Published in final edited form as:

Mar Biotechnol (NY). 2013 October ; 15(5): 520–525. doi:10.1007/s10126-013-9504-5.

Optimization of a method for chromatin immunoprecipitation assays in the marine invertebrate chordate *Ciona*

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Abstract

Chromatin immunoprecipitation (ChIP) assays allow the efficient characterization of the *in vivo* occupancy of genomic regions by DNA-binding proteins, and thus facilitate the prediction of *cis*-regulatory sequences *in silico* and guide their validation *in vivo*. For these reasons, these assays and their permutations (*e.g.*, ChIP-on-chip, ChIP-Sequencing) are currently being extended to several non-mainstream model organisms, as the availability of specific antibodies increases. Here we describe the development of a polyclonal antibody against the Brachyury protein of the marine invertebrate chordate *Ciona intestinalis* and provide a detailed ChIP protocol that should be easily adaptable to other marine organisms.

Keywords

Ascidian; *Brachyury*; chromatin immunoprecipitation; *Ciona*; *cis*-regulatory module; enhancer; transcription factor

INTRODUCTION

After serving as the model organisms of choice for embryological studies for well over a century (Chabry 1887; Conklin 1905), ascidians have rapidly entered their post-genomic era after the genomes of *Ciona intestinalis* and *Ciona savignyi* have been fully sequenced (Dehal et al. 2002; Vinson et al. 2005). Currently, there is a growing interest in studying the *in vivo* occupancy of genomic sequences that might work as enhancers, or *cis*-regulatory modules (CRMs), by transcription factors, both at the level of individual *Ciona* CRMs (*e.g.*, Kanda et al. 2009; Dunn and Di Gregorio 2009) or on a genome-wide scale (Kubo et al. 2010). Such studies, which mainly rely upon chromatin immunoprecipitation (ChIP), are instrumental for the reconstruction of the gene regulatory networks controlling the development of these simple chordates, and are informative for more complex organisms in this phylum (Delsuc et al. 2006; Davidson and Christiaen 2006; Lemaire 2009). For these reasons, we have optimized in *Ciona intestinalis* the following ChIP protocol, which should provide a useful reference for similar assays in other ascidian species and in related marine embryos. The

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CONFLICT OF INTERESTS

The Authors declare no conflict of interest.

method is used here in combination with a polyclonal antibody that was raised against the *Ciona* Brachyury (Ci-Bra) protein.

MATERIALS AND METHODS

Animal husbandry and embryo culturing

Adult *Ciona intestinalis* (*species A*; Caputi et al., 2007) were purchased from Marine Research and Educational Products (M-REP; Carlsbad, CA) and maintained in a refrigerated aquarium with recirculating artificial sea water at a temperature of 18°C. Detailed protocols describing fertilization, dechoriation and culturing of *Ciona* embryos have been published previously (Zeller et al. 2004; Christiaen et al. 2009). Dechorionated *Ciona intestinalis* embryos were grown in Petri dishes in filtered artificial sea water (FSW) until the desired stage(s) was reached, usually at temperatures ranging from 15°C to 21°C (Whittaker 1977).

Generation of a *Ciona* Brachyury polyclonal antibody

The full-length 1324-bp *Ci-Bra* cDNA (NCBI accession no. NM_001032487; Corbo et al. 1997) was PCR-amplified using as a template RNA extracted from mid-tailbud embryos, as previously described (Oda-Ishii and Di Gregorio 2007), cloned first into the pGEM-T vector (Promega, Madison, WI, USA), and then transferred to the BglII and EcoRI sites of the pRSET-B vector (Invitrogen, Carlsbad, CA, USA) in frame with a Histidine tag. The His-Ci-Bra protein was induced in bacteria at 25°C using 0.5 mM IPTG and purified essentially as previously published (Gazdaru et al. 2005). The purified protein was run on a 10% SDS PAGE gel and stained with Coomassie blue (Bio-Rad, Hercules, CA, USA).

The size of the purified tagged protein was ~53.5 KDa, as predicted (data not shown). Approximately 2 mg were sent to Covance Inc. (Princeton, NJ, USA) for the generation of polyclonal antibodies in rabbits.

