Chromatin ImmunoPrecipitation (ChIP)

As used for analysis of transcribing regions and DSB repair

NOTE 1: This is the Buratowski lab protocol, as modified by post-doc Philip Komarnitsky from the Struhl lab version. While the protocol as written uses a 250ml starting culture, it is commonly scaled down to 50ml.

NOTE 2: In all these years not one single person has gotten the protocol to work 1st time (and the one that did, it didn't work the 2nd, so doesn't count). People have also found the most amazing ways to screw up; there's no one place to look out for. However when it works the protocol is very consistent.

SAFETY: Red-hot needles, phenol and ultracentrifugation – be careful.

Protocol published in: Keogh & Buratowski (2004) Using chromatin immunoprecipitation to map cotranscriptional mRNA processing in Saccharomyces cerevisiae. *Methods Mol Biol* **257:**1-16 (**manuscript included in this protocol**).

Protocol used in: pretty much every paper to come out of the lab >2000, including (in a completely non-biased selection):

Keogh *et al* (2003) *Mol Cell Biol* **19:**7005-18
Krogan *et al* (2003) *Mol Cell* **12:**1565-76
Krogan *et al* (2004) *Proc Natl Acad Sci USA* **101:**15313-8
Krogan *et al* (2004) *Mol Cell* **16:**1027-34
Keogh *et al* (2005) *Cell* **123:**593-605
Keogh *et al* (2006) *Nature* **439:**497-501
Keogh *et al* (2006) *Genes Dev* **20:**660-5

PROTOCOL

1. To the 250ml culture ($OD_{600} \approx 0.5$; $\approx 10^7$ cells/ml) add 25ml 11% HCHO (freshly made from commercial 37% solution) so that the final [HCHO] = 1%. Make the 11% HCHO by adding 7.5 ml of 37% HCHO to 17.5 ml diluent (final concentration 0.1M NaCl, 1mM EDTA, 50mM HEPES-KOH, pH 7.5). Incubate 20 min at RT°, swirling briefly every 5 min.

Diluent (500 ml):		
	0.143 M NaCl	14.3 ml 5M NaCl
	1.43 mM EDTA	1.43 ml 0.5M EDTA
	71.43 mM HEPES-KOH	8.51 g HEPES
	Adjust pH with KOH	
	Water to 500 ml	

2. Add 37.5 ml of 3M glycine, 20 mM Tris (not pH-ed). Mix and incubate for another 5 min. You may have to dissolve the glycine in a rotating water bath at 50 °C prior to use.

3. Pellet the cells, wash 2x with 200 ml cold TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), once with 10 ml ice cold FA lysis buffer/**0.1% SDS**. Transfer to a 14 ml Falcon tube. At this point pelleted cells can be frozen at –70 °C.

2x FA lysis buffer (200ml):		
	100 mM Hepes-KOH, pH 7.5	4.77 g HEPES
	300 mM NaCl	12 ml 5M NaCl
	2 mM EDTA	0.8 ml 0.5M EDTA
	2% Triton X-100	4 ml 100% Triton
	0.2% Na Deoxycholate	4 ml 10% Na Deox.

4. Resuspend the pellet in 1 ml ice cold FA lysis buffer / **0.5% SDS**

5. Add 1.5ml small glass beads (**Sigma G8772**) and vortex in a round bottomed plastic tube (*eg*. 14ml Falcon 352059) tube for 8 cycles: 30" vortex max speed, 30" on ice. Ensure the beads don't settle (particularly in later cycles when the foam is quite impressive).

6. Pierce the bottom of the tube with a red-hot 22G needle and insert into a plastic Nalgene 30 ml centrifuge ("Oakridge") tube. Add 6.5 ml of ice cold FA lysis buffer / **0.1% SDS**. Collect lysate by centrifugation in SS-34 rotor at 4ºC @1000 rpm for 5 min.

7. Transfer the lysate to an ultracentrifuge bottle. Balance to two decimal places. Spin in the 50Ti rotor at 45,000 rpm @ 4 ºC for 20 min.

8. Decant the supernatant, resuspend the pellet in 8 ml of ice cold FA lysis buffer / **0.1% SDS**. **Note:** The presence of a glass-like transparent top layer on the pellet indicates chromatin is out of the cells (allegedly). Pellet cannot be completely resuspended so just use a wooden applicator to break it up as much as possible.

9. Centrifuge in the 50 Ti rotor : 45,000 rpm, 4ºC for 20 min.

10. Decant the supernatant, resuspend the pellet in 1.5 ml of ice cold FA lysis buffer / **0.1% SDS** and transfer to a 2 ml plastic cryovial.

11. **< THIS IS YOUR RESOLUTION STEP >** Place on ice and sonicate to shear the chromatin (CellBio sonicator, Goldberg lab, 4th floor): 5 pulses, 20" on, 20" off with a peak to peak power of 12µm. (**NB.** will have to determine this for other sonicators empirically).

12. Transfer to an ultracentrifuge tube, add 6.5ml of ice cold FA lysis buffer / **0.1% SDS**, and centrifuge in the 50Ti rotor at 45,000 rpm for 20 min.

13. Sheared chromatin is now in the supernatant. Freeze in 800µl aliquots at –70 ºC.

14. To test chromatin sonication yield and quality, take 100µl of the chromatin supernatant, perform decrosslinking (see below), extract with phenol-chloroform, resuspend in 200µl TE and run 20µl on an agarose gel.

IMMUNOPRECIPITATION AND DECROSSLINKING

15. Thaw chromatin solution and add 5M NaCl until the final concentration is 275 mM NaCl; this corresponds to 20 µl for 800 µl. Pre-wash 10 µl of protein A beads per reaction with 1 ml of FA lysis buffer / **0.1% SDS**, @ RT, 4 min on a rotator. Add 750µl of NaCl-adjusted chromatin solution to 10 µl of protein A (or other) beads and your favorite antibody. Save remainder as an INPUT sample.

