TKX1 Competent Cells

Instruction Manual

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Revision C.0

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TKX1 Competent Cells

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MATERIALS PROVIDED

Materials provided[a]	Quantity
TKX1 competent cells (clear tubes)[b]	5 × 200-µl aliquots
pUC18 control plasmid DNA (0.1 ng/µl in TE buffer[c])	10 µl
1.42 M β-mercaptoethanol	25 µl

[a] Provides enough reagents for 10 reactions.

[b] Transformation efficiency is $>5 \times 10^7$ cfu/µg of pUC18 DNA. This transformation efficiency is guaranteed when the cells are used according to the specifications outlined in this instruction manual.

[c] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

Store the cells immediately at –80°C
Do not place the cells in liquid nitrogen

ADDITIONAL MATERIALS REQUIRED

LB agar plates[§] with 12.5 µg/ml of tetracycline, 50 µg/ml of kanamycin and selecting antibiotic for plasmid encoding the target protein gene
Antibiotics
- Ampicillin stock (100 mg/ml in H_2O)
- Tetracycline stock [12.5 mg/ml in 50% (v/v) ethanol]
- Kanamycin stock (25 mg/ml solution of kanamycin sulfate in water, filter sterilized and stored at –20°C)

Indoleacrylic acid [2.5 mg/ml in 95% (v/v) ethanol]
TK induction medium[§]
LB broth[§] for target gene expression
IPTG inducer for target gene expression [IPTG stock is 100 mM in H_2O]

[§] See *Preparation of Media and Reagents.*

Revision C.0

INTRODUCTION

TKX1 competent cells are a tyrosine kinase (TK) derivative of the XL1-Blue MRF´ Kan strain [Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*5* (Kan^{r})].[1] TKX1 competent cells are resistant to kanamycin.

The XL1-Blue MRF´ Kan TK strain, abbreviated TKX1, harbors a plasmid-encoded, inducible tyrosine kinase gene (pTK). The TKX1 strain can be transformed with a plasmid (ColE1 origin) containing a DNA sequence encoding a phosphorylation target domain or protein. If the target protein is cloned as a fusion to an affinity tag, purification of large amounts of phosphorylated protein is relatively easy. The phosphorylated protein isolated from the TK strain can be used to screen expression libraries and to affinity purify or blot proteins that interact with the tyrosine-phosphorylated target.

HOST STRAIN AND GENOTYPE

For the *Escherichia coli* strain, the genes listed in the table below signify that the bacterium carries a mutant allele. The genes present on the F´ episome represent the wild-type bacterial alleles.

Host strain	References	Genotype
TKX1 strain	1, 2	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F´ *proAB lacI*^{q}*ZΔM15* Tn*5* (Kan^{r})] [pTK Tet^{r}]

TRANSFORMATION GUIDELINES

Important *Please read the following guidelines before proceeding with the* Transformation Protocol*.*

It is important to store the competent cells at –80°C to prevent a loss of efficiency. For best results, please follow the directions outlined in the following sections.

Storage Conditions

Competent cells are very sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Competent cells should be placed at –80°C directly from the dry ice shipping container. Cells stored in this manner should retain their guaranteed efficiency for at least 6 months.

Aliquoting Cells

When aliquoting, keep the competent cells on ice at all times. It is essential that the Falcon® 2059 polypropylene tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 100 µl of competent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of Falcon 2059 Polypropylene Tubes

It is important that Falcon 2059 polypropylene tubes are used for the transformation protocol, since other tubes may be degraded by the β-mercaptoethanol used in step 3 of the *Transformation Protocol*. In addition, the incubation period during the heat-pulse step is critical and has been calculated for the thickness and shape of the Falcon 2059 polypropylene tubes.

Use of β-Mercaptoethanol

β-Mercaptoethanol has been shown to increase transformation efficiencies two-to three-fold. This kit includes prediluted β-mercaptoethanol, which is ready to use. Using the β-mercaptoethanol within 3 months is recommended. Use 1.7 µl of the β-mercaptoethanol provided or a fresh 1:10 dilution (stock solution 14.2 M)/100 µl of cells.

Quantity of DNA Added

Add 1–5 µl of DNA for a final concentration of 1–100 ng of DNA in the transformation.

Length of the Heat Pulse

There is a defined "window" of highest efficiency resulting from the heat pulse in step 7 of the *Transformation Protocol*. Optimal efficiencies are observed when cells are heat-pulsed for 45–50 seconds. Heat-pulsing for at least 45 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease sharply when incubating for <45 seconds or for >60 seconds.

