Chapter 16

Chromatin Immunoprecipitation Analysis in Filamentous Fungi

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Abstract

Chromatin immunoprecipitation (ChIP) is used to map the interaction between proteins and DNA at a specific genomic locus in the living cell. The protein–DNA complexes are stabilized already in vivo by reversible crosslinking and the DNA is sheared by sonication or enzymatic digestion into fragments suitable for the subsequent immunoprecipitation step. Antibodies recognizing chromatin-linked proteins, transcription factors, artificial tags, or specific protein modifications are then used to pull down DNA–protein complexes containing the target. After reversal of crosslinks and DNA purification locus-specific quantitative PCR is used to determine the amount of DNA that was associated with the target at a given time point and experimental condition. DNA quantification can be carried out for several genomic regions by multiple qPCRs or at a genome-wide scale by massive parallel sequencing (ChIP-Seq).

Key words: Chromatin, Protein–DNA interactions, Antibody, Quantitative PCR, Filamentous fungi

1. Introduction

Chromatin, the complex of histone and non-histone proteins associated with nuclear DNA, is the natural substrate of all DNA-dependent processes such as replication, recombination, repair or transcription. Experimental approaches to study chromatin dynamics and modifications such as chromatin accessibility or "Chromatin immunoprecipitation" (ChIP) assays have been developed and applied also in filamentous fungi (1–4). The ChIP technique was pioneered by Varshavsky and colleagues (5, 6) and is used to detect and to quantify interactions between DNA and associated proteins in vivo. It aims to determine whether specific proteins such as histones or transcription factors are located at a given genomic region under a given experimental condition. The original method

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is based on analysis of DNA that is reversibly crosslinked to, and is co-precipitating with, protein complexes. In order to stabilize these interactions during the IP step, intact cells are usually treated with formaldehyde in their growth medium. Native ChIP procedures have been developed which omit the crosslinking step, but have so far not been applied in filamentous fungi. Time and concentration of formaldehyde crosslinking depends on the organism and needs to be optimized empirically for each medium and species.

Briefly, chromatin and other DNA-associated proteins (in cell lysates or intact cells) are temporarily crosslinked, then genomic DNA is sheared by sonification or enzymatic restriction to obtain smaller DNA fragments of similar size (usually between 300 and 500 bp). Antibodies recognizing epitopes in the protein of choice (target protein) are then added to the mix to immunoprecipitate DNA–protein complexes. Subsequently, the crosslinks are reversed, protein components are digested and DNA is isolated. Finally, the amount of precipitated DNA is determined by quantitative PCR in comparison to input DNA. The values indicate how much of the antibody target associates with the analyzed DNA locus at a certain time point under a given experimental condition. If many loci should be analyzed in parallel, a library of co-precipitated fragments can be generated and analyzed by sequencer-based fragment analysis (7) or multiple qPCRs. A genome-wide map of binding events can be generated by hybridizing the co-precipitated fragments to microarray probes (ChIP-Chip) (8) or by massive-parallel sequencing of libraries generated from precipitated fragments (ChIP-Seq) (9, 10). Systematic identification of DNA targets on a genome-wide level with extremely high resolution by ChIP-Seq has revolutionized mapping of DNA–protein interactions or the characterization of specific histone modifications associated with metabolic or developmental processes (11, 12). In filamentous fungi, these genome-wide techniques have been applied so far only for *Neurospora crassa* (13, 14).

In addition to determining genomic locations of target protein binding, ChIP analysis also provides the possibility to test for condition-specific protein modifications. Antibodies directed against epitopes such as protein phosporylation, acetylation, methylation, or ubiquitination are commercially available and have been used partially also in filamentous fungi (Table 1). Crucial to all ChiP experiments are controls that reveal the background of DNA co-precipitated by unspecific antibody binding (see below). When specific modifications of a protein are analyzed additional input controls are necessary. These controls include a separate ChIP with an antibody that recognizes a different epitope in the analyzed protein, optimally an epitope that is not subject to posttranslational modifications (PTMs). For example, to test for histone H3 N-terminal modifications such as methylation or phosphorylation, an antibody recognizing the conserved C-terminus of histone H3 is often used (see Table 1).