In parallel we also generated a GST-Ci-Bra protein by cloning the sequence encoding the C-terminal half of the Ci-Bra protein in the pGEX2T vector. We used this protein to purify the anti-His-Bra antibody from the immune sera by attaching it covalently to agarose-glutathione beads, using the GST Orientation Kit (Thermo Scientific, Rockford, IL, USA). The affinity resin that was reacted with the immune sera was washed twice with 0.5 M KCl-HEG buffer (0.5 M KCl, 25 mM HEPES-KOH pH 7.5, 0.5 mM EDTA pH 8.0, 0.1% NP-40, 10% glycerol), then extensively washed with wash buffer (20 mM Tris-HCl pH 7.5, 1 M NaCl, 1% Triton X-100). The antibody was eluted by adding elution buffer (0.2 M Glycine pH 2.2, 0.5 M NaCl) and the eluate was rapidly neutralized with 1M Tris, pH 8.0. The purified antibody was concentrated by ammonium sulfate precipitation and subsequently dialyzed against 50 mM KCl-HEG overnight, then quantified against BSA standards using a spectrophotometer.

Immunohistochemistry

Dechorionated *C. intestinalis* embryos were fixed at room temperature for 50 min. in 4% paraformaldehyde/PBS, washed twice in PBS for 5 min., then washed for 20 min. in 0.25% Triton X-100, 0.1% Tween-20 in PBS, and washed again once in PBS before being incubated overnight in PBS/1% BSA at 4°C in the presence of the Ci-Bra-specific antibody, as described in Zega et al. (2008). After a series of washes in PBS, the embryos were incubated overnight at 4°C, in the dark and with gentle rocking, with a goat anti-rabbit Alexa Fluor 546 fluorescent secondary antibody in PBS (Invitrogen, Carlsbad, CA, USA). The following day, the embryos were washed 5–6 times in PBS for a total of ~1 hr., mounted with mounting medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA, USA) and photographed using a Leica DMR fluorescent microscope.

Chromatin Immunoprecipitation (ChIP)

This method has been adapted to *Ciona* embryos using previously published protocols as a reference (Lee et al. 2006; Nelson et al. 2006). The ChIP protocol detailed here has been used for embryos at the mid-tailbud stage, grown at 15°C for 15 hrs., and has been employed successfully on both wild-type and transgenic embryos. For reference, a 100-mm diameter Petri dish containing 25 mL FSW holds about 5000 embryos and provides a ~25 μ L pellet, corresponding to roughly 10^7 cells.

1. Cross-linking—Embryos were fixed for 10 min. at room temperature on a rotating platform after adding fresh formaldehyde (Polysciences, Warrington, PA, USA) to a final concentration of 1% to the Petri dishes. After cross-linking was quenched in 2.5 M Glycine for 5 min., embryos were transferred to fresh Petri dishes containing PBS pH 7.4 (Life Technologies, catalog # 10010-023) and washed for 10 min. with gentle rotation, then transferred to 1.5-mL microfuge tubes, collected by brief centrifugation and washed once more in PBS.

Note: At this point, after carefully removing as much PBS as possible, embryos can be stored at -80°C as a pellet, if necessary.

2. Chromatin fragmentation—To fractionate chromatin, approximately 100 μ L of fixed embryos (*i.e.*, ~20,000 embryos, collected from four 100-mm Petri dishes) were sonicated in a final volume of 1 mL in IP buffer (Table 1) containing a protease inhibitor cocktail (Roche Applied Science, #1873580).

Note: As an alternative to the pre-made cocktail, we also used successfully a combination of protease inhibitors containing, per mL: 2.5 μ L of 200 mM PMSF (final concentration: 0.5 mM), 1 μ L of 10 μ g/ μ L leupeptin (final concentration: 10 μ g/mL), 1 μ L of 10 μ g/ μ L aprotinin (final concentration: 10 μ g/mL).

Sonication was carried out on ice in a 1.5-mL microfuge tube, 20 times for 12 sec. each using a Branson 250 sonicator at a power setting of 4 and 30% duty cycle.

Note: Sonication is a key step in this protocol. The optimal conditions required to obtain the desired fragment size may vary depending upon the sonicator employed, and should be determined by monitoring the fragmentation achieved using different conditions (*e.g.*, 14, 16, 18 times, etc.). As an alternative to sonication, the chromatin can be digested with micrococcal nuclease (MNase) (Gaffney et al., 2012).

