

A Rapid Induction Overexpression System for the Fission Yeast *Schizosaccharomyces pombe*

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[Abstract] The fission yeast *Schizosaccharomyces pombe* is an excellent genetically tractable model organism used in the study of conserved eukaryotic cellular biology. One genetic tool in the assessment of gene function is the *in vivo* overexpression of proteins. Existing overexpression tools have limitations of induction kinetics, dynamic range, and/or system-wide changes due to the induction conditions or inducer. Here, I describe the methodology for the use of a plasmid-based long non-coding RNA (lncRNA)-regulated overexpression system that is induced by the addition of thiamine. This system, termed the pTIN-system (thiamine inducible), utilizes the fast repression kinetics of the thiamine-regulated *nmt1*⁺ promoter integrated with the lncRNA regulated *tgp1*⁺ promoter. The advantages of the pTIN-system are rapid induction kinetics of gene expression, broad dynamic range, and tunable expression.

Keywords: Thiamine-induced, Overexpression, lncRNA-regulation, Fission yeast

[Background] Overexpression is a useful tool to study gene function. In the fission yeast *Schizosaccharomyces pombe*, many such tools exist, but each system has some disadvantages, including slow induction kinetics, narrow dynamic range, induction-based growth arrest, stress and/or system-wide transcriptional changes, self-limiting gene expression, and requirement of strain construction (addressed in Garg, 2020). The thiamine-repressed *nmt1*⁺ promoter is the most popular regulatable system used in fission yeast (Forsburg, 1993). This promoter system has a fast rate of repression but takes 14-20 h to induce mRNA expression (Maundrell, 1990). To transcend the limits of existing systems, I had designed the pTIN-system (Garg, 2020). Here, the *nmt1*⁺ promoter directs synthesis of the *nc-tgp1* lncRNA, which is 5'-adjacent to the *tgp1*⁺ promoter. The *tgp1*⁺ promoter in turn guides the synthesis of the gene of interest (GOI). In thiamine-starved conditions, lncRNA transcription interferes with the *tgp1*⁺ promoter and represses expression of the GOI. Inclusion of thiamine shuts off lncRNA synthesis and allows GOI expression. The pTIN-overexpression system has (i) rapid induction with maximal mRNA expression within an hour after the addition of thiamine, (ii) 60-fold dynamic range, and (iii) tunable expression by varying thiamine concentration in the medium (Garg, 2020). This protocol describes the utilization of the pTIN-system.

Materials and Reagents

A. Consumables

1. Test tubes (Fisherbrand, catalog number: 14-961-34, or equivalent)
2. Standard sterile serological pipettes (2, 5, 10, and 25 ml)
3. Micropipette tips (1,000-100 μ l, 200-20 μ l, 20-2 μ l, and 2.0-0.1 μ l)
4. 100 \times 15 mm Petri dishes (VWR, catalog number: 25384-088 or equivalent)
5. 15 and 50 ml centrifuge tubes (Corning, catalog numbers: 430766 and 430291, respectively, or equivalent)

B. Medium and growth conditions

Standard pombe minimal glutamate (PMG) medium, pH 5.6, supplemented as necessary with 250 mg/L each of adenine, uracil, leucine, histidine, and lysine (Sabatinos and Forsburg, 2010). Bacto agar should be added at a final concentration of 2% (w/v) for preparation of solid agar medium. Leu-PMG medium has all supplements mentioned except leucine. Add thiamine as specified in the methods. Thiamine must be added after cooling the media to 45-50°C after autoclaving for preparation of PMG agar medium with thiamine.

The base pTIN-system vectors, pTIN and pTIN7m, are available at: https://www.addgene.org/Stewart_Shuman.

pTIN ID#162885

pTIN7m ID#162886

Your GOI can be cloned into and overexpressed from these vectors.