16. Incubate on a rotator overnight @ 4 ºC

17. Pellet beads (microfuge 1100rpm, 4min, RTº) and wash once with 1ml of each of the following:

i) FA lysis buffer/**0.1% SDS/275 mM NaCl**, 4 min @ RT, rotator (250µl 5M in 10 ml)
ii) FA lysis buffer/**0.1% SDS/500 mM NaCl**, 4 min @ RT, rotator (700µl 5M in 10 ml)
iii) 10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% Na Deoxycholate, 4 min @ RT, rotator

iv) TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), 4 min @ RT, rotator

18. Elute IPed stuff by heating for 10min @ 65 °C in 250 µl of 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS (Elution buffer)

19. Pellet beads and transfer the supernatant to a fresh tube. Wash the beads with 250µl TE and add that to the supernatant. Add 180µl of TE to the 70µl leftovers (**INPUT**). To reverse the crosslinks add 10 µl of 20mg/ml pronase (Boehringer) per 250 µl of solution and incubate for 1hr @ 42 °C, then 4 - 5 hrs @ 65 °C.

20. Add 50 µl 4M LiCl and vortex.

21. Extract with 400µl Phenol-Chloroform-Isoamyl alcohol (25:24:1 PCI) and then 300µl chloroform. In each case, vortex well and microfuge 12000rpm (max speed), 10 min, RT°. Precipitate the DNA by adding 1µl of 20 mg/ml glycogen (Boehringer), 1ml 100% EtOH (-20°C). Incubate for ≥1 hour, -80°C. Microfuge max speed, 10 min, RT°.

22. Discard supernatant and wash pellet with **100%** EtOH. Dry on bench, resuspend in 200µl TE (10mM Tris-HCl, **1mM** EDTA all to pH 8). Store @ -20 °C.

PCR ANALYSIS

23. Use 1/50th of the IP and 1/1000th of input per reaction ~ 4 µl of each

Each 10 µl PCR reaction will contain 4µl DNA + 6 µl PCR mix

Mix (per x reactions):	1x	20x	28x	36x
10 µM intergenic primer mix (internal std)	0.25	5.0	7.0	9.0
2.5 mM each dNTP mix	0.4	8.0	11.2	14.4
10x Platinum Taq buffer	1.0	20.0	28.0	36.0
50 mM MgCl	0.3	6.0	8.4	10.8
Platinum Taq polymerase (Gibco BRL)	0.1	2.0	2.8	3.5
α-[^{32}P]-dATP (sp. activity 10 mCi/ml)	0.03	0.6	0.9	1.1
PCR ddH_2O	3.67	75.0	103.0	132.0
Total	5.75	116.0	160.0	206.0

Plus 0.25 µl of 10 µM each primer mix per reaction

PCR cycle: 1 cycle of 90 s @ 94 °C, then 26 cycles of 30 s @ 94 °C, 30 s @ 55 °C, 1 min @ 72 °C, and finally 4 min @ 72 °C (**cycle name; QUANT-PK**).

24. Analyse PCR products on an 8 % polyacrylamide - TBE gel (36ml ddH_2O, 12ml 5x TBE, 15ml 30% acrylamide, 140µl 30% APS, 105µl TEMED). Run at 200V, first dye should run approx. 4-5 cm into the gel. Adsorb gel to filter paper, dry down and expose to phosphoimager plate for 5 min @ RT (this is approximately equivalent to 90m exposure to blue film).

NOTE 3: After resolution by PAGE, PCR products were quantitated using a Fujix BAS 2040 PhosphoImager and allied Fuji *ImageGauge* software. The Input sample was used to calculate the Normalization Value (NV) between each specific primer pair and the control "No-ORF" primer pair (*). This ratio compensates for any

variation in PCR efficiency and label content by converting the signal from different primer pairs into normalized units of the control primer pair. This generates the Corrected Value (CV) for each specific primer pair in each immunoprecipitation. Finally each CV is divided by the No-ORF (*) signal from each immunoprecipitation to give a relative value (x-fold compared to the * internal control) that allows trend comparison across samples to be performed.

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Using chromatin immunoprecipitation to map co-transcriptional mRNA processing in *Saccharomyces cerevisiae*

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Abstract

The Chromatin Immunoprecipitation (ChIP) technique has been used to determine where and under which conditions DNA binding proteins associate with specific DNA sequences. Proteins are cross-linked *in vivo* with formaldehyde, and chromatin is then isolated and sheared. The protein of interest is then immunoprecipitated, and the associated DNA sequences identified via PCR. Although originally designed to assay DNA binding proteins, mRNA processing factors associated with transcription complexes can also be monitored using this technique.

Keywords: Chromatin Immunoprecipitation, epitope tagging, Polymerase Chain Reaction, TAP (Tandem Affinity Purification) tag.

Abbreviations

ChIP, Chromatin Immunoprecipitation; IP, Immunoprecipitates; ORF, open reading frame; PCR, Polymerase Chain Reaction; RNApII, RNA polymerase II; TAP; Tandem Affinity Purification; TBP, TATA binding protein.

1. Introduction

Synthesis of mRNA by RNA polymerase II (RNApII) is a complex process involving the transient association of large protein complexes with DNA (***1,2***). Much work in the field has concentrated on *in vitro* reconstitution, examining the role of individual proteins or complexes in different steps of the transcription cycle. However, a more complete understanding necessitates study of this process in the natural chromosomal environment.

This chapter describes chromatin immunoprecipitation (ChIP), a method that allows one to determine where and when a particular protein is located near specific DNA sequences (***3-6***). Chromatin immunoprecipitation has been used extensively in the budding yeast *Saccharomyces cerevisiae*, but the technique has been adapted successfully to many species (***3,4,7-10***). In its simplest form the protein of interest is cross-linked to chromatin, which is then isolated and sheared to a specific desired size. The protein is then immunoprecipitated along with any associated DNA. The chromatin is de-crosslinked and specific DNA sequences assayed using PCR (**Figure 1**). The exquisite sensitivity of PCR and the availability of complete genomic sequences have made the technique very powerful.

Formaldehyde is the cross-linking agent of choice for these experiments (*Section 3.2*). It is easy to handle, water-soluble, and active over a wide concentration range. Most importantly, it readily traverses biological membranes, allowing cross-linking to be performed on intact cells (***3,11***). Formaldehyde crosslinks primary amino groups such as those on lysines and the bases adenine, guanine and cytosine. Protein-protein and protein-DNA cross-links are formed between groups within distances of approximately 2 Å. These modifications are reversible: extended incubation at 65^{o}C breaks the protein-DNA bonds while the protein-protein cross-links can be reversed by boiling (***3***).