3. Reverse cross-linking and DNA purification of the input sample—After sonication, 30 μ L of the sonicated sample (whole-cell extract, WCE) were transferred to a 2-mL microfuge tube for reverse cross-linking, to be used as the WCE input sample, while the remainder (970 μ L) was stored at -80°C . The WCE input sample was first mixed with 210 μ L of DNase-free water and 60 μ g of RNase-A (from a 10 mg/mL stock) and incubated at 37°C for 30 min. to remove RNA that might interfere with the visualization of short DNA fragments on agarose gel. The chromatin was then reverse cross-linked by incubating overnight at 65°C in the presence of 30 μ g of proteinase K (from a 10 mg/mL stock), 125 mM NaHCO_3 and 1.25% SDS. After addition of 1.5 mL PB buffer (QIAGEN, Valencia, CA, USA; catalog #27104), the sample was incubated for 1 hr. at room temperature on a nutator, then purified using the QIAprep Spin Miniprep columns (QIAGEN). The WCE input sample was applied to a column, then centrifuged at 13,200 rpm for 1 min. The column was then washed first with 0.6 mL of PB buffer and subsequently with 0.75 mL of PE buffer (QIAGEN), followed by centrifugation as described above. The purified DNA was subsequently eluted by incubating twice with 50 μ L of Elution Buffer (QIAGEN) for 10

min., followed by centrifugation at 13,200 rpm for 1 min. 10 μ L of the purified WCE input were used to check the DNA fragmentation, while the remaining amount was stored at -20°C , to be used later for qPCR. The size of the DNA fragments was assessed by electrophoresis using a 1.5% agarose gel and staining with ethidium bromide to a final concentration of 0.5 $\mu\text{g}/\text{mL}$. If the fragment size was found to range between 200 bp and 800 bp, then the 970- μ L WCE sample(s), previously stored at -80°C , was used for immunoprecipitation.

4. Immunoprecipitation—For the immunoprecipitation, 50 μ L of Dynabeads ProteinA (Invitrogen, Carlsbad, CA, USA) were pre-treated by washing with 950 μ L IP buffer (Table 1), and then by incubating with 950 μ L of IP buffer containing 1 mg BSA and 2 μg sonicated salmon sperm DNA for 3 hrs. at 4°C with rotation.

Note: Salmon sperm DNA or other carrier DNAs should not be used in the blocking solution if ChIP-Seq experiments are planned, in order to avoid contamination in the sequencing steps.

After the 3-hr incubation, the beads were separated from the IP buffer using the DYNAL magnetic rack (Invitrogen), according to the manufacturer's instructions, then quickly washed twice with fresh IP buffer at 4°C .

The 970- μ L WCE samples were thawed on ice, then centrifuged at 13,200 rpm for 15 min. at 4°C and the supernatant was transferred to fresh microfuge tubes. For preclearing non-specific background, the samples were incubated with 50 μ L of the pre-treated beads and 0.5 μg normal rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 6 hrs. at 4°C on a nutator.

Note: After the first 3 hrs. of incubation, begin pre-treatment of two additional 50- μ L aliquots of beads in 1.5-mL microfuge tubes, as described above.

After preclearing, the WCE samples were separated from the beads using the magnetic rack and the beads were discarded. Precleared WCE samples were then divided equally into the two tubes containing the 50- μ L aliquots of pre-treated beads, and incubated overnight at 4°C on a nutator with 0.25 μg of normal rabbit IgG and the specific antibody of choice, respectively.

Note: The beads should not be allowed to dry out; for this reason, we recommend removing the IP buffer just before adding the precleared WCE samples to the beads.

We have used this protocol with the His-Ci-Bra antibody on wild-type embryos (Fig. 1) and with GFP antibodies on transgenic embryos carrying the *Ci-Bra>Ci-Bra::GFP* transgene (data not shown).

Note: to minimize background signal, we recommend purifying the antibody (e.g., as described above).

Dynabeads bound to the immunoprecipitated DNA were then washed 5 times for 10 min. on a nutator at 4°C with 1.5 mL of ice-cold RIPA buffer (Table 1), followed by two 2-min. washes with 1.5 mL of ice-cold TE buffer (Table 1). After transferring the tubes to the magnetic rack the supernatant was carefully aspirated and the tubes were removed from the rack. Subsequently, the beads were incubated in 300 μ L of TE buffer containing 60 μg of RNase-A at 37°C for 30 min., then washed again with 1.5 mL of TE buffer.

Note: at this point most of the supernatant should be carefully removed.

5. Reverse cross-linking and DNA purification of the immunoprecipitated sample

The immunoprecipitated DNA was eluted by incubating twice for 45 min. with 150 μ L of freshly made bicarbonate/SDS buffer (Table 1), at room temperature. The eluted sample (300 μ L) was reverse cross-linked by addition of 30 μ g of Proteinase K and overnight incubation at 65°C, purified as described above, then subjected to PCR and/or qPCR in parallel with the WCE input sample.

Note: Proteinase K incubation can be shortened to 2 hrs.