Table 1
List of antibodies used for ChIP in filamentous fungi

Antibodies against protein tags	Company	Product number	References	Organism	Comment
S-tag antibody	Abcam	ab19321	(16)	*A. nidulans*	ChIP antibody directed against S-tagged KapK. Subsequent amplification of the precipitated DNA at the NirA binding site to examine in vivo interaction of these two proteins involved in nitrate assimilation
HA tag antibody	Abcam	ab9110	(17)	*A. nidulans*	ChIP antibody directed against HA-tagged GATA factor AreA to determine protein binding to promoter sites of genes involved in nitrate assimilation
anti-GFP	Abcam	ab290	(14)	*N. crassa*	Identification of centromeric DNA by ChIP-seq of DNA associated with tagged versions of centromere foundation proteins and one kinetochore protein
Antibodies against histones, histone marks and non-histone proteins	**Company**	**Product number**	**References**	**Organism**	**Comment**
anti-H3K4me2	Abcam	ab32356	(18)	*A. nidulans*	Determination of histone H3K4 dimethylation associated with monodictyphenone and emodin production in background of a *cclA* gene deletion
	Upstate	07-030	(14)	*N. crassa*	Determination of histone H3K4 dimethylation at centromeric loci identified by ChIP-seq
anti-H3K4me3	Abcam	ab1012	(18)	*A. nidulans*	Examination of histone H3K4 trimethylation associated with monodictyphenone and emodin production in background of a *cclA* gene deletion
	Active Motif	AR-0169	(14)	*N. crassa*	Determination of histone H3K4 trimethylation at centromeric loci identified by ChIP-seq

(continued)

Table 1
(continued)

Antibodies against histones, histone marks and non-histone proteins	Company	Product number	References	Organism	Comment
anti-H3K9me3	Abcam	ab8898	(18)	*A. nidulans*	Examination of histone H3K9 trimethylation associated with monodictyphenone and emodin production in background of a *cclA* gene deletion
			(19)	*A. nidulans*	Investigation of the role of H3K9 histone methyltransferase ClrD in the regulation of sterigmatocystin cluster genes
				F. graminearum	Determination of histone H3K9 trimethylation within the aurofusarin and the *Tri5* gene clusters upon deletion of the heterochromatin protein 1 homolog
			(20)		
				N. crassa	Determination of histone H3K9 trimethylation at centromeric loci identified by ChIP-seq
	Active Motif	39161	(14)		
anti-5me-C	Diagenode	MAb-5MECYT-100	(14)	*N. crassa*	Correlation between DNA methylation and heterochromatic marks of centromeric DNA
Anti-histone H3 (acetyl K9)	Abcam	ab4441	(21)	*A. nidulans*	Examination of histone H3 acetylation associated with SAGA-dependent activation of secondary metabolism gene clusters
Anti-acetyl-histone H3 (Lys14)	Upstate	07-353	(4)	*N. crassa*	Quantification of specific histone H3K14 acetylation underlying blue light response in *N. crassa*
Anti-histone H3 (acetyl K14)	Abcam	ab46984	(21)	*A. nidulans*	Examination of histone H3 acetylation associated with SAGA-dependent activation of secondary metabolism gene clusters

Anti-acetyl-histone H3 (binding to acetylated K9 and K14)	Upstate	06-599	(4)	*N. crassa*	Quantification of histone H3 acetylation underlying blue light response in *N. crassa*
			(22)	*A. nidulans*	Investigation of the effect of histone acetylation in the proline assimilation cluster
			(17)	*A. nidulans*	Determination of acetylation activities mediated by the GATA factor AreA involved in nitrate assimilation
			(18)	*A. nidulans*	Examination of histone H3 acetylation associated with monodictyphenone and emodin production in background of a *cclA* gene deletion
			(19)	*A. nidulans*	Determination of acetylation levels of H3 at the promoter site of AflR, the specific activator of secondary metabolite cluster genes, in background of different regulator deletions
			(21)	*A. nidulans*	Examination of histone H3 acetylation associated with SAGA-dependent activation of secondary metabolism gene clusters
			(1)	*N. crassa*	Analysis of histone H3 acetylation state at *frq* locus encoding a factor involved in regulation of clock and light-controlled genes
Anti-hyperacetylated histone H4 (Penta)	Upstate	06-946	(23)	*A. parasiticus*	Correlation between AflR binding to promoters of aflatoxin biosynthesis genes and levels of acetylated histone H4
Anti-acetyl-histone H4	Upstate	06-866			
Anti-tetra-acetylated histone H4 (K5, K8, K12, K16)	Upstate	Not specified in publication	(1)	*N. crassa*	Examination of histone H4 acetylation state at *frq* locus encoding a factor involved in regulation of clock and light-controlled genes
Anti-dimethyl R26 histone H3	Abcam	ab8047	(23)	*A. parasiticus*	Did not detect the predicted size protein in *A. parasiticus* whole-cell protein extracts in Western blot analysis. No longer included in Abcam product range
Histone H4 (symmetric di methyl R3)	Abcam	ab5823	(23)	*A. parasiticus*	Did not detect the predicted size protein in *A. parasiticus* whole-cell protein extracts in Western blot analysis. Correlation between AflR binding to promoters of aflatoxin biosynthesis genes and levels of methylated histone H4
Antibody specific to AflR	Non-commercial		(24, 23)	*A. parasiticus*	Correlation between AflR binding to promoters of aflatoxin biosynthesis genes and levels of acetylated histone H4