After cross-linking, the yeast cells are mechanically lysed (*Section 3.3*). However DNA fragments in these lysates are too long to determine the precise genomic location of chromatin–associated proteins. Sonication is a rapid and straightforward way to shear the chromatin fragments and generate the smaller sized fragments desired. By controlling the

sonication time and strength one can generate relatively uniform size populations and increase the resolution of the technique (***12***). In our experience, the maximum resolution achievable is approximately 200 bp.

Once the technique is established, the main variable encountered is the immunoprecipitation step (*Section 3.4*). Not all primary antibodies are amenable to the relatively stringent conditions employed. This variable can be avoided by epitope-tagging the protein of interest (***13***), although it must be shown that the tag does not interfere with the function of the protein. Epitope tagging of genomic loci in *S.cerevisiae* is a relatively straightforward process (***14,15***), which greatly increases the utility of the technique in this species.

In our experience, the HA (human influenza virus hemagglutinin) epitope and protein A tags work well in chromatin immunoprecipitation. The small HA-epitope (YPYDVPDYA) is recognized by the commercially available 12CA5 monoclonal antibody, which binds with equal efficiency to Protein A or Protein G Sepharose (***16***). The HA-epitope works well in most locations within the tagged protein. However, it is best to use three or more copies of the epitope for maximum efficiency. Although the protein A module is larger, it has some advantages. For immunoprecipitation, relatively inexpensive IgG Agarose is used. Also, the popular Tandem Affinity Purification (TAP) tag (***17***) contains one copy of the Protein A module, and many TAP-tagged strains are already available. Although the TAP tag was originally designed for purification of tagged proteins, we find that it works well in chromatin immunoprecipitation.

After reversal of crosslinking, the PCR step (*Section 3.5*) allows one to ask whether specific DNA sequences are bound to the protein under study. Each reaction contains two or more primer pairs. We strongly recommend including a control primer pair that amplifies a non-transcribed region (i.e. no open reading frame, marked with an asterisk in **Figures 2 and 3**). This serves as an internal negative control for background and PCR efficiency, and this signal can be used to normalize separate ChIP experiments. In addition, the reaction can contain one or more primer pairs that amplify a specific region of interest (**Figures 2 and 3**). Primers are designed primarily on the basis of location, but are typically 24 – 30 mers with an annealing temperature of $\approx 55^{o}$C. A BLAST search of the primer sequences against the entire genome is recommended to

assure that hybridization is specific to the desired region. It is also worthwhile to use one of the many available computer programs that tests primer sequences for internal hairpins, primer-dimers, etc.

PCR products are easily resolved on a non-denaturing polyacrylamide or agarose gel. Of course, if multiple primer pairs are used in the same reaction, the amplified products must be of different sizes. The inclusion of radiolabeled nucleotide in the reactions allows quantitation of the two or more products (the negative control and specific sequences) in each tube. If a protein crosslinks to a specific DNA sequence there should be an increase in the relative abundance of this PCR product compared to the control standard (**Figure 3**). For accurate quantitation, it is extremely important that the PCR reactions are assayed while still in the exponential phase.

A schematic of the protocol is shown in **Figure 2**, which also indicates at which points the procedure can be safely interrupted. A typical ChIP experiment (assuming the current availability of all strains and materials) takes four to five days. Up to the preparation of PCR-ready samples, we generally deal with no more than 12 cross-linked samples at once, a bottleneck imposed in our case by the ultra-centrifugation steps on day two (see Sections 3.3.3 and 3.3.5). The PCR throughput is determined by the capacity of the thermocycler(s).

Although the length of the protocol can be daunting, it is relatively simple to master if each step has been controlled. For the worker trying to establish the technique, it is useful to initially perform the analysis with previously characterized factors. As a transcription lab, we generally use the crosslinking of TBP and Rpb3 as controls. The former should crosslink specifically to promoters, the latter at promoters and throughout coding regions ***(6, 18-19)***. These positive controls can verify the quality of the chromatin and the proper execution of the protocol. These patterns serve as points of comparison for crosslinking of new factors. It is important to analyze occupancy at multiple genes (see **Note 1**) before any specific observations can be generalized.

Chromatin immunoprecipitation has been used for mapping various factors involved in DNA-related processes, including replication, chromatin modifications, and transcription. However, we and others have found that other factors associated with transcription complexes but not directly associated with DNA, such as the mRNA

capping enzyme and other mRNA processing factors, can also generate a signal in ChIP experiments. Such crosslinking is strongly indicative of cotranscriptional mRNA processing.

2. Materials

2.1 Growth of yeast cells

1. Appropriate growth media
2. Incubator shaker

2.2 Formaldehyde crosslinking and chromatin preparation

2.2.1 Equipment -

1. Preparative centrifuge (Sorvall RC5B+ or equivalent)
2. Ultracentrifuge (Beckman Coulter Optima LE-80K or equivalent)
3. Beckman Ti50 Rotor
4. Ultracentrifuge tubes (10.4 ml polycarbonate, Beckman #335603 or equivalent)
5. Microcentrifuge (Ependorf 5415C or equivalent)
6. Centrifuge flasks / tubes (preparative)
7. 14ml round-bottom Falcon tube (Falcon #2059 or equivalent)
8. Acid washed glass beads, 425 - 600μ (Sigma, G-8772)
9. Glass Pasteur pipettes (VWR 14672-380 or equivalent)
10. 2ml vials (Corning #430289 or equivalent)
11. Probe Sonicator with microprobe tip (MSE 2/76 Mk2 or equivalent)

2.2.2 Reagents -

1. 37% Formaldehyde (HCHO): Molecular Biology grade; VWR EM-FX0415-5.
2. Glycine Stop solution: 3M Glycine, 20 mM Tris base, do not adjust the pH.
3. Diluent pH 7.5: 150 mM NaCl, 1.5 mM EDTA, 70 mM HEPES, adjust pH with KOH.
4. TBS: 20 mM Tris-HCl pH 7.5, 150 mM NaCl.

