TALO8 Purification

Reference: Unnikrishnan A, Gafken P, Tsukiyama T. Dynamic changes in histone acetylation regulate origin of DNA replication. Nature Struct Mol Biol 2010, 17:430-437.

Questions: Tsukiyama lab web site (http://labs.fhcrc.org/tsukiyama/protocols.html) or Toshi Tsukiyama (ttsukiya@fhcrc.org)

MATERIALS

REAGENTS

Yeast strain harboring pRS406-CMV-lacI-3FLAG and TALO8 (**see the end of this protocol for important info**)

Yeast media: synthetic media without tryptophan

200 mM phenylmethanesulfonyl fluoride (PMSF) in 100% methanol

Buffer H 150: 25mM HEPES KOH pH 7.6, 2mM $MgCl_2$, 0.5mM EGTA, 0.1mM EDTA, 10% glycerol, 150mM KCl, 0.02% NP40

100x Protease inhibitors: 100mM PMSF, 200μM pepstatin, 60μM leupeptin, 200mM benzamidine, 200μg/ml chymostatin A in 100% methanol. Store at -20 °C.

100x Phosphatase inhibitors: 200mM imidazole, 100mM sodium fluoride, 115mM sodium molybdate, 100mM sodium orthovanadate, 400mM sodium tartarate dihydrate in H_2O.

1000x Phosphatase inhibitors: 2.5mM (-)-p-bromotetramisole oxalate, 0.5mM cantharidin, 500nM microcystin in DMSO

1000x Histone deacetylase inhibitors: 500μM Trichostatin A (Sigma), 25mM Sirtinol (Calbiochem) in DMSO

Dynabeads Protein G (Invitrogen)

anti-FLAG M2 antibodies (Sigma)

0.1M sodium phosphate pH7.0

0.1M sodium phosphate pH 7.0, 0.01% Tween-20

0.2M triethanolamine pH 8.2 (Sigma)

20mM Dimethyl pimelimidate (Sigma) in 0.2M triethanolamine, pH 8.2

50mM Tris-HCl pH 7.5

PBST: Phosphate buffered saline with 0.01 %Tween-20

Buffer H 300: Same as Buffer H 150, except with 300mM KCl

Rinse Buffer: 25mM HEPES KOH pH 7.6, 2mM $MgCl_2$, 10% glycerol, 150mM KCl

Elution Buffer: 50mM Ammonium bicarbonate, 0.1% Rapigest (Waters Corporation) (or 0.1% SDS, if the final purpose is not mass spec)

EQUIPMENT

Swing bucket centrifuge (Beckman J6B or equivalent)

Bead beater or blender

Magnetic particle concentrator (MPC)

Ultra centrifuge

PROCEDURE

Growing and harvesting cells

1) Grow yeast cells harboring TALO8 and pRS406-CMV-lacI-3FLAG to an appropriate cell density (OD 660=0.7~1.2) in media lacking tryptophan.

2) Spin cells down at 6,000 rpm for 5 minutes at 4 °C.

3) Suspend cells in ~20x volume of ice cold water supplemented with 2mM phenylmethanesulfonyl fluoride (PMSF) and pellet them as in (2).

4) Suspend cells in ~10x volume of Buffer H 150 freshly supplemented with 1x protease inhibitors, phosphatase inhibitors and histone deacetylase inhibitors, and pellet them in 50ml Falcon tubes at 4000 rpm for 5 minutes at 4 °C.

PAUSE POINT Whole cell extracts can be prepared immediately or the cell pellet can be frozen in liquid nitrogen and stored at -80 °C.

Preparation of whole cell extract

5) Suspend cells in an equal volume of Buffer H 150 freshly supplemented with 1x protease inhibitors, phosphatase inhibitors and histone deacetylase inhibitors.

6) Aliquot equal volumes of cell suspension and zirconia/silica beads to fill up screw-capped 2 ml tubes. Beat cells for 3-5 minutes until "majority" of the cells are broken as assessed under a light microscope.

7) Puncture holes at the bottom and top of the tubes, and place them onto 12 x 75 mm tubes. Recover the cell extract by spinning the tubes at 1,000 rpm for 3 minutes.

Alternatively, frozen cell pellet in (4) can be ground in a blender in the presence of dry ice for 20 minutes. Frozen ground cells are then thawed in Buffer H 150 freshly supplemented with 1x protease inhibitors, phosphatase inhibitors and histone deacetylase inhibitors.
CRITICAL STEP

8) Clarify the cell extract by centrifuging them at 27,000 rpm for 90 minutes in Beckman SW41 or equivalent at 4 °C.

9) Soluble cell extract is drawn out through a syringe.
CRITICAL STEP

PAUSE POINT The cell extract can be used immediately in purification or be frozen in liquid nitrogen and stored at -80 °C.