(continued)

Table 1
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Antibodies against histones, histone marks and non-histone proteins	Company	Product number	References	Organism	Comment
HP1 antibody	Abcam	ab24726	(19)	*A. nidulans*	Examination of heterochromatin protein 1 homolog occupancy at promoters of different genes involved in sterigmatocystin production
Anti-histone H3	Upstate	06-755	(4)	*N. crassa*	ChIP antibody directed against N terminus of histone H3 to quantify modifications of the histones detected by an additional ChIP antibody relative to histone presence.
anti-Histone H3—C term	Abcam	ab1791	(22) (17) (18) (19) (21) (20)	*A. nidulans* *A. nidulans* *A. nidulans* *A. nidulans* *A. nidulans* *F. graminearum*	ChIP antibody directed against C-terminus of histone H3 to quantify modifications of the histone detected by an additional ChIP antibody relative to histone presence
anti-WC-1 anti-WC-2	Non-commercial		(1) (13)	*N. crassa* *N. crassa*	Antibody directed against transcription factors White Collar-1 (WC-1) and White Collar-2 (WC-2), respectively, to determine binding of the WC proteins to elements within the promoter of *frq*, an additional factor involved in regulation of clock and light-controlled genes WC-1 and WC-2 dimerize to form the White Collar Complex (WCC), a regulator of gene expression in response to light and the circadian clock (25, 26). A genome-wide ChiP-Seq approach was performed to identify WCC targets in downstream regulatory pathways
anti-CSW-1	Non-commercial		(1)	*N. crassa*	Localization of CSW-1, which is required for chromatin remodeling, at promoter sites of the *frq* gene, that encodes a factor involved in regulation of clock and light-controlled genes

In contrast to in vivo footprinting which exclusively reveals direct DNA–protein interactions (15) ChIP detects all proteins interacting with DNA, either through their own DNA-binding domain or in a complex with DNA-binding factors. This indirect interactions need to be taken into account for interpretation of the ChIP results. On the other hand, these indirect interactions also provide the opportunity to test by ChIP protein–protein interactions between a target protein and a DNA-binding factor, when the DNA recognition sequence of the DNA-binding protein is known. This method has, for example, been used to map the interactions between the nuclear export in KapK with its cargo protein NirA, a DNA-binding transcription factor mediating nitrate induction (2).

Crucial for the success of ChIP experiments are the available antibodies. The specificity of these antibodies should be determined prior to ChIP experiments. This step is usually carried out in Western analysis of crude protein extracts of an isogenic mutant strain not expressing the target protein, if such a mutant is viable. When a tag should be used to precipitate the target protein the control strain should be isogenic without expressing the tagged version of the target protein. Control strains for ChIP against PTMs of the target usually carry replacements of amino acids that receive the tested modification, although for many proteins such replacements lead to loss of cell viability. In all these cases, where such mutations are lethal the use of a second-site epitope in the target protein is advisable for a control (e.g., histone H3 C-terminus). After Western analysis of crude protein extracts a preliminary ChIP analysis is carried out to test the antibody for its specificity under ChIP conditions. The experimentators should note that ChIP conditions represent native interactions between the epitope of a protein and the antibody used for IP, whereas Western analysis is usually carried out with cell extracts containing denatured proteins. To clarify if the antibody also specifically interacts with the protein of choice under non-denaturating conditions, a Western analysis of immunoprecipitated protein samples can be performed. If a signal is detected only in IP samples with the specific antibody and not in the control samples (only resin without antibody, mutants strain, strain harboring untagged version of target protein) the specificity of the antibody is satisfying for ChIP.