5. 2x FA Lysis buffer: 100mM HEPES.KOH pH 7.5, 300mM NaCl, 2mm EDTA, 2% Triton X-100, 0.2% sodium deoxycholate.
6. 1X FA lysis buffer / 0.1% SDS
7. 1X FA lysis buffer / 0.5% SDS
8. 5M NaCl
9. Protein A Sepharose CL-4B (Amersham Pharmacia Biotech, 17-0780-01)
10. Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech, 17-0618-01)
11. Rabbit IgG Agarose (Sigma, A-2709)

2.3 Immunoprecipitation and Decrosslinking

2.3.1 Reagents -

1. TBS: 20mM Tris-HCl, pH 7.5, 150 mM NaCl.
2. 2x FA Lysis Buffer (see above).
3. 5M NaCl.
4. Wash 1: 1x FA Lysis buffer / 0.1% SDS / 275mM NaCl).
5. Wash 2: 1x FA Lysis buffer / 0.1% SDS / 500mM NaCl).
6. Wash 3: 10 mM Tris-HCl pH8.0, 1 mM EDTA, 0.25M LiCl, 0.5% NP40, 0.5% sodium deoxycholate).
7. Wash 4: TE pH 8.0, 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
8. Elution Buffer: 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS.
9. Pronase (20 mg/ml) (Roche, 165 921).
10. 4M LiCl.
11. PCI: phenol / chloroform / isoamyl alcohol, 25:24:1.
12. Glycogen (10 mg/ml) (Roche, 901 393).
13. 100% EtOH.

2.4 PCR analysis

2.4.1 Equipment –

1. Thin walled PCR tubes (0.5ml).
2. PCR machine with heated lid (MJ Research PTC-100 or equivalent).

3. Vertical polyacrylamide electrophoresis system.

4. Detection and quantitation system; eg. Phosphorimager plates and analysis system or autoradiography film (Kodak X-OMAT AR or equivalent) and developing system.

2.4.2 Reagents -

1. dNTP mix: 2.5 mM dATP, dTTP, dCTP, dGTP.
2. Platinum Taq (Invitrogen 10966-034, 5U/μl or equivalent – see **Note 2**).
3. 10x Platinum Taq Reaction buffer.
4. 50 mM $MgCl_2$.
5. 10 μM primer mixes.
6. α-[^{32}P]dATP (specific activity 10 mCi/ml)
7. 6x Gel Loading Buffer: 0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 30% Glycerol.

3. Methods

A schematic of the protocol is presented in **Figure 2**. The protocol can be stopped for long term storage at the indicated points. If performed straight through it takes four days. The protocol as described supplies sufficient chromatin for an **Input** sample and ten immunoprecipitations (**IP**). Each immunoprecipitation can be used for approximately 50 PCR amplifications. If multiple analyses of a single preparation are not required, the protocol can be scaled back accordingly, although as a rule we keep all volumes as indicated until step *3.3.5*.

3.1 Growth of yeast cells

Grow a seed culture overnight to confluence. At the beginning of Day 1, dilute the overnight culture to an $OD_{\lambda 600} \approx 0.15$ and grow under the appropriate conditions to $OD_{\lambda 600} \approx 0.65 – 0.8$ (see **Note 3**). For a wild type strain at the optimal growth temperature

(30°C) in YPD media with 2% glucose as carbon source, this will take approximately five hours. Cells are in exponential growth phase during crosslinking.

3.2 Formaldehyde crosslinking

3.2.1 To 250 ml culture add 25 ml 11% HCHO (freshly made from commercial 37% solution and Diluent) such that the final formaldehyde concentration is 1%. Incubate 20 min at room temperature with gentle mixing.

3.2.2 Add 37.5 ml Glycine Stop solution and incubate for a further five min with gentle mixing. Although the Stop solution can be made in advance it often precipitates – redissolve crystals by heating to >50°C with stirring before use. Alternatively, make the solution fresh before each experiment.

3.2.3 Pellet cells by centrifugation at 3000 rpm in a Sorvall GS-4 rotor or equivalent. All steps from this point on are performed on ice with pre-cooled solutions unless indicated otherwise. The cell pellet should be washed by resuspending and repelleting twice with 100 ml TBS and once with 10 ml FA lysis buffer / 0.1% SDS. Transfer to a 14ml round-bottom Falcon tube, pellet cells and aspirate buffer. Pellets can be stored at this point at –80°C.

3.3 Preparation of Chromatin

3.3.1 Resuspend the cell pellet in 1ml FA lysis buffer / 0.5% SDS. Add an equal volume of acid washed glass beads. Vortex vigorously 10 cycles of 30 sec mixing, 30 sec on ice. A large amount of foaming occurs, so ensure the beads don't settle during vortexing.

3.3.2 Add 6.5 ml FA lysis buffer / 0.1% SDS and puncture the tube bottom and cap with a 22G needle. Insert the Falcon into a 30 ml Nalgene Oakridge tube and centrifuge in the GS-4 rotor at 1000 rpm for 4 min. Collect the lysate in the Oakridge tube and discard the Falcon tube containing the glass beads.

3.3.3 Resuspend the loose pellet and transfer the entire lysate to an ultracentrifuge tube. Centrifuge at 45000 rpm in a Ti50 rotor for 10 min at 4°C. Discard the supernatant and resuspend the pellet in 8 ml FA lysis buffer / 0.1% SDS (see **Note 4**). Centrifuge this at

45000 rpm, 4°C, for 20 min. Discard the supernatant, resuspend the pellet in 1.5 ml FA lysis buffer / 0.1% SDS and transfer to a 2 ml microcentrifuge tube.

3.3.4 Sonicate the suspension to break the chromatin into fragments of approximately 200 – 500 bp (see **Note 5**). For this step, the conditions used need to be determined empirically for each sonicator. For an MSE Sonifier (2/76 Mk2) fitted with a microprobe tip, five or six 20 sec pulses of constant output (amplitude 12) are sufficient. Keep the sample tubes on ice throughout sonication and allow 20 sec breaks between pulses to prevent excessive warming. Ensure the sonicator tip is fully immersed in the solution to reduce foaming.

3.3.5 Transfer to an ultracentrifuge tube, add 6.5 ml FA lysis buffer / 0.1% SDS and centrifuge 45000rpm, 4°C, for 20 min. Carefully remove the supernatant containing the sheared chromatin and freeze in 800 μl aliquots at –80°C. Also set aside one 100 μl aliquot to be used as an **Input** sample (see *3.4.6*).