TRANSFORMATION PROTOCOL

1. Thaw the TKX1 competent cells on ice.

2. Gently mix the competent cells. Aliquot 100 µl of the competent cells into a prechilled 15-ml Falcon 2059 polypropylene tube.

3. Add 1.7 µl of β-mercaptoethanol provided with this kit or a fresh 1:10 dilution (of a 14.2 M stock) of β-mercaptoethanol [diluted in distilled water (dH_2O)] to the 100 µl of competent cells, giving a final concentration of 25 mM.

4. Swirl the contents of the tube gently. Incubate the polypropylene tube on ice for 10 minutes, swirling the tube gently every 2 minutes.

5. Add 1–5 µl (1–100 ng) of DNA to the to the polypropylene tube containing 100 µl of cells and swirl gently. As an optional transformation efficiency control, add 1 µl of the pUC18 control plasmid to another 100-µl aliquot of the competent cells and swirl gently.

6. Incubate the two polypropylene tubes on ice for 30 minutes.

7. Heat-pulse the tubes in a 42°C water bath for 45 seconds. The duration of the heat pulse is critical for obtaining the highest efficiencies.

8. Incubate the tubes on ice for 2 minutes.

9. Add 0.9 ml of **SOC medium**[§] and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.

10. Use a sterile spreader to plate ≤200 µl of the transformation mixture on the appropriate antibiotic plates.[§,‖] For the pUC18 control plasmid transformation, plate 10 µl of the transformation on LB–ampicillin–tetracycline agar plates.[§]

If desired, the transformed cells may be concentrated by centrifuging at 1000 rpm for 10 minutes. Resuspend the pellet in 200 µl of SOC medium and plate immediately.

Expected Results

Bacterial strain	Amount of transformation plated	Expected colony number (pUC18 control)	Efficiency (cfu/µg of pUC18 DNA)
TKX1 strain	10 µl	>50	$>5 \times 10^7$

[§] See *Preparation of Media and Reagents.*

[‖] When spreading bacteria onto the plate, tilt and tap the spreader to remove the last drop of cells. If plating <100 µl of the transformation mixture, pipet cells into a 200-µl pool of SOC medium. If plating ≥100 µl, the cells can be spread directly onto the plates.

INDUCTION PROTOCOL

Introduction

A two-step protocol has been shown to effectively produce large amounts of phosphorylated fusion protein. Expression of the kinase **target** gene is induced, and the target protein is allowed to accumulate before expression of the kinase gene is induced. Induction of expression of the kinase target protein gene depends on the promoter under which the target gene cloned. After induction of the target protein gene promoter, the cells with accumulated target protein are harvested and then resuspended in kinase induction media. The *tk* gene is induced and allowed to phosphorylate the target protein for 2 hours. The cells are then harvested.

The *tk* gene is controlled by the *trp* promoter. Under noninducing conditions, including tryptophan (Trp) in the media (growth in rich media) represses expression of the *trp* operon. Induction of kinase gene expression is carried out by growing the cells in Trp starvation media in the presence of indoleacrylic acid (IAA). Trp starvation induces the *trp* operon. Adding IAA to the medium induces expression further.[3] Indoleacrylic acid is an analog of the co-repressor tryptophan and competes with tryptophan for binding to the *trp* repressor protein. The IAA–*trp* repressor complex is unable to bind to the *trp* operator and represses transcription, thereby allowing high levels of expression.[4]

Induction of Expression of the Target Protein Gene

Before performing a large-scale purification, We advise checking target protein expression in the culture by carrying out a small pilot experiment to establish optimal conditions for expression. Protein expression can be monitored during growth and induction by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Parameters that may be evaluated include optical density of culture at the time of induction, concentration of inducing agents (e.g., IPTG concentration) and the period of time cells are grown under inducing conditions.

Production of Phosphorylated Target Protein in TKX1 Cells

An example of conditions which resulted in the production of a large amount of phosphorylated target protein is given as follows. The target protein is glutathione *S*-transferase (GST) fusion. The gene is under the control of the *tac* promoter on a plasmid carrying the *lacI*^{q} gene. We recommend inducing expression by adding IPTG to 0.1 mM and growing cells in the presence of 2% (w/v) glucose, enabling increased control of the *tac* promoter. After a 2-hour growth period in the presence of IPTG, collect the cells and induce the *tk* gene by growing in Trp starvation media plus IAA for 2 hours.