The presented step-by-step protocol is an optimized procedure for ChIP in filamentous fungi. It can be directly applied to *Aspergillus nidulans* and *Fusarium graminearum*. Moreover, in Table 1 we have summarized all antibodies that have been successfully used so far for ChIP in filamentous fungi. It should be noted that these organisms grow apically in submerged cultures and on solid media and thus their cells have different age and may be in

different metabolic states at the time of harvesting. Aerial cultures on solid media also represent cells of different age and after longer incubation differentiation into conidia or sexual structures occurs. The precise time required for entry into differentiation stages depend on the individual species. For interpretation of ChIP results, these crucial facts need to be considered.

2. Materials

2.1. Reagents Stocks

1. 1 M HEPES–KOH pH 7.5.
2. 5 M NaCl.
3. 1 M Tris-HCl pH 8.
4. 0.5 M EDTA pH 8.
5. 20% SDS.
6. 20% Triton X-100.
7. 4 M LiCl.
8. Ig epal CA630.
9. 1% Na-deoxycholate.
10. 1 M $NaHCO_3$.
11. 100× Fungal protease inhibitor mix (Promega).
12. Salmon sperm DNA/protein A agarose slurry (Upstate).
13. Promega BSA assay.
14. 1 M Tris–HCl pH 6.5.
15. Proteinase K (1 mg/mL, MBI).

2.2. Reagents Setup

1. *Sonication buffer* 50 mM HEPES–KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 1× Fungal protease inhibitor.
2. *ChIP dilution buffer* 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1× fungal protease inhibitor.
3. *Low salt wash buffer* 150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.
4. *High salt wash buffer* 500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.
5. *LiCl salt wash buffer* 0.25 mM LiCl, 0.5% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8, 0.5% Ig epal CA 630.
6. *TE buffer* 1 mM EDTA, 10 mM Tris-HCl pH 8.
7. *Resuspension buffer* SDS 1%, $NaHCO_3$ 0.1 M.

3. Methods

This protocol has been optimized for *A. nidulans* cells from liquid cultures, additional specifications for solid cultures are provided. Changes in the protocol that specifically concern *F. graminearum* and *Fusarium fujikoroi* liquid cultures are indicated by (a). Bold numbering from 1 to 27 indicates the sequential steps of the procedure.

3.1. Chemical Crosslinking of Mycelia

1. Add formaldehyde to a final concentration of 1% to the submerged culture and shake the flaks for 15 min at the same temperature at which growth was performed (the "desired temperature"). For aerial cultures, grow mycelia on cellophane and peel off the cellophane with the mycelia from the solid medium plate and transfer the mycelium together with the cellophane to a Petri-dish containing 20 mL liquid minimal media and 1% of formaldehyde. Slightly shake the mixture for 15 min at the desired temperature. Important: If you need to couple ChIP data with transcriptional data you may reserve part of the mycelia for RNA extraction before formaldehyde treatment (see also Notes 1–3 for general considerations in experimental setup).
2. Add glycin to a final concentration of 125 mM and incubate for 5 min at the desired temperature. Filtrate mycelia from submerged cultures and freeze them immediately in liquid nitrogen. The mycelia from the aerial cultures should have detached by now from the cellophane. Filtrate the mycelia and freeze it immediately in liquid nitrogen.

3.2. Sonication

3. Grind 100 mg of mycelia with mortar and pestle in liquid nitrogen, transfer it to a 2 mL Eppendorf tube and add 1 mL of sonication buffer.
4. As stated above, sonication conditions need to be optimized empirically for each type of equipment and organism. Any sonication equipment may be used, such as sonication probes or water baths with sonication function, just make sure the temperature within the sonicated sample stays low by constantly keeping the samples on ice. The company DIAGENODE designed a cooled sonicator for ChIP and the conditions for sonication of *A. nidulans* and *F. graminearum* cells are provided here as an example. Using the Bioruptor™200 from DIAGENODE the samples are sonicated in cycles of 2 min "on" and 1 min "off" during a total treatment time of 15 min at the maximal power. The samples should be always on ice. The sonication time and power should be determined empirically

when using different sonicators. Keep the samples on ice after the sonication procedure (see also Note 4).