3.4 Immunoprecipitation and Decrosslinking

3.4.1 Prepare the appropriate resin:antibody complexes (see **Note 6**). As an example, for immunoprecipitation of a HA-epitope tagged protein, prebind 10 μl of pre-equilibrated Protein-A Sepharose beads (TE pH 8.0) and 5 μl 12CA5 ascites in 100 μl TE pH 8.0 for 30 min at 25°C with gentle mixing. Collect the complexes by centrifugation at 2000 rpm in a microcentrifuge, discard the supernatant and wash the resin with 100 μl of TE pH 8.0. Resuspend the antibody-bound resin in 20 μl TE pH 8.0.

3.4.2 Thaw chromatin aliquots for immunoprecipitation on ice. Add 5M NaCl to a final concentration of 275 mM (20 μl for 800 μl aliquot from *3.3.5*) (see **Note 7**). Add the resin:antibody complex from above and bind overnight with gentle rolling at 4°C.

3.4.3 Collect the beads by centrifugation at 2000 rpm in a microcentrifuge and discard the supernatant. Add 1.4 ml of Wash Buffer 1 and place on a rotator for four min at 25°C. Collect the beads by centrifugation, discard the supernatant and wash sequentially with Wash Buffers 2, 3 and 4. Discard the supernatant following the last wash.

3.4.4 Add 250 μl Elution Buffer to the washed beads and elute at 65°C for 10 minutes. Collect the beads by centrifugation and transfer supernatant to a new microcentrifuge

tube. Wash the beads with 250 µl TE pH 8.0, collect the beads by centrifugation and combine this wash with the previous eluate. This sample is the **IP** (**Figure 2)**.

3.4.5 To reverse the crosslinks add 20 µl of Pronase (20 mg/ml) and incubate for one hr at 42°C followed by 4 hr at 65°C.

3.4.6 To prepare the **Input** sample add 400 µl TE pH 8.0 to 100 µl of chromatin from step *3.3.5*. Add 20 µl of Pronase (20 mg/ml) and incubate for one hr at 42°C and 4 hours at 65°C.

3.4.7 Add 50 µl 4M LiCl per tube and vortex. Sequentially extract with 400 µl PCI and 300 µl chloroform. At each step mix by vortexing, separate phases by centrifugation and collect the upper aqueous layer. To precipitate DNA add 1µl Glycogen (10 mg/ml) and 2.5 volumes of 100% EtOH per tube. Incubate for one hour to overnight at -80°C. Centrifuge at 4°C to collect DNA and wash precipitate with 1 ml 100% EtOH. Remove the liquid, dry pellet at 25°C for 10 min. Resuspend the **IP** in 200 µl TE (pH 8.0) and the **Input** in 400 µl TE (pH 8.0). Store at -20°C. These are stable for at least six months.

3.5 PCR analysis

3.5.1 Each primer pair is generally selected to be 250 - 300bp apart and designed to have similar melting temperatures (≈55°C) (see **Note 1**). As an internal control each reaction also contains another primer pair (the intergenic primer pair in **Figure 1**) that amplifies a ≈180 bp product from a non-transcribed region.

3.5.2 Thaw reaction components on ice and vortex well before use. Each 10 µl PCR reaction contains 4 µl DNA (**IP** or **Input**) from *3.4.7* and 6 µl PCR mix. With these small sample volumes, it is preferable to use a heated lid PCR machine rather than a mineral oil overlay.

3.5.3 Prepare a PCR master mix on ice (5.75 µl per reaction consisting of 0.25 µl 10µM Intergenic primer mix (internal standard), 0.4 µl 2.5mM dNTP mix, 1 µl 10x Platinum Taq buffer, 0.3 µl 50 mM $MgCl_2$, 0.1 µl Platinum Taq (5U/µl) (see **Note 2**), 0.03 µl α-[^{32}P]dATP, 3.67 µl ddH_2O). To each individual reaction add 0.25 µl of the specific

primer pair (10 μm) and 4 μl of the chromatin sample to be tested. Centrifuge briefly in a microcentrifuge to collect the mix at the bottom of the tube.

3.5.4 Each sample is PCR amplified by the following protocol; 1 cycle of 94°C for 90 sec, followed by 26 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec. Reactions are completed by an additional incubation at 72°C for 10 min (see **Note 8**).

3.5.5 When the PCR is complete, add 2 μl 6x Gel Loading buffer to each tube and briefly centrifuge to mix. The PCR products are separated on an 8% vertical polyacrylamide gel with 1x TBE buffer at 180 volts for 3 hr. We generally use 0.8 or 1.5 mm thickness gels, although the thicker gels are easier to handle. PCR products of 180 – 300 bp migrate approximately with the Xylene Cyanol FF dye. The unincorporated NTPs (including the radioactive dATP) will run near the bottom of the gel, and this strip can be cut off to reduce background and contamination. The gel is dried onto Whatman 3MM paper and exposed to phosphorimager plates or autoradiography film.

3.5.6 PCR products can be quantitated using a phosphoimager. To determine whether a specific protein is associated with a particular sequence, the relative abundance of the PCR product from the region in question is compared to a reference (**Figure 3**). The efficiency of amplification and labeling for each primer pair relative to the internal negative control pair (no-ORF control in Figure 3) is calculated from their ratio in the **INPUT** sample. This ratio is termed the Normalization Value (**NV**). The intensity of each specific primer pair band from each IP is then divided by the relevant NV to give the Corrected Value (**CV**). The CV is then divided by the No-ORF signal from each IP to give the Occupancy Value (**OV**). An OV greater than one theoretically indicates the presence of the immunoprecipitated protein at the relevant region or DNA. However, we generally feel most confident when the OV is >2. For a strongly expressed gene, the typical OV seen for TBP is between four to six. Some variability is seen between experiments.

4. Notes

1. We have found that the best signals come from genes transcribed at higher levels (>30 mRNA copies / hour). If the researcher is interested in determining occupancy at a specific yeast locus, a useful resource is provided by the Young lab at the Whitehead institute (http://web.wi.mit.edu/young/expression). This database indicates the transcription frequency of most yeast genes. Some primers that we use to determine occupancy at a number of strongly expressed genes (including the PMA1 gene shown in **Figures 2 and 3**) are listed in **Table 1**.

2. We have found that not all thermostable polymerases work well for ChIPs. Platinum Taq (Invitrogen) is used in our laboratory although other labs successfully use other "hot start" formulations.

