoplasmic cRNA and nuclear cDNA injections. This new technique takes advantage of the reliability of cytoplasmic injection without the requirement for an *in vitro* transcription step. The method relies on an autonomous cDNA transcription system, driven by the prokaryotic T7 promoter of the bacteriophage T7 DNA dependent RNA polymerase (T7-RNAP). Thanks to the optimization in critical steps of T7-RNAP purification, this new procedure turns out to be less time-consuming

and is more cost-effective than the two classical expression techniques. Proper functional protein expression have been assessed by different methodologies such as metabolic labelling and electrophysiological recordings on different proteins forming K^+, Na^+ and Ca^{2+} channel subunits of different species ***(28)*** (see notes 1 to 5). We present here the protocols required for the use of this T7 RNAP expression technique.

2. Materials

2.1. For T7 RNA polymerase purification

1. *E. coli* strain of BL21 (DE3) (InVitrogen).
2. pMR-T7-RNAP plasmid ***(29)***.
3. Luria Bertani (LB) medium: 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl.
4. Ampicillin.
5. Isopropylthio-β-D-galactoside (IPTG) (Sigma, St Louis, MI, USA).
6. Tris-Buffer Saline (TBS): 150 mM NaCl, 25 mM Tris-HCl, pH 7.4.
7. Benzaminide.
8. Phenylmethylsulfonyl fluoride (PMSF).
9. Triton X-100 (Sigma, St Louis, MI, USA).
10. Resuspension buffer: 150 mM NaCl, 25 mM Tris-HCl, pH 7.4 with 0.1% Triton X-100, 1 mM benzamidine and 0.23 mM PMSF.
11. Sepharose beads coupled to iminodiacetic acid (Sigma, St Louis, MI, USA).
12. Disposable Poly-Prep® Chromatography columns (Bio-Rad, Hercules, CA, USA).
13. Spectra/Por® Membrane (Biovalley, Conches, France).
14. Activation buffer: 50 mM $NiSO_4$.
15. Loading buffer: 40 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl, pH 7.9.
16. Elution buffer: 400 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl, pH 7.9.
17. Coomassie blue staining solution (Bio-Rad, Hercules, CA, USA).

18. Gel destaining solution: 50% ethanol, 10% acetic acid.

19. Bradford assay using commercial reagents (Bio-Rad, Hercules, CA, USA).

2.2. For in vitro T7 RNAP Western blot detection and transcriptional activity

1. SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) equipment.
2. Nitrocellulose membrane (Hybond® ECL, Amersham Biosciences, Bucks, UK).
3. Transfer buffer: 48 mM Tris, 39 mM glycine, 20% methanol and 0.0375% SDS.
4. Luminescent reagent (Western Lightning™ Chemiluminescence Reagent Plus) (Perkin-Elmer, Boston, MA, USA).
5. Monoclonal anti-T7 antibody (Novagen, Madison, WI, USA).
6. Monoclonal anti-mouse IgG antibody conjugated to peroxydase (ZYMED, San Francisco, CA, USA).
7. Blocking solution: 150 mM NaCl, 50 mM NaH_2PO_4, 5% milk, 0.05% Tween 20; pH 7.4.
8. Hyperfilm™ ECL™ (Amersham Biosciences, Bucks, UK).
9. Transcription buffer is from the mMessage mMachine™ kit (Ambion, Cambridgeshire, UK).
10. Agarose electrophoresis grade (GIBCO BRL, Paisley, Scotland).

2.3. For Xenopus oocyte experiments (preparation, maintenance, injection and metabolic labelling)

1. Mature *Xenopus laevis* female frogs (CRBM, Montpellier, France).
2. Anaesthetics: 3-aminobenzoic acid ethyl ester (Sigma, St Louis, MI, USA).
3. Collagenase type IA (Sigma, St Louis, MI, USA). Store at -20°C.
4. Ca^{2+}-free Barth's medium: 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 15 mM HEPES, pH 7.4 with NaOH. Filter sterilize and store at 4°C.