5. Centrifuge for 1 min at 15871 × *g* at 4°C. Recover the supernatants in new Eppendorf tubes and keep them on ice.
6. To pre-clear the samples, add 40 μL Salmon sperm DNA/protein A agarose slurry (Upstate) and turn the tubes on a rotary shaker for 1 h at 4°C. Spin for 30 s at 15871 × *g*. Transfer the supernatant to a new tube.
7. Measure the protein content (e.g., by Bradford assay).
8. Dilute the sample with ChIP dilution buffer to a concentration of 200 μg protein/mL in a final volume of 1.2 mL.

3.3. Antibody Incubation

9. Take 1 mL of the quantified chromatin sample and add 2 μg of antibody. A 1:100 dilution in respect to 200 μg of protein is recommended (see also Note 5).
10. Incubate over night at 4°C on a rotary shaker.
11. Reserve 100 μL of sonicated and quantified chromatin for use as non-precipitated input control and freeze it at −80°C.

3.4. Precipitation of the Protein–Antibody Conjugate

12. Any protein-A conjugate carrier may be used at this step (e.g., protein A-sepharose) but in our experience it is more convenient to use paramagnetic particles as protein A carrier as the separation between pellet and supernatant is more easily achieved with these beads. Add 30 μL of Protein A beads for immunoprecipitation (e.g., Dynabeads®, Invitrogen). The chromatin–Dynabeads complexes are incubated for 1 h at 4°C on a rotary shaker.
13. Place the samples on the magnetic rack and carefully remove and discard the liquid phase. It is important not to disturb the chromatin–beads complex and therefore vacuum pumps should be avoided to remove the supernatant.
14. Add 1 mL of low salt washing buffer, invert the tubes gently and place them again on the magnetic rack. Remove and discard the remaining liquid.
15. Add 1 mL of high salt wash buffer, gently invert the tubes twice and place them again on the magnetic rack. Remove and discard the remaining liquid.
16. Add 1 mL of LiCl wash buffer, gently invert the tubes twice and place them again on the magnetic rack. Remove and discard the remaining liquid. When you work with paramagnetic protein A beads *skip* this last step because the *LiCl washing buffer* interferes with the magnetic function of the beads.
17. Wash the chromatin–Dynabeads complex twice in 1 mL of TE buffer; remove and discard the TE buffer.

3.5. Resuspension of the Chromatin

18. Add 50 µL of fresh Resuspension buffer and incubate at 65°C for 15 min.
    (a) *For Fusarium species* use a tenfold diluted Resuspension buffer (resulting in SDS 0.1%, $NaHCO_3$ 0.01 M)
19. Centrifuge at 15871 × *g* for 2 min. Transfer the *SUPERNATANT* to a new tube. Repeat this step (final volume recovered 100 µL).