3. The appropriate growth conditions will depend on the experiment. Published conditions exist for the analysis of samples following temperature shift to either induce heat shock genes, inactivate temperature sensitive alleles, inducing genes on alternative carbon sources, etc. For rapid temperature shift grow 250 ml of cells in a 1L flask to an OD≈ 0.5 then add an appropriate volume of pre-warmed medium to immediately bring the culture to the desired temperature. Flasks are then incubated at this temperature for the duration of the experiment. For the induction of GAL genes grow cells in media containing 2% Raffinose / 0.5% Glucose to $OD_{\lambda 600} \approx 0.3$. Add Galactose to 2% to induce and incubate for a further two to four hours prior to cross-linking and analysis.

4. The chromatin / debris pellet is resuspended with a pasteur pipette flame-sealed and bent at the end. It is not possible to completely resuspend this material, so the pellet should be disrupted as much as possible.

5. The efficiency of sonication determines the resolution of the technique, so efficiency at this point is extremely important. The sonication conditions will have to be

determined empirically for each sonicator. Average fragment size after sonication can be determined by gel electrophoresis. Samples should be heat treated to reverse cross-links and extracted with phenol / $CHCl_3$ to remove protein prior to gel analysis. To demonstrate efficient chromatin sonication it is useful to examine the chromatin on an agarose gel after reversal of crosslinking. Another useful control is immunoprecipitation with a well-characterized antibody specific for a given region. As an example, we use anti-TBP (TATA binding protein), since TBP is recruited specifically to the promoter. We have designed primers to overlapping regions near the promoter of ADH1 (**Table 1**), moving sequentially 3' into the coding sequence. If anti-TBP appears to crosslink to coding sequence products, removed from the TATA box by >300 bp, it is an indication that the sonication conditions are less than optimal.

6. The resin : antibody complexes used for each immunoprecipitation are obviously dependent on the protein under study. We routinely use Protein A or Protein G Sepharose dependent on the antibody isotype. IgG-Agarose resin works well for Protein A (TAP)-tagged proteins. The amount of antibody in each experiment is determined empirically. For HA-tagged proteins, 5 μl of 12CA5 ascites fluid is used for each immunoprecipitation.

7. The immunoprecipitation and wash conditions are dependent on the antibody used. The conditions described are standard, although we have found that lower affinity interactions, such as those with IgM antibodies, are not always stable to these stringent treatments. In these cases, lower stringency conditions can be used. The NaCl (Section *3.4.2*) can be omitted and wash buffers 1 - 3 can be replaced with 1xFA lysis buffer / 0.1% SDS.

8. For accurate quantitation it is essential that PCR products are analyzed when the amplification is still in the exponential phase. The conditions described are used for single copy genes. For the study of multiple copy loci (such as ribosomal genes) the number of PCR cycles can be reduced accordingly.

Figure Legends

Figure 1. Chromatin Immunoprecipitation schematic

Protein X is localized in the region of the promoter (TATA) during transcription, but not throughout the open reading frame (ORF) or at the 3' UTR (AATAAA). Following formaldehyde crosslinking, the cells are lysed and the chromatin isolated and sheared to smaller fragments by sonication. Protein X remains crosslinked and associated with the promoter region chromatin throughout these manipulations. Protein X is further purified by immunoprecipitation, the crosslinks reversed and the associated chromatin isolated. The specific DNA sequences bound to Protein X can be assayed by PCR with specific primers.

Figure 2. Chromatin Immunoprecipitation protocol

An overview of the steps in the technique are shown. The points at which the protocol can be safely interrupted and samples stored are indicated. The panels at bottom show a representative PCR analysis of an input sample and immunoprecipitation, in this case performed with the promoter-localized TATA-binding protein (TBP). Six specific primer pairs throughout YFG (Your Favorite Gene) are depicted (upper band in each case). Each tube also contains a second primer pair (*) specific to a smaller non-transcribed region of DNA which acts as an internal standard and negative control. The increased intensity of the primer pair 1 band, corresponding to the promoter in the IP panel, indicates specific occupancy of TBP at this location.

Figure 3. Quantitating occupancy by ChIP

After PAGE, PCR products are quantitated by phosphoimager (we use a Fujix BAS 2040 PhosphoImager and the allied Fuji *ImageGuage* software). The experiment depicted utilizes six primer pairs that amplify different regions of the PMA1 gene. A graphical location of each primer is shown in the top panel and the specific sequence of each given in **Table 1**. The Input sample is used to calculate the Normalization Value

(**NV**) between each specific primer pair (numbered 1 – 6) and the control "No-ORF" primer pair (*). This ratio compensates for any variation in PCR efficiency and label content by converting the signal from different primer pairs into normalized units of the control primer pair. This operation generates the Corrected Value (**CV**) for each specific primer pair in each immunoprecipitation as shown. Finally each CV is divided by the No-ORF (*) signal from each immunoprecipitation to give the Occupancy Value (**OV**).

In the experiment shown, three different proteins are localized along the constitutively transcribed PMA1 gene. Immunoprecipitation of TBP and the large RNA polymerase II subunit Rpb1 demonstrate that TBP is localized at the promoter and Rpb1 throughout the gene as expected. We can see that the factor Bur1 is recruited in the region of the promoter and present thoughout the coding sequence, but shows displacement in the region of the 3' UTR (**19)**.