5. Barth's medium: 88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 15 mM HEPES, pH 7.4 with NaOH. Filter sterilize and store at 4°C.
6. Defined Nutrient Oocyte Medium (DNOM). Filter sterilize and store at 4°C. Stable for 2 weeks.
7. Falcon 24-well plates (Becton-Dickinson, Le pont de Claix, France).
8. Nanoliter injector (WPI, Sarasota, Florida, USA).
9. [^{35}S]-L-methionine at 1175 Ci/mmol (Perkin-Elmer, Boston, MA, USA).
10. Glycerol (Sigma, St Louis, MI, USA).
11. dNTPs and Cap analogue (Ambion, Cambridgeshire, UK).
12. Homogenization buffer: 1% Triton X-100, 50 mM Tris-HCl, pH 6.8.
13. Protease inhibitor cocktail Complete^{TM} (Boehringer-Mannheim, Mannheim, Germany).

3. Methods

The methods described here allows i) a single-step purification of large and pure amounts of recombinant T7 RNAP, ii) the co-injection into the cytoplasm of *Xenopus* oocytes of T7-driven plasmids and purified T7-RNAP, and iii) the characterization by biophysical and biochemical means of the expressed proteins.

3.1. Expression plasmids

3.1.1. Expression vector

The cDNA encoding for the full-length wild-type T7 RNAP has been previously cloned in the pMR-78 vector ***(29)*** and is presented in **Fig. 1**. The expression of the insert is under the control of the Ptac promoter, which is inducible by IPTG. This vector has a specific *bla* gene that confers ampicillin resistance for colony selection and growth. It is designed to express an N-terminal his-tagged T7 RNAP for purification with a Ni^{2+} / iminodiacetic acid affinity column.

3.1.2. cDNA constructs

The cDNAs used should contain a T7 promoter at the 5' end of the gene of interest. For protein production in oocytes, the cDNA should encode a Kozak's sequence for initiation of translation ***(30)*** and a stop codon. cRNA stability, and thus protein yield, can be greatly improved by the presence of a poly A tail sequence at the 3' end of the gene of interest ***(31)***.

3.2. Protein induction

3.2.1. E. coli. transformation and plasmid selection

E. coli strain BL21(DE3)pLysS competent cells were transformed with the pMR-T7-RNAP plasmid and plated on selective Luria Bertani (LB)-agarose dishes containing 0.1 mg/ml ampicillin. A single colony was grown overnight at 37°C under agitation in 5 ml of LB supplemented with 0.1 mg/ml ampicillin. Half of this culture was used to extract the plasmid DNA and check its identity by the use of restriction enzymes. The other half of the culture was used to inoculate a larger bacterial culture (40 ml of LB with 0.1 mg/ml ampicillin) which was grown overnight at 37°C under agitation until reaching saturation.

3.2.2. Induction and bacteria harvesting

1. Dilute 40 ml of the overnight culture into 400 ml of standard LB medium supplemented with 0.1 mg/ml ampicillin.
2. Once the OD_{600} reached 0.6 (about 2-3 hours later), induce protein synthesis by adding a final concentration of 1 mM isopropylthio-β-D-galactoside (IPTG) to the culture medium for 4 h.
3. Harvest the bacteria by centrifuging at 4,000 g for 10 min at 4°C and resuspend the pellet in 10 ml of resuspension buffer. Use conical 15 ml polypropylene Falcon tubes.

3.3. Protein purification

3.3.1. Cell disruption

1. Sonicate four times the resuspended pellet in bursts of 30 sec at maximum power using a microtip probe. The Falcon tube should be immersed on ice. Special care should be taken to avoid heating the preparation.
2. Centrifuge the lysate at 14,000 g for 15 min. Keep the supernatant, containing the soluble T7 RNAP fraction of interest, on ice for subsequent treatment.

3.3.2. Protein purification

1. Add 5 ml of suspended iminodiacetic acid-sepharose beads to a disposable 10 ml polypropylene column with a final bed volume of 2.5 ml.
2. Wash the column with 3 bed volumes of H_2O.
3. Charge the column with 5 bed volumes of activation buffer (50 mM $NiSO_4$).
4. Equilibrate the column with 3 bed volumes of loading buffer.
5. Load the supernatant bacterial preparation of T7 RNAP onto the column.
6. Wash the column with 20 ml of loading buffer.
7. Elute the immobilized T7 RNAP by adding 15 ml of elution buffer (imidazole, 400 mM; NaCl, 500 mM; Tris-HCl, 50 mM; pH 7.9) and collect fractions of 1 ml.

3.3.3. Detection of T7 RNAP (Coomassie blue staining and Western blotting)

The quality of the purification procedure can be assessed by gel electrophoresis and immunoblotting as shown in ***Fig. 2***. The purity and amount of T7 RNAP is analyzed as described hereunder:

1. Load 3 µl of each eluted fraction on a 5-15% gradient SDS-PAGE.
2. Stain the gel with Coomassie blue staining solution (Bio-Rad).