3.6. Reverse Crosslinking and Recovery of DNA

20. Add 4 µL NaCl (5 M) and incubate for 4 h at 65°C.
21. Treat 100 µL of the input sample that was reserved at step 11 with 4 µL NaCl (5 M), 4 h at 65°C. These incubation steps (20 and 21) can be extended overnight and afterwards samples can be stored at –20°C.
22. 100 µL samples of the resuspendend chromatin are incubated with 2 µL EDTA (0.5 M), 4 µL 1 M Tris–HCl pH 6.5, and 2 µL Proteinase K (1 mg/mL, MBI) for 1 h at 45°C. This step serves to reverse crosslinking and to digest the associated proteins.
23. DNA is purified from the samples most easily with a PCR purification kit (QIAgen). Other purification procedures may be used after an optimization procedure. Using these kits, the samples are eluted in 100 µL of an 1:10 dilution of EB (QIAgen). The DNA can be stored for up to 2 months at –20°C.
    (a) *For Fusarium species*, the DNA is purified in our hands best by the Cetyltrimethylammonium bromide (CTAB) method instead of PCR purification kit (reason unknown). Start with the addition of 400 µL CTAB buffer (100 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, 2% (w/v) CTAB, 2% (v/v) β-Mercaptoethanol freshly added). Then the samples are incubated by shaking for 1 h at 65°C. 400 µL CI buffer (Chloroform:Isoamylalcohol 24:1) are added and after mixing of the two phases by vigorously inverting the tubes the samples are centrifugated for 10 min at 15871 × *g*. 450 µL of the upper phase are transferred to a new tube and incubated with 20 µg RNAse A for 30 min at 37°C. The DNA is precipitated by the addition of 500 µL Isopropanol and centrifugation for 10 min at 15871 × *g*. The liquid is poured off and the DNA adhering to the tube wall is washed by the addition of 1 mL 75% (v/v) ethanol. After centrifugation for 10 min at 15871 × *g* the liquid is poured off. Remaining drops are spun down and removed by the use of a pipette. The pellets are dried for 10 min at 65°C (with open lids) and the DNA is eluted by the addition of 50 µL of an 1:10 dilution of EB (QIAgen).

The samples are now ready to be analyzed. Quantitative PCR is the most common method to quantify the relative abundance of a target DNA region. This relative abundance generally directly correlates with the abundance of a given antibody epitope (protein or PTM) at the tested genomic location, but exceptions exist to this general rule (see also Note 6).

3.7. Quantification of the co-precipitated DNA

24. Dilute the input control DNA 100-fold in water.
25. Set up real-time PCRs in duplicate with the diluted input DNA. For primer selection, see Note 7.
26. The concentration of specific DNA fragments can vary considerably depending on which protein was immunoprecipitated. Therefore, empirical testing over several dilutions of the final eluates (step 23) may be necessary to find the optimal template concentration for qPCR. For general transcription factors as such as the nitrogen regulator AreA or histone proteins 5 µL of a non-diluted and 1:10 diluted aliquot of the final eluate has proven as initially suitable concentrations for qPCR (unpublished observations). For pathway-specific regulators such as the nitrate activator NirA, where much less targets are present in the genome, only non-diluted samples of the eluates have resulted in successful amplification (unpublished observation). All quantitative PCR protocols can be used for ChIP qPCR and suppliers may also provide specially adapted programs for read-out of ChIP experiments. For the protocol described here, DNA quantification is carried out on a myIQ cycler (Biorad) using Platinum® SYBR® Green qPCR SuperMix-UDG. Processing of ChIP samples for microarray analysis or massive parallel sequencing is described in the literature (8, 10, 11, 12, 13, 14).

4. Notes

1. *Experimental conditions and repetitions.* Two main forms of fungal culture can be used to study different aspects of metabolism and/or development: submerged cultures or aerial cultures. Since ChIP contains many experimental steps and slight variations in each step may lead to significantly different results in experimental repetitions, it is advisable to work with several technical as well as biological repeats. In order to assess the variability among identical experimental setups at least two biologically independent experiments should be performed and both should be analyzed by three technical repeats. However, not too many samples should be processed at the

same time because incubation times with reagents at the different steps should be as equal as possible.

2. *Optimization of the amount of input material.* In order to assure that crosslinking of the mycelia is homogeneous, it is important that the amount of biomass is in the range given below and as similar as possible in all experiments. For the aerial cultures, spores are inoculated on the top of a cellophane disk that is placed onto the medium of choice (minimal medium, complete medium). For the submerged cultures is important to inoculate the same amount of spores in each flask.
3. *Optimization of chemical crosslinking.* In order to capture the DNA–protein interaction in a cellular context, formaldehyde is used as a crosslinking agent. The time of crosslinking for both submerged and aerial cultures needs to be empirically determined when setting up ChIP for the first time for each organism. This step may be problematic, as the extent of crosslinking may vary from sample to sample. It is crucial that all the samples are in contact with formaldehyde for the same amount of time. Longer or shorter periods of individual samples within an experimental series will result in significant variations of ChIP results.
4. *Optimization of sonication efficiency.* Shearing of the chromatin is a critical step in successful implementation of ChIP. Shearing efficiency can be simply tested by agarose gel electrophoresis. It is recommended that the chromatin is sheared to a size range between 300 and 1,000 bp, with the majority of fragments around 500 bp. If the chromatin is not sheared to this extent, the resolution of the ChIP assay will decrease. For example, if primers are designed for a binding site and control site primers are binding within a region of 1,200 bp, inefficient shearing will result in the detection of binding at both loci. If the sonication produces bigger fragments, try to increase the first power of the sonicator and then to increase the time. When submerged cultures and aerial mycelial samples are compared (e.g., ChIP to elucidate binding of sporulation regulators) it needs to be assured that the average fragment sizes of the shared chromatin are in the same range. We have observed that aerial culture samples require a longer sonication time or increased sonication power.
5. *Optimization of the antibody (Ab) amount used for immunoprecipitation.* The crosslinked DNA–protein complex is specifically immunoprecipitated using an Ab against the protein-of-interest or a tag fused to the protein of choice. The quality and specificity of the Ab contributes immensely to the outcome of the ChIP procedure. Prior to the ChIP experiment it is advisable to test the specificity of the Ab by conducting a Western