1. Adenine (Sigma, catalog number: A2786)
2. Uracil (catalog number: U0750)
3. Leucine (Fisher, catalog number: BP385-100)
4. Histidine (Sigma, catalog number: H5659)
5. Lysine (Fisher, catalog number: BP386-100)
6. Bacto agar (BD, catalog number: 214010)
7. Thiamine hydrochloride (Sigma, catalog number: T1270)
8. Bacto agar (BD, catalog number: 214010)

C. Yeast competent cells and transformation

1. Tris base (Fisher, catalog number: BP152)
2. EDTA (Sigma, catalog number: E5134)
3. Lithium acetate (LiOAc) (Sigma, catalog number: L4158)
4. PEG 3350 (Sigma, catalog number: P4338)
5. Glycerol (Fisher, catalog number: BP229)

6. Salmon sperm DNA (ssDNA) (Sigma, catalog number: D1626)
7. LiOAc-mix (see Recipes)
8. PEG-mix (see Recipes)
9. 50% Glycerol/LiOAc-mix (see Recipes)
10. Salmon sperm DNA (ssDNA) solution (see Recipes)
11. 2,000× Thiamine hydrochloride (see Recipes)

Equipment

1. Incubator shaker (New Brunswick Innova 44 or equivalent)
2. Micropipettes (1,000-100 μ l, 200-20 μ l, 20-2 μ l, and 2.0-0.1 μ l)
3. Standard glass Erlenmeyer flasks (volume of culture should not exceed half the volume of the flask)
4. Incubators (Nuair IR autoflow or equivalent)
5. Test tube roller (New Brunswick, TC-7 with 25 mm test tube holder or equivalent)
6. Microcentrifuge (Eppendorf 5425 or equivalent)
7. Microcentrifuge tubes (Crystalgen, catalog number: L-2052 or equivalent)
8. Glass beads (Fisher Scientific, catalog number: 11-312C or equivalent)
9. Cooling centrifuge (Thermo Scientific Sorvall BP8 with HAEMAFlex 6 swinging bucket rotor or equivalent)
10. Centrifuge bottles (Thermo Scientific, catalog number: 3120-0250 or equivalent)

Procedure

A. Generation of yeast competent cells for long-term storage:

1. Grow 100 ml of your fission yeast strain auxotrophic for leucine in PMG medium supplemented with leucine, histidine, lysine, adenine, and uracil at 30°C till they reach mid to late-log phase (A_{600} 0.5 to 0.9). Use shaking conditions of 220 rotations per minute.
Note: Cells must be grown in medium lacking thiamine. Do not grow cells in YES medium (Sabatinos and Forsburg, 2010), which contains thiamine.
2. Transfer cells to a 250 ml centrifuge bottle and spin cells at 4,500 $\times g$ for 5 min at room temperature. Remove supernatant, resuspend in 5 ml of sterile water, and transfer suspension to a 15 ml centrifuge tube. Spin cells at 4,500 $\times g$ for 3 min.
3. Remove supernatant and resuspend in 2 ml of LiOAc-mix.
Note: Resuspend using 5 ml serological pipette (or greater volume).
4. Split sample into two 1.7 ml microcentrifuge tubes and spin cells at 4,500 $\times g$ for 2 min at room temperature.
5. Resuspend cells in LiOAc-mix in a final volume (cells + LiOAc-mix) of 1 ml for each aliquot.

6. Add 0.5 ml of 50% glycerol/LiOAc-mix for each aliquot and mix by inverting the tubes or gentle pipetting.
7. Split sample into 300 μ l aliquots and store in standard 1.7 ml microcentrifuge tubes at -80°C .
Note: Each 300 μ l aliquot is sufficient for four transformations. Smaller or larger aliquots can be made. Each aliquot can be thawed (on ice) and re-frozen a maximum of two times. Limit the time in between thaw and re-freeze to maintain competence. In the interim, dry ice may be used for freezing cells before transfer to -80°C . Transformation efficiency decreases with each freeze-thaw cycle (empirical observation).