3. Destain the gel with destaining solution and check for a protein band with a molecular weight of 98 kDa, corresponding to purified T7 RNAP. Determine the most concentrated elution fractions and analyze them by Western blotting. In our experience, the two most concentrated fractions correspond to elution fractions 3 and 4.
4. For immunodetection, first transfer the proteins from the gel to a nitrocellulose membrane (Hybond® ECL). Perform the transfer during 1h30 at 400 mA in transfer buffer.
5. Block the membrane overnight at 4°C in blocking solution.
6. Incubate the membrane for 30 min at 37°C with an anti-T7 monoclonal antibody (from Novagen) previously diluted 5000-fold in the same blocking solution.
7. After 4 washing steps of 10 min in TBS, incubate the membrane for 30 min with the secondary antibody conjugate (anti-mouse IgG coupled to peroxydase from ZYMED diluted 1/5000).
8. Wash again the membrane 4 times with TBS.
9. Reveal the peroxydase activity by a 1 min incubation of the membrane with the chemiluminescent reagent (Perkin-Elmer) and its exposure to a Hyperfilm™ ECL™ film (Amersham Biosciences). ***Fig. 2*** shows a representative example of immunodetection of purified T7 RNAP.

3.3.4. Dialysis of purest and most concentrated fractions

1. According to a visual examination of SDS-PAGE analysis, pool together the purest and most concentrated elution fractions for dialysis and buffer exchange (up to 4 to 6 ml in volume).
2. Load the pooled eluted T7 RNAP fractions into a dialysis tube (Spectra/Por® Membrane from Biovalley) with a molecular weight cut-off of 10,000 Da.
3. Dialyse the fractions overnight at 4°C against 1 l of TBS. Repeat the operation once for two hours by replacing the external TBS solution.

4. Recover the dialysed sample which is ready for protein dosage.

3.3.5. Yield analysis

Determine the protein concentration of the dialyzed T7 RNAP sample using a Bradford assay following manufacturer's instructions (Bio-Rad commercial reagents). Expect total T7 RNAP quantities to be in the range of 20-40 mg.

3.3.6. Aliquoting and storage

Purified T7 RNAP is stable for two weeks at 4°C and can be used as such without further aliquoting. For long-term storage, it is recommended to adjust the concentration of the sample to 10 mg/ml with TBS and by adding 40% glycerol. Small volumes aliquots of the sample (10 to 100 µl) should be produced and kept at -20°C.

3.4. Activity of purified T7 RNAP

The activity of the purified T7 RNAP can be evaluated by an *in vitro* transcription assay.

1. Add to 40 µl of transcription buffer (mMessage mMachine™ kit from Ambion), 100 ng of purified T7 RNAP and 2 µg of a linearized cDNA clone carrying a T7 promoter.
2. Carry out the reaction for 2 hours at 37°C.
3. Check cRNA production after gel electrophoresis onto an agarose gel containing formaldehyde ***(32)***.

3.5. Oocyte preparation, maintenance and injection

3.5.1. Oocyte preparation

Stage V and VI oocytes are prepared according to the procedure previously described by De Waard and Campbell ***(33)*** which is further described hereunder:

1. Maintain mature *Xenopus laevis* female frogs under a cycle of 12 h light / 12 h dark in water tanks at 16°C.
2. Choose a healthy frog, place it into a small and secure container, add water for partial body immersion, and add 0.03% (w/v) of 3-aminobenzoic acid ethyl ester to the water to anesthetize the animal. Partial body immersion is important to avoid drowning of the animal. Leave the frog under anaesthetics until it does not react anymore to a leg pinch test.
3. Surgically remove some of the ovaries by making a small incision in the abdomen. Place the ovaries in Ca^{2+}-free Barth's solution. The incision is sutured immediately thereafter and the frog left to recover in the secure tank after washing out the anaesthetics. The animal will return to the water tank after full recovery from surgery and should not be reused for at least two months after the operation.
4. Isolate individual oocytes by an enzymatic digestion of follicle membranes for 2 hours with 2 mg/ml of collagenase IA (Sigma) in Ca^{2+}-free Barth's solution. Select only stages V and VI oocytes, wash them several times with Ca^{2+}-free Barth's solution to remove any remaining enzymatic activity.
5. Visually inspect with a microscope that all oocytes are correctly defolliculated. If not, a manual defolliculation is required at this stage.
6. Wash all defolliculated oocytes again with regular Barth's solution.