1. Transform TKX1 competent cells with the plasmid encoding the kinase target protein.

2. Select a single transformant colony from a freshly streaked plate and inoculate 100 ml of 2× YTG broth[§] containing 100 µg/ml of ampicillin, 12.5 µg/ml of tetracycline and 50 µg/ml of kanamycin in a 250-ml Erlenmeyer flask.

   Note *The volume of the culture should represent only 20–25% of the capacity of the flask to ensure adequate aeration (i.e., 20 ml in a 100-ml flask).*

3. Incubate for 12–15 hours at 37°C with vigorous shaking.

4. Dilute the culture 1:10 into fresh 2× YTG broth containing 100 µg/ml of ampicillin and 12.5 µg/ml of tetracycline.

5. Grow the culture at 37°C with shaking until the OD_{600} reaches 1–2.

6. Add 100 mM of IPTG to a final concentration of 0.1 mM and continue to incubate for 2 hours (or until an OD_{600} is achieved to maximize target production).

7. Measure the OD_{600} and spin down the cells at 2000 × *g*.

8. Resuspend the cells in TK induction media[§] to an OD_{600} of 0.5.

9. Continue to grow the cells for 2 hours at 37°C.

10. Harvest the cells by centrifugation at 2000 × *g* for 10 minutes. Store the cell pellets at –80°C until analysis.

Analyses of Extracts

Phosphorylation of the target gene can be evaluated by antiphosphotyrosine antibody immunoblotting. After resolution of *E. coli* protein extracts or purified target protein by SDS–PAGE, proteins can be electrotransferred to a membrane and blotted with any one of several commercially available antiphosphotyrosine antibodies.

§ See *Preparation of Media and Reagents.*

CONTROL INDUCTION

This optional control induction experiment should be performed to evaluate induction of the *tk* gene.

1. Streak the TK strain on a LB–tetracycline–kanamycin agar plate.[§] Grow overnight at 37°C.

2. Select a single transformant colony from the streaked plate and inoculate 50 ml of 2× YTG broth containing 50 µg/ml of kanamycin and 12.5 µg/ml of tetracycline.

3. Incubate overnight at 37°C with shaking.

4. Remove 5 ml of the overnight culture and spin down the cells for 10 minutes.

5. Resuspend the cell pellet in 50 ml of fresh 2× YTG broth containing 50 µg/ml of kanamycin and 50 µg/ml of tetracycline.

6. Grow the culture at 37°C to an OD_{600} of 0.6–1.0.

7. Spin down the cells in two polypropylene tubes at 1000 × *g*.

8. For an **uninduced** sample, resuspend one cell pellet in 2× YTG broth containing 50 µg/ml of kanamycin and 12.5 µg/ml of tetracycline to an OD_{600} of 0.5.

   For an **induced** sample, resuspend the other cell pellet in 1× TK induction medium.

9. Incubate the two cultures for 2 hours at 37°C.

10. Measure the OD_{600} of the final cultures and harvest the cells by centrifugation at 1000 × *g* for 15 minutes at 4°C. Store the pellets at –80°C while preparing the soluble protein extracts.

[§] See *Preparation of Media and Reagents.*

Preparation of Control Cell Extracts

1. Resuspend the frozen cell pellets in PLC lysis buffer.[§]

   Notes *The volume of PLC lysis buffer should be adjusted so that all of the samples contain approximately the same number of cells per ml of lysis buffer. Generally, the volume of lysis buffer should be approximately 1/12th the volume of the final culture. For example, resuspend pellets from 50-ml cultures in 4 ml of PLC lysis buffer. To approximate protein concentration, spin down the final culture volume to generate frozen cell pellets, which are then multiplied by the final* OD_{600} *to obtain the same results for each sample.*

   After lysates are prepared, a protein determination can be made for a more accurate comparison of samples analyzed by performing SDS–PAGE.

2. After resuspending pellets to the same density, transfer a 1-ml aliquot from the tube containing the induced sample and a 1-ml aliquot from the tube containing the uninduced sample to two microcentrifuge tubes placed on ice. Freeze the remainder of the samples at –80°C.

3. While keeping the samples on ice as long as possible, sonicate each 1-ml aliquot for 15 seconds at the minimum setting, placing the samples back on ice for 2 minutes between each burst. Perform this sonication cycle 5 times.