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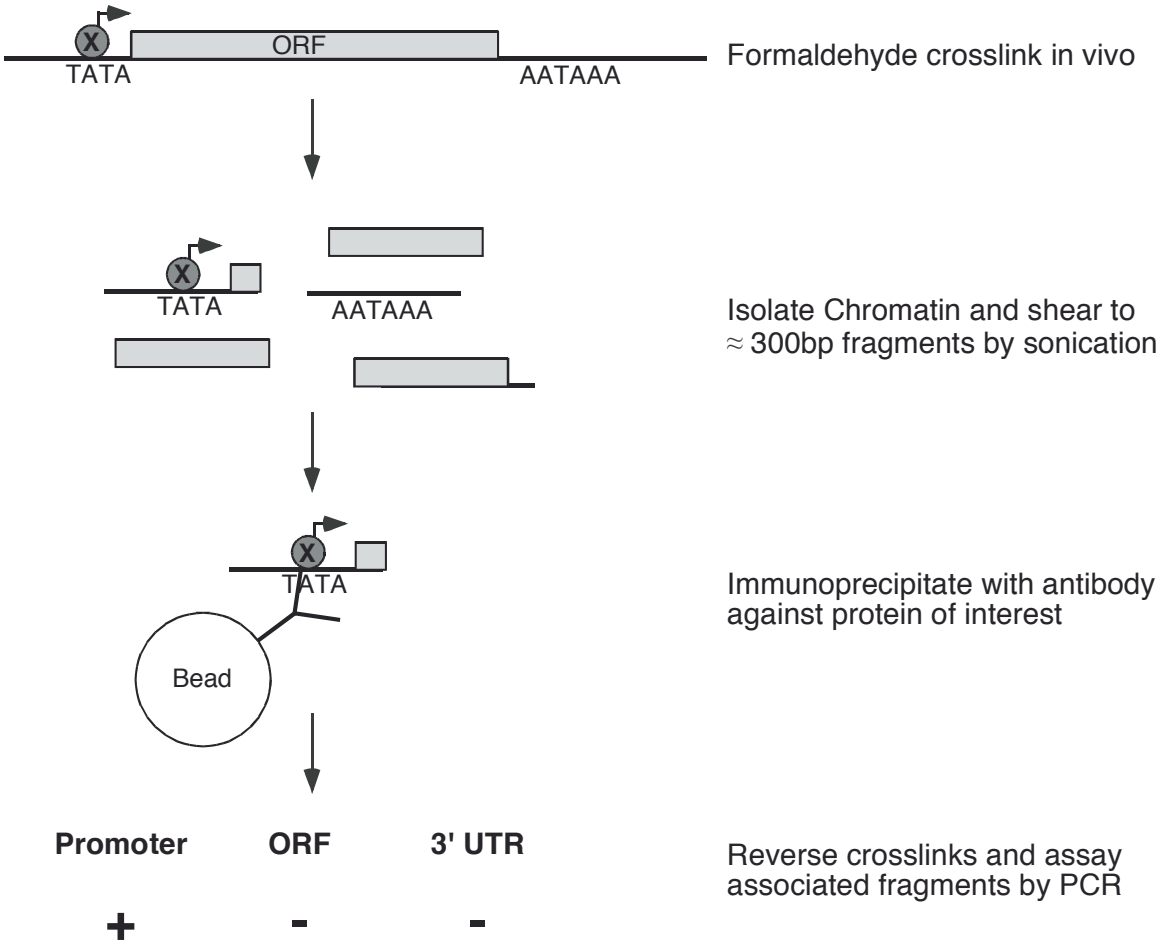
Table 1 Selection of primers used for ChIP on the constitutively expressed PMA1 and ADH1 and the galactose inducible GAL1 gene.

Name / location*	**Oligo Sequence**
ADH1 p, - 235	TTCCTTCCTTCATTCACGCACACT
ADH1 p, -18	GTTGATTGTATGCTTGGTATAGCTTG
ADH1 cds1, +146	ACGCTTGGCACGGTGACTG
ADH1 cds1, +372	ACCGTCGTGGGTGTAACCAGA
ADH1 cds2, +844	TTCAACCAAGTCGTCAAGTCCATCTCTA
ADH1 cds2, +1018	ATTTGACCCTTTTCCATCTTTTCGTAA
ADH1 3'UTR top	ACCGGCATGCCGAGCAAATGCCTG
ADH1 3'UTR bottom	CCCAACTGAAGGCTAGGCTGTGG
PMA1 p, -370	GGTACCGCTTATGCTCCCCTCCAT
PMA1 p, -70	ATTTTTTTTCTTTCTTTTGAATGTGTG
PMA1 cds1, +168	CGACGACGAAGACAGTGATAACG
PMA1 cds1, +376	ATTGAATTGGACCGACGAAAAACATAAC
PMA1 cds2, +1010	GTTTGCCAGCTGTCGTTACCACCAC
PMA1 cds2, +1235	GCAGCCAAACAAGCAGTCAACATCAAG
PMA1 cds3, +2018	CTATTATTGATGCTTTGAAGACCTCCAG
PMA1 cds3, +2290	TGCCCAAAATAATAGACATACCCCATAA
PMA1 cds4, +584	AAGTCGTCCCAGGTGATATTTTGCA
PMA1 cds4, +807	AACGAAAGTGTTGTCACCGGTAGC
PMA1 3'UTR top	GAAAATATTTGGTATCTTTGCAAGATG
PMA1 3'UTR bottom	GTAAATTTGTATACGTTCATGTAAGTG
GAL1 p, -190	GGTAATTAATCAGCGAAGCGATG
GAL1 p, +54	TGCGCTAGAATTGAACTCAGGTAC
GAL1 cds1 +427	CCGGAAAGGTTTGCCAGTGCTC
GAL1 cds1 +726	CGGAGTAGCCTTCAACTGCGGTTTG
GAL1 cds2 +1039	GAAGAGTCTCTCGCCAATAAGAAACAGG
GAL1 cds2 +1331	GAACATTCGTAAAGTTTATCGCAAG
GAL1 3'UTR +1764	CCACAAACTTTAAAACACAGGGAC
GAL1 3' UTR +2079	CCTCCTCGCGCTTGTCTACTAAAATC
Chr V no-ORF up	GGCTGTCAGAATATGGGGCCGTAGTA
Chr V no-ORF down	CACCCCGAAGCTGCTTTCACAATAC

*Primers are used in pairs. Numbers are relative to the first nucleotide of the open reading frame initiation codon. P refers to promoter, CDS to coding sequence, UTR to untranslated region, and ORF to open reading frame.