3.5.2. Oocyte maintenance

1. Stage V and VI oocytes can be kept for extremely long periods of time (> 3 weeks) if incubated in Defined Nutrient Oocyte Medium (DNOM) at a temperature range of 16-18°C before and after injection. The composition of DNOM has been defined by Eppig and Dumont ***(34)***. It has been optimized for oocyte survival and maintenance, and basically contains amino acids, carbohydrates, salts, vitamins, antibiotics, amphotericin B, phenol red for pH control and polyvinylpyrrolidone to avoid oocyte attachment to the plastic surface. We strongly

recommend the use of DNOM instead of Barth's solution for oocyte maintenance as expression and survival rates were significantly improved.

2. Distribute 10 oocytes in each well of a Falcon 24-well plate and hermetically seal the plate with parafilm to avoid water evaporation and DNOM concentration.

3.5.3. Oocyte injection

1. *Xenopus* oocytes can sustain up to 50 nl injection in the cytoplasm. For reliability results, we use a nanoliter injector of WPI based on the displacement of an oil / aqueous solution interface.
2. Mix the cDNA of interest with purified T7 RNAP and adjust the concentration with water. In our hands ***(28)***, maximal expression level is reached by injecting 5 ng of cDNA and 5 ng of T7 RNAP in each oocyte. Making the assumption that the average volume of an oocyte is about 1 µl, and that each oocyte is injected with a total volume of 50 nl, then the injected solution should contain 100 ng/µl of cDNA and 100 ng/µl of T7 RNAP. These concentrations should represent the upper limits though some adaptation may be required for each plasmid. With a single purification of T7 RNAP, more than 2,000 oocytes can be injected. We successfully expressed various voltage-gated K^+ channels, a voltage-gated Na^+ channel and a Ca^{2+} channel auxiliary subunit with this technique ***(28)***. With T7 RNAP aliquots stored at -20°C, oocytes will also contain 0.02% glycerol. This is not a problem as these cells can contain up to 0.05% glycerol without noticeable effect on protein expression ***(28)***.
3. After injection, maintain the oocytes 3 to 6 days at 18°C in DNOM before proceeding with other functional tests. It should be emphasized that cell survival is excellent with this technique. We routinely keep healthy oocytes for 2 to 3 weeks after injection. The aim of this chapter is not to describe the functional tests used to reveal the expression of the protein of interest. However, we describe a rapid biochemical test to assess the presence of the protein that one wishes to express in oocytes.

3.6. Metabolic labelling

This procedure should be used to determine that protein synthesis is effective. It can also be used to optimize T7 RNAP and cDNA concentrations (see notes 1 and 2).

1. Coinject 0.2 µCi of [^{35}S]-methionine *per* oocyte along with the mixture of cDNA and T7 RNAP.
2. After injection, maintain oocytes for 24 hours in Barth's medium.
3. Resuspend 20 oocytes for each experimental condition in 1 ml of ice-cold buffer A (50 mM Tris, 1% Triton X-100, pH 6.8) supplemented with Complete^{TM}, a protease inhibitor cocktail, and then mechanically homogenize the cells with a 1 ml pottter (Bioblock).
4. Incubate the homogenate at 4°C for 30 min under agitation and centrifuge 20 min at 10,000 g.
5. Collect the supernatant with a Pasteur piper making sure to avoid the lipid layer.
6. Analyse the proteins by SDS-PAGE and autoradiogram analysis.

4. Notes

1. For voltage-gated K^+ channel expression, the effective range of T7 RNAP concentration was between 1 and 100 ng/µl. Maximal protein expression is reached at 50 ng/µl. A similar concentration dependence was observed with two different plamids (SP72 and pGEM3) encoding tow different proteins (*Shaker* B channel and calcium channel β_3 subunit) suggesting that it may be generalized. We recommend the use of 100 ng/µl T7 RNAP as a first assay of the technique, though some minor optimization may be required.

2. A cDNA dose-response curve has also been realized ***(28)***. Expression appeared around 20 ng/µl of cDNA, whereas it saturated at concentrations above 100 ng/µl. Here, again one should start with 100 ng/µl of cDNA, although, again, higher concentrations of cDNA can be attempted.