4. Microcentrifuge the samples at 4°C for 10 minutes.

5. Mix 1 µl of the supernatant with SDS–PAGE loading buffer[§] and boil for 5 minutes.

6. Load onto a 4–20% (w/v) acrylamide gel with biotinylated protein size markers.

7. Resolve the soluble proteins by performing electrophoresis.

8. Electrotransfer the proteins to PVDF membrane and immunoblot with an antiphospho-tyrosine antibody. Use appropriate detection reagents to detect specific binding.

   Figure 1 illustrates an antiphosphotyrosine antibody immunoblot of soluble protein extracts generated from cultures of the TK parent strains and the induced and noninduced TK strains.

[§] See *Preparation of Media and Reagents*.

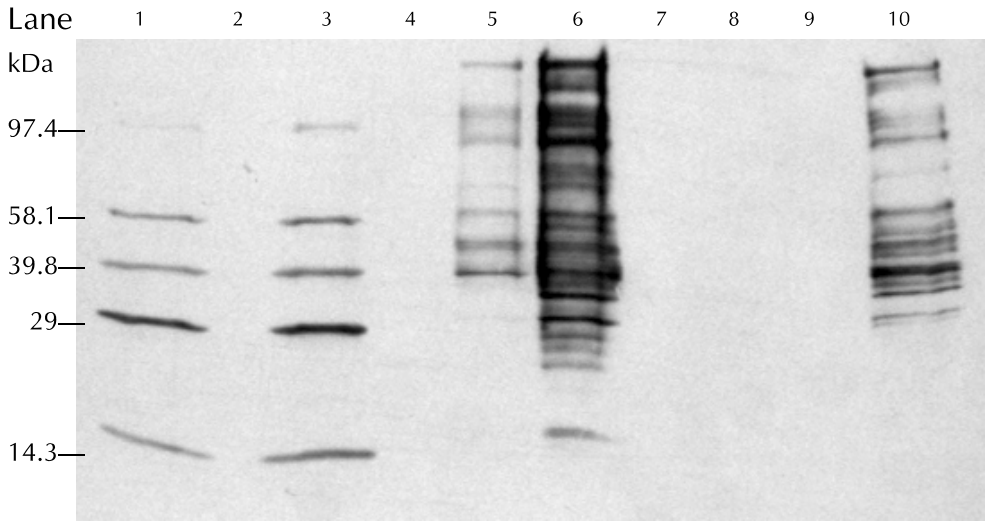

Figure 1 Detection of kinase expression by immunoblotting with antiphosphotyrosine antibody. Lanes 1 and 3 contain 10-µl per lane of a mixture of biotinylated protein size markers (9 µl markers prepared with 1 µl β-mercaptoethanol). Lanes 4–6 and 8–10 contain soluble protein extract derived from the BL21(DE3) strain (lane 4); the TKB1 strain uninduced (lane 5); TKB1 strain induced (lane 6); XI1-Blue MRF´ Kan strain (lane 8); TKXI strain uninduced (lane 9); and TKXI strain induced (lane 10). (Lanes 2 and 7 are empty lanes.)

Summary of Control Induction and Preparation of Control Cell Extracts

1. Prepare the soluble protein extracts and membrane as previously described.

2. Block the membrane in blocking buffer[§] plus TBST buffer[§] for 1 hour at room temperature.

3. Incubate the membrane with 1:1000 diluted αPY20 antiserum in BSA blocking buffer[§] for 1 hour at room temperature.

4. Wash the membrane again in TBST buffer for 30 minutes, replacing the buffer with fresh buffer every 5 minutes.

5. Incubate the membrane with rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) diluted to 1:1000 and 10 ml of streptavidin HRP for 1 hour in 1× BSA blocking buffer at room temperature.

6. Wash the membrane again in TBST buffer for 30 minutes, replacing the buffer with fresh buffer every 5 minutes.

[§] See *Preparation of Media and Reagents.*

7. Detect specific binding by immersing the membrane in 9 ml of appropriate detection reagents for 1 minute. Drain the reagents.

8. Wrap the membrane in plastic wrap and expose to Kodak® X-OMAT® AR film for ~1 second.

PREPARATION OF MEDIA AND REAGENTS

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Adjust to pH 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Adjust pH to 7.0 with 5 N NaOH
Add deionized H_2O to a final volume of 1 liter
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

LB–Ampicillin–Tetracycline Agar (per Liter)

(Use for reduced satellite colony formation)
1 liter of LB agar
Autoclave
Cool to 55°C
Add 100 mg of filter-sterilized ampicillin
Add 12.5 mg of filter-sterilized tetracycline
Pour into petri dishes (~25 ml/100-mm plate)
.