B. Yeast transformation

1. Thaw competent cells (on ice), ssDNA, and plasmid DNA (concentration of approximately 1-2 mg/ml).
2. For each transformation, mix 3 μ l ssDNA, 2 μ l plasmid DNA, and 70 μ l cells. A mix of ssDNA and cells can be made before making aliquots for each transformation.
3. Incubate at room temperature for 10 min (shaking not required).
4. Add 290 μ l PEG-mix and mix by gently inverting tubes multiple times till uniform.
5. Incubate samples at 30°C for 1 h (shaking not required).
6. Heat shock at 42°C for 5 min.
7. Cool samples at room temperature for 5 min (shaking not required).
8. Spin for 1 min at $4,500 \times g$ at room temperature.
9. Remove supernatant and resuspend cells in 100 μ l sterile water at room temperature.
10. Gently spread sample(s) on selective agar plate(s) using glass beads and incubate at 30°C till the appearance of single colonies (3-4 days). Leucine dropout or Leu-PMG agar medium is used for selecting the pTIN-system plasmids.
11. Patch single transformants on Leu-PMG agar and incubate at 30°C for 1-2 days. It is best to use freshly grown transformants, but agar plates may be stored at 4°C for up to 5 days.

C. Induction and expression from the pTIN-system in yeast

1. Inoculate patched transformants in Leu-PMG medium and grow for 5-6 h at 30°C .
2. Dilute cells in Leu-PMG or Leu-PMG with 15 μM thiamine liquid medium and grow for the requisite amount of time needed for the experiment. The mRNA is expressed to peak levels by 1 h, and half-maximal protein expression, as assayed by Pho1 (cell surface acid phosphatase) reporter activity, is achieved by 5.6 h (Garg, 2020). To achieve lower target gene expression, thiamine concentration can be varied between 0.01 and 0.3 μM . Concentrations $\geq 0.3 \mu\text{M}$ lead to maximal derepression of the target gene (Garg, 2020). [15 μM thiamine has been widely utilized to repress the *nmt1⁺* promoter (Forsburg, 1993) and is thus used for maximal derepression from the pTIN-system]. Cells can be used for the assay of choice after the desired period of overexpression.
3. For growth analysis, continue from step 1. Dilute cells to 0.1 A_{600} and make serial dilutions (e.g.,

5-fold dilution series) in Leu-PMG medium. Spot 3 μ l of the serial dilutions on Leu-PMG Agar or Leu-PMG Agar containing 15 μ M thiamine. Incubate plates at the desired temperatures. Incubation time can be adjusted until the desired colony size is achieved. Incubation duration must be standardized to allow the formation of similar colony sizes of wild-type cells when grown at different temperatures.

Data analysis

Representative data: Representative data and details of experiments used to validate the system are provided in the original published research article of this method (Garg, 2020). These can be accessed through the following link: <https://rnajournal.cshlp.org/content/26/11/1743.long>.

Recipes

Yeast competent cells and transformation

Sterilize stock solutions (10 \times TE [Tris-EDTA buffer], 1 M LiOAc, 50% PEG 3350, 62.5% Glycerol, and ssDNA) by autoclaving. The thiamine hydrochloride solution should be filter-sterilized.

1. LiOAc-mix
(10 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM lithium acetate)
1 ml 10 \times TE
1 ml 1M LiOAc
8 ml water
2. PEG-mix
(40% PEG 3350, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM lithium acetate)
8 ml 50% PEG 3350
1 ml 10 \times TE
1 ml 1 M LiOAc
3. 50% Glycerol/LiOAc-mix
(50% glycerol (v/v), 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 100 mM lithium acetate)
80 ml 62.5% Glycerol
10 ml 10 \times TE
10 ml 1 M LiOAc
4. Salmon sperm DNA (ssDNA)
10 mg/ml in water
5. 2,000 \times Thiamine hydrochloride
10 mg/ml in water (30 mM stock solution)

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Competing interests

Patent Application No.: PCT/US21/40051; International Filing Date: July 1, 2021; Title: lncRNA-regulated gene expression system; Applicant: Memorial Sloan Kettering Cancer Center; Inventor: Garg, Angad

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