analysis of immunoprecipitated samples. This step ensures that the Ab also recognizes the epitope under non-denturating conditions. Optimally, this analysis should include controls such as precipitating with the resin only or precipitating in a genetic background in which the target protein is not present or is not tagged.

6. *Accessibility of the antibody to target epitope*. Consider that the accessibility of the Ab to a protein residing within a large protein complex can vary considerably depending on the composition of a potentially dynamic complex at different conditions, in different strains or at different genomic locations. Thus, measuring low amounts of target protein under a certain condition may not always mean that the protein amount is low but that the accessibility if the Ab to its epitope is low. Also, the presence of one protein modification (e.g., histone H3 phosphorylated at serin 10, H3P10) could have an influence on the interaction of the Ab with the neighboring epitope (e.g., trimethylated H3K9). As Ab preparations differ considerably in specificity and titer, the amount of Ab used to immunoprecipitate the crosslinked protein–DNA complexes needs to be empirically determined every time a new batch of Ab is used. All antibodies which were reported in the published literature to be suitable for ChIP in filamentous fungi are summarized in Table 1.
7. *Optimization of oligonucleotides*. Primers for qPCR should be designed in a way that the amplicon is between 50 and 150 bp long. Double-check whether the targeted regions have extensive secondary structures as this will significantly decrease the efficiency of the PCR. In order to increase the specificity of detection it is advisable to choose primer T_m above 60°C.