Coupling anti-FLAG M2 antibody with magnetic beads

11) Typically, the antibody-conjugated beads are prepared immediately before use. For each liter of cells from which extract was prepared, 25 μl of Dynabeads Protein G beads slurry and 11.5 μg of anti-FLAG M2 antibodies are used. Concentrate magnetic beads on a magnetic particle concentrator (MPC), then suspend and concentrate beads twice in 0.5 ml of 0.1M sodium phosphate pH 7.0.

12) Mix antibody and magnetic beads in 0.1M sodium phosphate pH7.0 and gently shake them at **room temperature for 30 minutes**

13) Concentrate and suspend beads twice in 0.5 ml of 0.1M Sodium Phosphate pH 7.0, 0.01% Tween-20.

14) Suspend and concentrate the beads twice in 1ml 0.2M triethanolamine pH 8.2.

15) Suspend the beads in 1ml of 20mM Dimethyl pimelimidate, 0.2M triethanolamine pH 8.2, and incubate them for **30 minutes at room temperature** with constant rotational mixing.

16) Concentrate and suspend the beads in 1ml 50mM Tris-HCl pH 7.5 and incubate for **15 minutes at room temperature** with constant rotational mixing.

17) Wash the beads three times in 1ml PBST. The beads are ready for use in purification.
CRITICAL STEP

Purification of TALO8 from cell extract

18) Incubate antibody-conjugated beads and cell extract at **4 °C for 3 hours** with constant rotational mixing. Take small aliquots from the whole cell extract prior to mixing with beads for western blots and DNA analyses to monitor purification efficiency.

19) Concentrate beads on an MPC and transfer the supernatant (unbound materials) into a fresh tube. Save small aliquots of unbound materials for western blots and DNA analyses to monitor purification efficiency, and freeze the rest in liquid nitrogen and save them for troubleshooting purposes if required. Suspend the beads in Buffer H 150 and transfer them into a siliconized 1.7 ml microfuge tube.
CRITICAL STEP

20) Suspend and concentrate the beads three times in 1ml Buffer H 150 freshly supplemented with protease inhibitors, phosphatase inhibitors, histone deacetylase inhibitors and 2mM dithiothreitol (DTT).

21) Suspend the beads in 1m Buffer H 300 freshly supplemented with protease inhibitors, phosphatase inhibitors, histone deacetylase inhibitors and 2mM DTT and at rotate them at 4 °C for 5 minutes. Concentrate the beads on MPC, and repeat this wash step three more times, for a total of four times.

22) Suspend and concentrate the beads three times in 1 ml of Rinse Buffer.

23) Mix beads with 50 μl Elution Buffer and agitate them vigorously for 30 minutes at room temperature. Concentrate the beads on an MPC, transfer the supernatant into a fresh microfuge tube. Take small aliquots from the eluted samples for western blots and DNA analyses to monitor purification efficiency, then immediately freeze the rest in liquid nitrogen and store them at -80 °C. Elution is performed a total of four times, with the first two done for 30 minutes each and the subsequent two for 15 minutes each. Freeze the beads in liquid nitrogen and store them at -80 °C for troubleshooting purposes if required.

TROUBLESHOOTING
CRITICAL STEP

24) Determine the yield and purity of the sample by DNA preparation and SDS-PAGE gel electrophoresis followed by silver staining. Typical yield of TALO8 from 10 liter culture at OD_{660}=0.7 is about 2-4 μg of chromatin (1-2 μg of core histones).
TROUBLESHOOTING

TROUBLESHOOTING TABLE

PROBLEM
Step 23 Yield of TALO8 is too low.

SOLUTION
Low yield of TALO8 can be caused by inefficiency in either cell breakage in Step 6, binding of TALO8 to antibody-coated magnetic beads in Step 18, or elusion of TALO8 from beads in Step 23. To monitor cell breakage, examine cells under microscope before high-speed spin to clarify cell extract. The binding and elution efficiencies of TALO8 should be monitored in two ways. Western blotting and DNA preparation using the starting materials, unbound extract, eluates and the beads after the final elution determine how much lacI protein and TALO8, respectively, were bound and eluted during the process. In case the efficiency of elution is low, increase the volume of Elution Buffer to 100 μl.

PROBLEM
Step24 The amount of contaminants in eluted TALO8 fractions is too high.

SOLUTION
If purification is successful, histone proteins should be the major bands in silver stained gel. High levels of contamination can be caused by inefficient washing of beads in Steps 20-21. We have also found that excess levels of lacI would bring

down a large amount of proteins non-specifically. If the level of free lacI in eluates is too high, the expression construct for lacI (pRS406-CMV-lacI-3FLAG) needs to be modified to achieve optimal levels of expression.