Here, we should emphasize that the quality of the cDNA is a critical factor for the success of this technique. A reliable index of cDNA quality is to achieve an *in vitro* coupled

transcription/translation assay. If the protein is readily produced *in vitro* by this technique, then it should also be produced with the T7 RNAP procedure in oocytes. If the assay using coupled *in vitro* transcription/translation is not effective, two conclusions can be drawn: the cDNA construct is either defective, in which case it should be improved, or the cDNA is of poor quality, and a new plasmid preparation and purification is thus required. We have used standard commercial columns for plasmid preparation (Promega, Qiagen, …) without encountering any problems.

3. We have not noticed any significant difference in protein expression time with the cRNA injection technique. Our observations suggest that the additional transcription step required by cDNA injection, compared to direct cRNA injection, does not significantly slow down protein expression in oocytes. The expression time in oocytes is generally short (1-2 days). For instance, we obtained a maximal expression level 2 days only after injection for a voltage-gated K^+ channel. What seems to delay expression level is the time required for cell trafficking. The appearance of multi-subunit calcium channel complexes in the plasma membrane of oocytes can take 4-7 days and reflect the time required for translocation to the plasma membranne. These expression times will vary depending on the nature of the protein being expressed.

4. We have successfully used this expression technique by also using commercial T3 and SP6 RNA polymerases along with the appropriate plasmids instead of purified T7 RNAP ***(28)***. The main drawback of commercial reagents is that their concentration is not provided by the manufacturer. However, using 10-fold diluted commercial T3 or SP6 RNAP (Ambion source) turned out to be fully functional. The use of commercial sources of RNA polymerases is thus perfectly valid for this expression technique if one does not want to perform the affinity purification of RNA polymerase. We can only recommend to either obtain the concentration value of the polymerase or to perform a dose-response curve in order to define the ideal dilution of the commercial reagent. Care should

also be taken to assess the cell toxicity of high concentration of glycerol for those dilution values that are extremely low.

5. We have frequently observed that the level of protein translation is smaller with the T7 RNAP technique than with direct cytoplasmic RNA or nuclear cDNA injections. Also, very large proteins appeared more difficult to synthesize with this technique. It has been observed that DNA injected into the cytoplasm is less stable than DNA injected in the nucleus ***(35)***. One hypothesis is that the cytoplasm of oocytes may have an endonuclease activity that would be absent from, or ineffective in, their nuclei. This may well explain the observed limitations of our T7 RNAP technique, though it should be emphasized that we did not observe any major differences in translation efficiency between circular and linear cDNA. A clear improvement in protein yield was observed if a mix of dNTPs and cap (3.22 mM dATP, dCTP and dUTP; 0.64 mM dGTP; and 2.58 mM cap analog) is co-injected along with purified T7 RNAP and the plasmid of interest ***(28)***. This may well represent a technical solution for expression problems encountered with larger proteins. Finally, it should be emphasized that the cytoplasm is not the normal locus for DNA transcription, and one should expect the technique to be drastically improved if the mix of cDNA / T7 RNAP is further injected into the nucleus.

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Figure legends

Fig. 1. Structure of pMR-T7 RNAP expression vector adapted from Arnaud and collaborators ***(29)***. Ptac, tac promoter; RBS1-MC-RBS2, minicistron flanked by two ribosome binding sites (RBS); $(His)_6$T7 RNAP, T7 RNAP coding region tagged with poly-histidine motif; rrnB T1 T2, strong transcription terminators; *bla*, gene conferring ampicillin resistance; pMB ori/M13 ori-, plasmid origin of replication; *lacI*^{q}, gene encoding for the lacI^q repressor.

Fig. 2. Expression, purification and analysis of T7 RNAP.

(A) Schematic representation of the protocol used for pMR-T7 RNAP transformation, induction of the expression, purification and analysis.

(B) *Left*, Coomassie Blue staining of a gradient 5-15% SDS-Polyacrylamide gel illustrating the various steps for T7 RNAP expression in bacteria and purification. Lanes 1 and 2 correspond, respectively, to crude lysate *E. coli* BL21 cells transformed with pMR-T7 RNAP, in the absence of induction (NI) or after induction (I); lanes 3 and 4: pellet (P) and supernatant (S) after sonication and centrifugation of bacterial suspension; lanes 5 to 7 correspond, respectively, to the 1st, 10th and 20th column wash (W1, W10 and W20); lanes 8 to 12 correspond to elution fractions 2, 5, 8 and 11 (E2, E5, E8, E11), respectively. *Right*, Western blot of the second elution fraction (1 µg) with an anti-T7 RNAP monoclonal antibody.