X
ORF
TATA
AATAAA
Formaldehyde crosslink in vivo
X
TATA
AATAAA
Isolate Chromatin and shear to
≈ 300bp fragments by sonication
X
TATA
Bead
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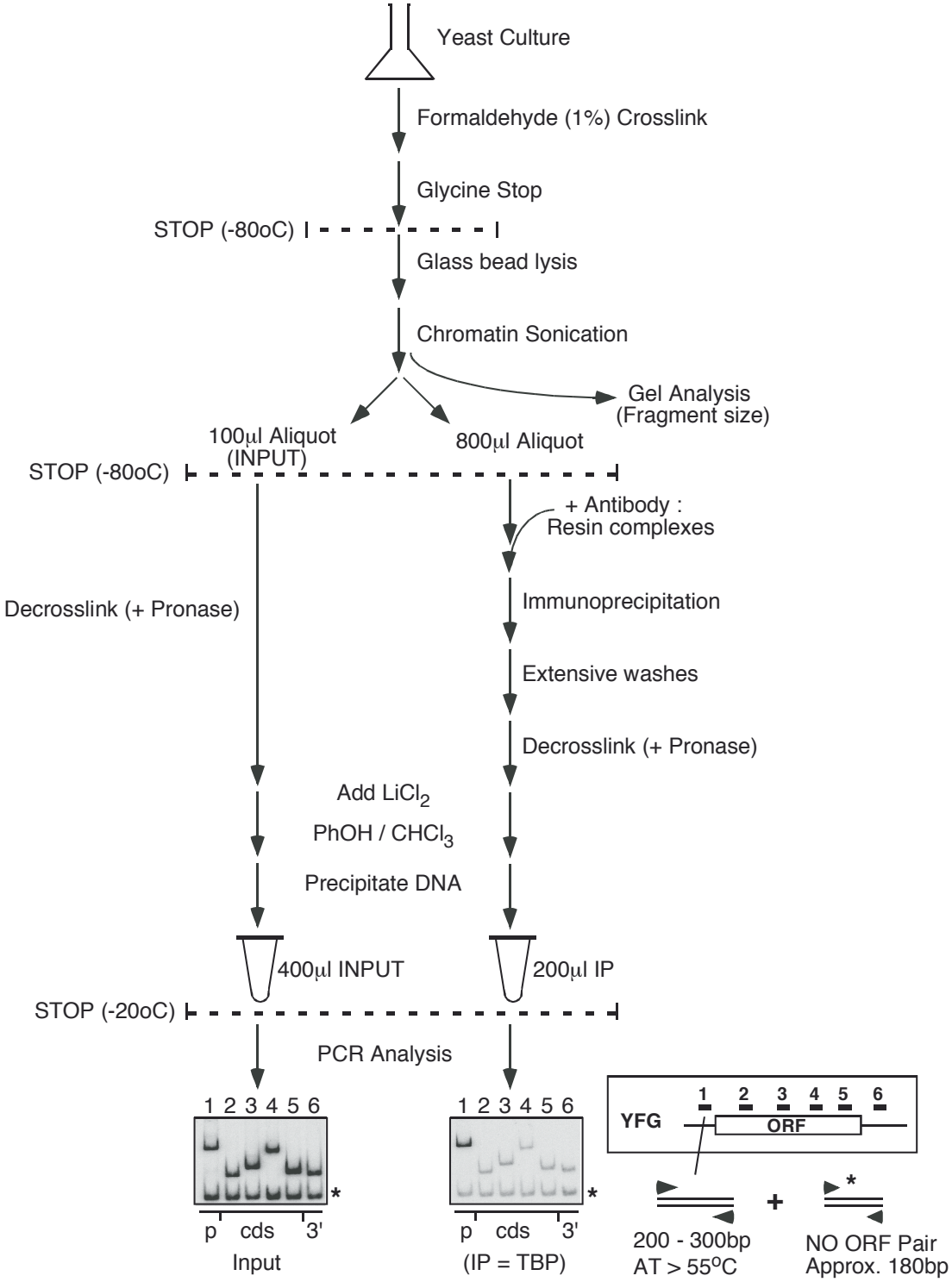
against protein of interest
Promoter
ORF
3' UTR
+
-
-
Reverse crosslinks and assay
associated fragments by PCR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Glycine Stop
STOP (-80oC)
Glass bead lysis
Chromatin Sonication
Gel Analysis
(Fragment size)
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(INPUT)
800µl Aliquot
STOP (-80oC)
+ Antibody :
Resin complexes
Decrosslink (+ Pronase)
Immunoprecipitation
Extensive washes
Decrosslink (+ Pronase)
Add LiCl2
PhOH / CHCl3
Precipitate DNA
400µl INPUT
200µl IP
STOP (-20oC)
PCR Analysis
1 2 3 4 5 6
p cds 3'
Input
1 2 3 4 5 6
p cds 3'
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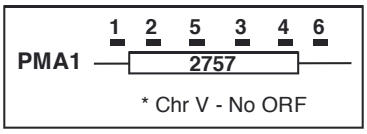
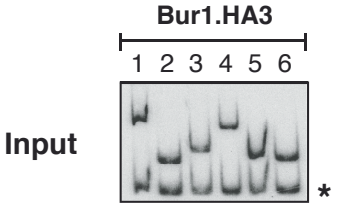
YFG
1 2 3 4 5 6
ORF
200 - 300bp
AT > 55oC
NO ORF Pair
Approx. 180bp



Step 1 - Use Input to derive Normalization Value (NV)



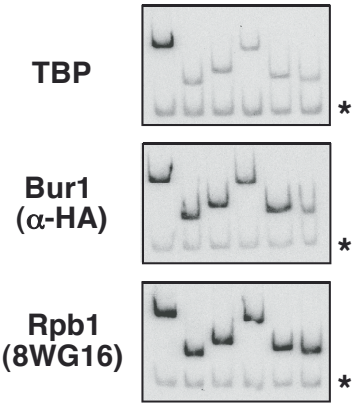
$$\frac{\text{Specific primer pair}}{\text{No-ORF (*) primer pair}} = \text{NV}$$

Specific NV

Primer	Bur1
1	0.84
2	1.03
3	0.99
4	1.26
5	0.71
6	0.84

Step 2 - Use each NV to derive the Corrected Value (CV)

$$\frac{\text{Specific primer pair}}{\text{NV}} = \text{CV}$$



Calculated OV for each IP

Primer	TBP	Bur1	Rpb1
1	4.0	6.5	6.9
2	1.0	5.7	6.0
3	1.0	4.5	5.0
4	1.0	5.0	5.9
5	1.0	4.7	5.5
6	1.0	1.9	5.0

Step 3 - Use CV to determine Occupancy Value (OV)

$$\frac{\text{Each specific CV}}{\text{Specific No-ORF (*)}} = \text{OV}$$