CRITICAL STEPS

Step 7 Examine the extract under microscope to make sure that the majority of cells are disrupted.

Step9 Avoid taking up soft, fluffy precipitates on the top of firmly packed precipitates.

Step 17 Cross-linking of FLAG M2 antibody to beads is not essential for purification, but significantly reduces the amount of contaminating proteins in eluates.

Step 19 Make sure that the vast majority of beads are concentrated to the wall so that they are not lost. At the same time, do not leave the beads on MPC for more than a few minutes. Otherwise, the beads will clump and will increase background.

Step23 Alternatively, TALO8 can be eluted by 0.5mg/ml 3xFLAG peptide, which allows elution of the template in a native state.

pUC-TALO8 and pRS406-CMV-LacI-3FLAG

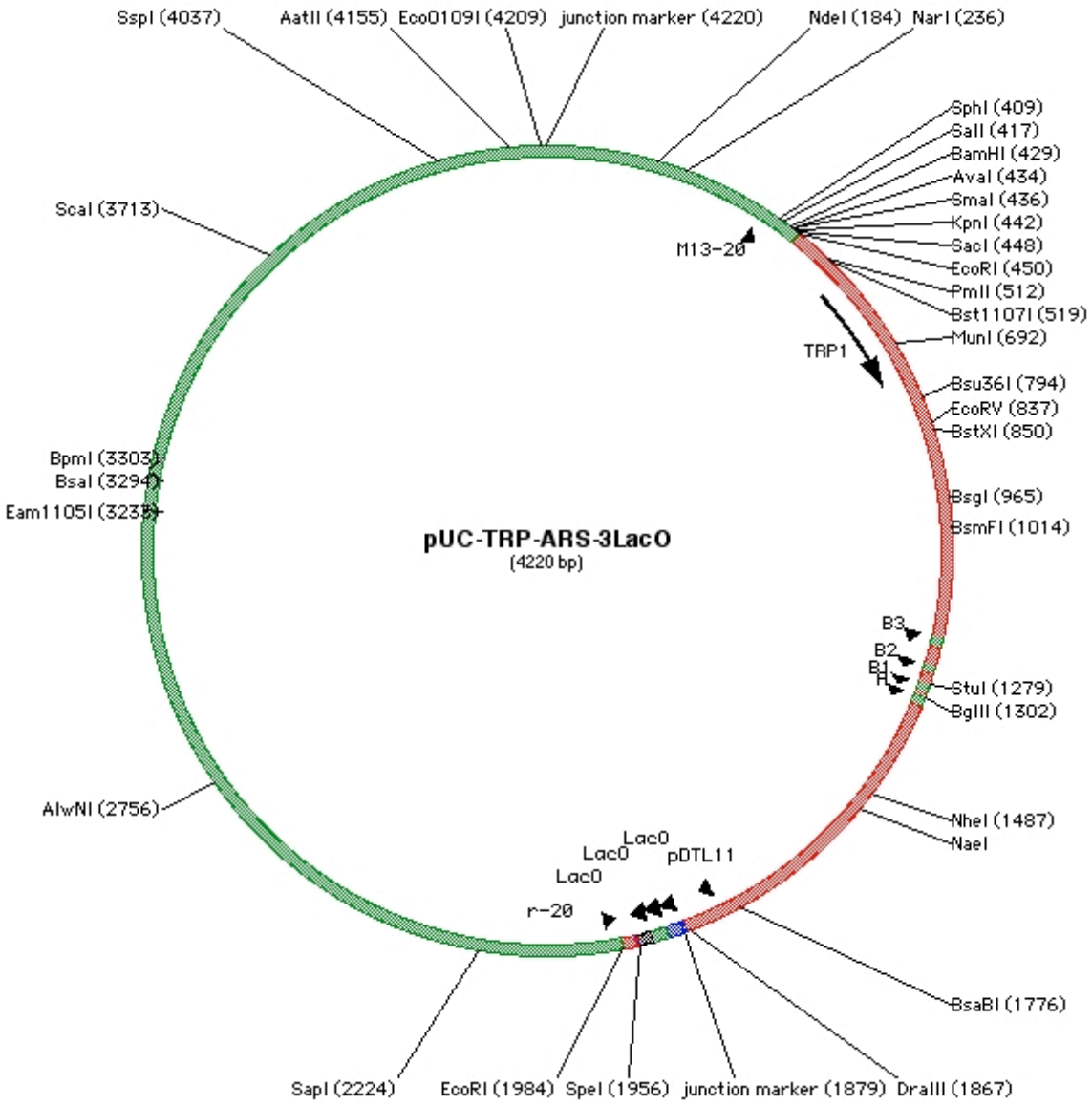
We propagate pUC-TALO8 using Stbl2 coli strain (Invitrogen) and grow at 30 °C in the presence of 1 mM IPTG in both plate and liquid media, in order to keep eight copies of LacO. Upon maxi-prep, we test the copy number of LacO by PCR using the following primers:

R-20: CAGCTATGACCATGATTACG
pDTL11: AATGCGAGATCCGTTTAAC

To transform yeast, we remove pUC backbone by cutting pUC-TALO8 with EcoRI, isolating ~1.7 kb fragment, and self-ligating it in vitro. We then directly transform yeast with the ligation mixture.