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References

1. Belden WJ, Loros JJ, Dunlap JC (2007) Execution of the circadian negative feedback loop in Neurospora requires the ATP-dependent chromatin-remodeling enzyme CLOCKSWITCH. Mol Cell 25:587–600
2. Bernreiter A, Ramon A, Fernández-Martínez J, Berger H, Araújo-Bazan L, Espeso EA, Pachlinger R, Gallmetzer A, Anderl I, Scazzocchio C et al (2007) Nuclear export of the transcription factor NirA is a regulatory checkpoint for nitrate induction in *Aspergillus nidulans*. Mol Cell Biol 27:791–802
3. Gonzalez R, Scazzocchio C (1997) A rapid method for chromatin structure analysis in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res 25:3955–3956
4. Grimaldi B, Coiro P, Filetici P, Berge E, Dobosy JR, Freitag M, Selker EU, Ballario P (2006) The *Neurospora crassa* white collar-1 dependent blue light response requires acetylation of histone H3 lysine 14 by NGF-1. Mol Biol Cell 17:4576–4583
5. Solomon MJ, Varshavsky A (1985) Formaldehyde-mediated DNA-protein cross-linking: a probe for *in vivo* chromatin structures. Proc Natl Acad Sci USA 82:6470–6474
6. Solomon MJ, Larsen PL, Varshavsky A (1988) Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. Cell 53:937–947
7. Basheer A, Berger H, Reyes-Dominguez Y, Gorfer M, Strauss J (2009) A library-based method to rapidly analyse chromatin accessibility at multiple genomic regions. Nucleic Acids Res 37:e42
8. Pillai S, Chellappan SP (2009) ChIP on chip assays: genome-wide analysis of transcription factor binding and histone modifications. Methods Mol Biol 523:341–366
9. Collas P, Dahl JA (2008) Chop it, ChIP it, check it: the current status of chromatin immunoprecipitation. Front Biosci 13:929–943
10. Ma W, Wong WH (2011) The analysis of ChIP-Seq data. Methods Enzymol 497:51–73
11. Barski A, Zhao K (2009) Genomic location analysis by ChIP-Seq. J Cell Biochem 107: 11–18
12. Jothi R, Cuddapah S, Barski A, Cui K, Zhao K (2008) Genome-wide identification of in vivo protein-DNA binding sites from ChIP-Seq data. Nucleic Acids Res 36:5221–5231
13. Smith KM, Sancar G, Dekhang R, Sullivan CM, Li S, Tag AG, Sancar C, Bredeweg EL, Priest HD, McCormick RF et al (2010) Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for *Neurospora* white collar complex. Eukaryot Cell 9: 1549–1556
14. Smith KM, Phatale PA, Sullivan CM, Pomraning KR, Freitag M (2011) Heterochromatin is required for normal distribution of *Neurospora crassa* CenH3. Mol Cell Biol 31:2528–2542
15. Wolschek MF, Narendja F, Karlseder J, Kubicek CP, Scazzocchio C, Strauss J (1998) In situ detection of protein-DNA interactions in filamentous fungi by *in vivo* footprinting. Nucleic Acids Res 26:3862–3864
16. Bernreiter A, Ramon A, Fernandez-Martinez J, Berger H, Araujo-Bazan L, Espeso EA, Pachlinger R, Gallmetzer A, Anderl I, Scazzocchio C et al (2007) Nuclear export of the transcription factor NirA is a regulatory checkpoint for nitrate induction in *Aspergillus nidulans*. Mol Cell Biol 27:791–802
17. Berger H, Basheer A, Bock S, Reyes-Dominguez Y, Dalik T, Altmann F, Strauss J (2008) Dissecting individual steps of nitrogen transcription factor cooperation in the *Aspergillus nidulans* nitrate cluster. Mol Microbiol 69:1385–1398
18. Bok JW, Chiang YM, Szewczyk E, Reyes-Dominguez Y, Davidson AD, Sanchez JF, Lo HC, Watanabe K, Strauss J, Oakley BR et al (2009) Chromatin-level regulation of biosynthetic gene clusters. Nat Chem Biol 5:462–464
19. Reyes-Dominguez Y, Bok JW, Berger H, Shwab EK, Basheer A, Gallmetzer A, Scazzocchio C, Keller N, Strauss J (2010) Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. Mol Microbiol 76:1376–1386
20. Reyes-Dominguez Y, Boedi S, Sulyok M, Wiesenberger G, Stoppacher N, Krska R, Strauss J (2011) Heterochromatin influences the secondary metabolite profile in the plant pathogen *Fusarium graminearum*. Fungal Genet Biol 49(1):39–47
21. Nutzmann HW, Reyes-Dominguez Y, Scherlach K, Schroeckh V, Horn F, Gacek A, Schumann J, Hertweck C, Strauss J, Brakhage AA (2011) Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. Proc Natl Acad Sci USA 108: 14282–14287
22. Reyes-Dominguez Y, Narendja F, Berger H, Gallmetzer A, Fernandez-Martin R, Garcia I,

Scazzocchio C, Strauss J (2008) Nucleosome positioning and histone H3 acetylation are independent processes in the *Aspergillus nidulans prnD-prnB* bidirectional promoter. Eukaryot Cell 7:656–663

23. Roze LV, Arthur AE, Hong SY, Chanda A, Linz JE (2007) The initiation and pattern of spread of histone H4 acetylation parallel the order of transcriptional activation of genes in the aflatoxin cluster. Mol Microbiol 66:713–726
24. Roze LV, Miller MJ, Rarick M, Mahanti N, Linz JE (2004) A novel cAMP-response element, CRE1, modulates expression of nor-1 in *Aspergillus parasiticus*. J Biol Chem 279: 27428–27439
25. Ballario P, Vittorioso P, Magrelli A, Talora C, Cabibbo A, Macino G (1996) White collar-1, a central regulator of blue light responses in *Neurospora*, is a zinc finger protein. EMBO J 15:1650–1657
26. Linden H, Macino G (1997) White collar 2, a partner in blue-light signal transduction, controlling expression of light-regulated genes in *Neurospora crassa*. EMBO J 16:98–109