LB–Tetracycline–Kanamycin Agar (per Liter)

1 liter of LB agar
Autoclave
Cool to 55°C
Add 12.5 mg of filter-sterilized tetracycline
Add 50 mg of filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)
Store plates in a dark, cool place or cover plates with foil if left out at room temperature for extended time periods, as tetracycline is light-sensitive

SOB Medium (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Autoclave
Add 10 ml of 1 M $MgCl_2$ and 10 ml of 1 M $MgSO_4$/liter of SOB medium prior to use
Filter sterilize

SOC Medium (per 100 ml)

SOB medium
Add 1 ml of a 2 M filter-sterilized glucose solution or 2 ml of 20% (w/v) glucose prior to use
Filter sterilize

PLC Lysis Buffer

1% Triton® X-100
150 mM NaCl
10% glycerol
50 mM *N*-(2-Hydroxyethyl)piperazine-*N*´(2-ethanesulfonic acid) (HEPES) (pH 7.5)
1 mM [ethylene-bis (oxyethylenenitrilo)]-tetraacetic acid (EGTA)
1.5 mM $MgCl_2$
1 mM EDTA
0.2 mM sodium vanadate ($NaVO_4$)
0.2 mM phenylmethylsulfonyl fluoride (PMSF) (MW 174.2)
[mix 100 mM stock 100% (v/v) ethanol and store at 4°C]
100 mM sodium fluoride (NaF)
10 mM pyrophosphate
10 µg/ml aprotinin
(mix 2 mg/ml stock in water and use at a final concentration of 2 µg/ml)
10 µg/ml leupeptin
(MW 475.6)
[mix 20 µg/ml (soluble up to 50 mg/ml) in water; stable 1 month at –20°C]

Modified 5× M9 Medium (per Liter)

30 g of dibasic sodium phosphate (Na_2HPO_4)
15 g of monobasic potassium phosphate (KH_2PO_4)
5 g of ammonium chloride (NH_4Cl)
2.5 g of NaCl
15 mg $CaCl_2$
Autoclave

1× TK Induction Medium (per Liter)

200 ml of modified 5× M9 medium
1 ml of 1 M $MgSO_4 \cdot 7H_2O$ (autoclaved)
10 ml of 20% (w/v) glucose (filter sterilized)
5 ml of 20% casamino acids (filter sterilized)
0.1 ml of 0.5% thiamine-HCl (filter sterilized)
4 ml of 2.5 mg/ml indoleacrylic acid stock
1 ml of 100 mg/ml ampicillin [antibiotic selection for **target plasmid** (filter sterilize the stock solution and store at –20°C)]
1 ml of 12.5 mg/ml tetracycline [mix tetracycline in 50% (v/v) ethanol and store at –20°C]
Dilute to 1 liter with sterile water

TBS Buffer

10 mM Tris-HCl (pH 7.5)
100 mM NaCl

TBST Buffer

0.1% (v/v) Tween® 20 in TBS buffer

Blocking Buffer

1% (w/v) BSA in TBS buffer

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

6× SDS Gel-Loading Sample Buffer

7 ml of 4× Tris-HCl/SDS (pH 6.8) (see below)
0.93 g of dithiothreitol (DTT)
3.6 ml of glycerol
1 g of SDS (electrophoresis grade)
1.2 mg of Bromphenol Blue
Add H_2O to 10 ml
Store 0.5-ml aliquots at –70°C

4× Tris-HCl/SDS (0.5 M Tris-HCl containing 0.4% SDS)

Dissolve 6.05 g of Tris base in 40 ml of water
Adjust to pH 6.8 with1 N HCl
Add water to 100 ml
Filter sterilize through a 0.45-µm filter
Add 0.4 g of SDS
Store at 4°C

2× YT Broth (per Liter)

10 g of NaCl
10 g of yeast extract
16 g of tryptone
Adjust to pH 7.5 with NaOH
Add H_2O to a final volume of 1 liter
Autoclave

1× BSA Blocking Buffer (40 ml)

4 ml of 10× buffer A (see below)
0.4 ml of 10% (v/v) Tween-20
0.4 g of BSA
dH_2O to 40 ml

10× Buffer A

10 mM Tris-HCl (pH 7.5)
100 mM NaCl

2× YTG Broth

2× YT broth supplemented with 2% (w/v) glucose

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3. Morse, D. E., Mosteller, R., Baker, R. F. and Yanofsky, C. (1969) *Nature* 223(201):40-3.
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ENDNOTES

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.