As for pRS406-CMV-LacI-3FLAG, we cut the plasmid by digestion with BstBI within *URA3* and integrate it at the *ura3* locus.

The map and sequence of pUC-TALO3 (the same as pUC-TALO8, but has only three copies of LacO)



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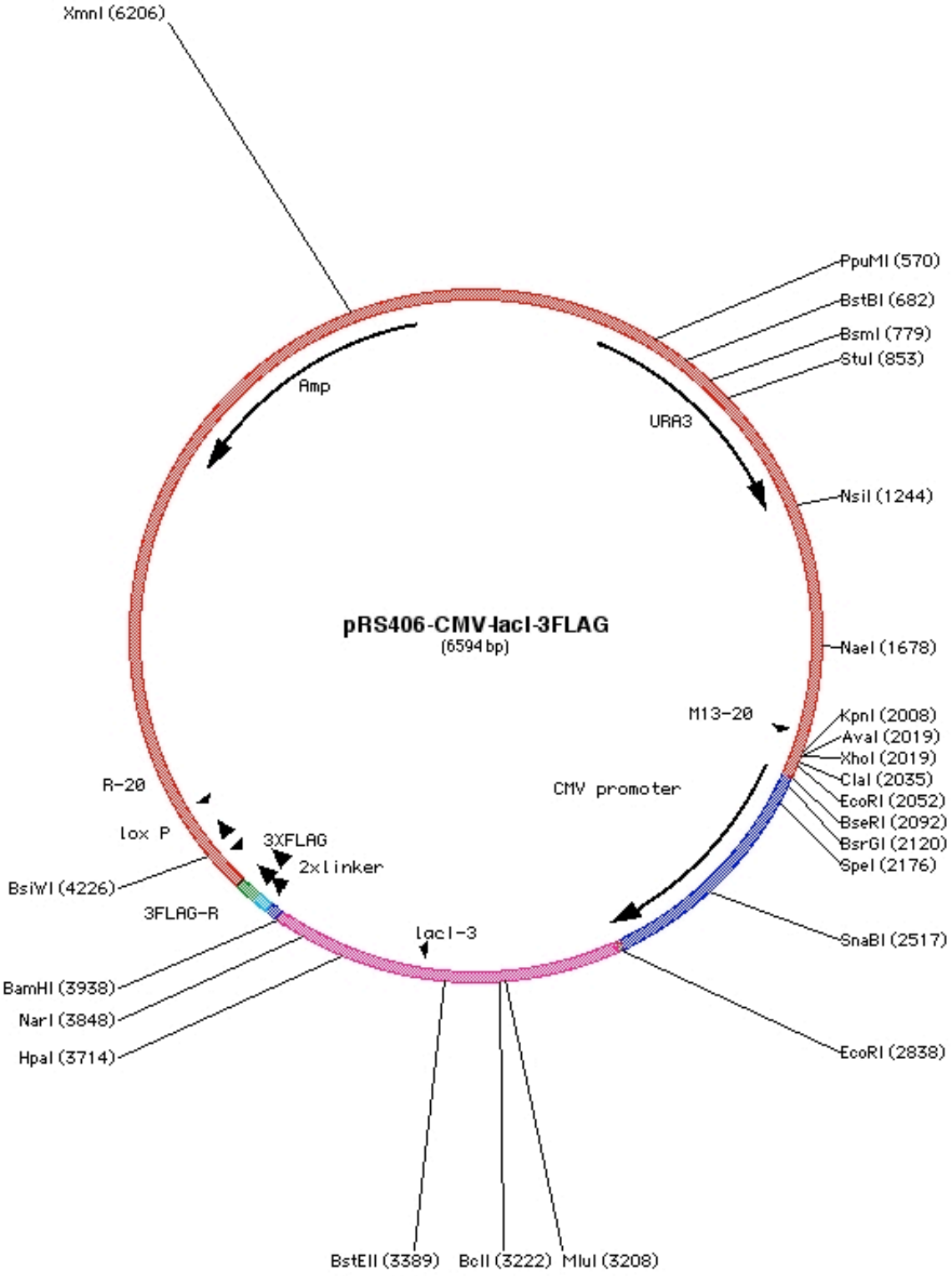
The map and sequence of pRS406-CMV-LacI-3FLAG



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