RESULTS AND DISCUSSION

Ci-Bra encodes a sequence-specific transcription factor that in *Ciona* and other ascidians, such as *Halocynthia*, is specifically expressed in the notochord (Corbo et al. 1997; Yasuo and Satoh 1993). Brachyury plays a pivotal role in notochord formation in all chordates analyzed so far, and is evolutionarily conserved not only across the chordate phylum, but also among other deuterostomes and in protostomes, such as the fruit fly *Drosophila*, where the *Brachyury* ortholog, *Brachyenteron*, is expressed in the hindgut and is required for the morphogenesis of this structure (Singer et al. 1996; Swalla 2006). In addition to being a major developmental regulator, Brachyury is also a specific marker and a causative agent of chordoma, a notochord-derived tumor (Vujovic et al. 2006; Yang et al. 2009). For these reasons, the identification of genes targeted by this transcription factor is of particular interest.

We first tried to raise polyclonal antibodies directed against a short synthetic peptide custom-designed from the Ci-Bra protein sequence, however this approach was unsuccessful because the resulting antiserum produced mostly background signal in preliminary tests (data not shown). We then tried the bacterial expression, recovery and purification of full-length and truncated versions of Ci-Bra, and the most satisfactory results were obtained with the full-length His-tagged Ci-Bra.

Fig. 1 shows both the results of an immunofluorescence experiment and the detection of ChIP results. The antibody raised against the full-length His-tagged Ci-Bra protein was first tested by immunohistochemistry (Fig. 1A–C) and by Western blot (data not shown). The same antibody was then affinity-purified (see Methods) and used for ChIP assays with primers specifically designed to amplify a 134-bp fragment of the *Ci-tune* notochord CRM. The *Ci-tune* notochord CRM is part of a *Ciona* genomic region that contains two Ci-Bra binding sites, which we have previously shown to be bound by Ci-Bra *via* electrophoretic mobility shift assays (Passamaneck et al. 2009). Thus, the *Ci-tune* notochord CRM provided us with a reliable positive control. We obtained similar results when the *Ci-tropomyosin-like* notochord CRM, another direct Ci-Bra target (Di Gregorio and Levine 1999) was used as a positive control (data not shown). The ChIP results were first visualized by agarose gel electrophoresis followed by ethidium bromide staining (Fig. 1D). Subsequently, qPCR experiments (Fig. 1E) allowed us to obtain an accurate quantification of the results.

CONCLUSIONS

The straightforward protocol provided here and the availability of an ascidian Brachyury antibody pave the way for studies of the genes regulated by this transcription factor in *Ciona*, and possibly in other ascidian species, and should aid the application of ChIP assays in other marine organisms. In *Ciona*, numerous early target genes of Ci-Bra have been identified through genome-wide ChIP-chip analyses by employing anti-GFP antibodies on embryos expressing a Ci-Bra-GFP fusion (Kubo et al. 2010). This approach has been very successful in early *Ciona* embryos, and has yielded numerous target genes not only for Ci-

Bra, but also for several other transcription factors (Kubo et al. 2010). The availability of a specific Ci-Bra antibody now allows the identification of notochord genes controlled by this factor at later developmental stages. In addition, the combination of this specific antibody and of the step-by-step protocol described here facilitates the validation of Ci-Bra occupancy at the level of individual genomic loci of interest, as well as the prediction of late-acting, Ci-Bra-dependent notochord *cis*-regulatory elements.

Acknowledgments

We are indebted to Prof. Fiorenza De Bernardi (Univ. of Milan, Italy) for the immunofluorescence protocol. This work was supported by grant NIH/NIGMS GM100466 along with supplemental funding from the American Recovery and Reinvestment Act award R01HD050704-05S1 to ADG, and by grants from the American Cancer Society (RSG-08-042-01-DDC) and the Charles A. Frueauff Foundation to YN. HA was supported in part by a postdoctoral fellowship from the Japan Society for the Promotion of Science (JSPS).

Abbreviations

bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CRM	<i>cis</i> -regulatory module
DAPI	4',6-diamidino-2-phenylindole
hr(s)	hour(s)
min	minute(s)
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
qPCR	quantitative PCR
sec	second(s)
wt	wild-type

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Figure 1. Use of the *Ciona* Brachyury antibody for immunofluorescence and ChIP assays

(A–C). *Ciona intestinalis* mid-tailbud embryo immunostained with the Ci-Bra antibody. (A). Counterstaining with DAPI visualizes nuclei. (B, C). The Ci-Bra antibody specifically stains the nuclei of the 40 notochord cells. (D, E). Results of ChIP assays on the *Ci-tune* notochord CRM, as detected by PCR (D) and qPCR (E). In (D), lanes were loaded as follows: Lane 1: DNA ladder (1 Kb Plus DNA Ladder, Invitrogen, cat #10787-018). Lane 2: PCR product (134 bp) obtained from chromatin immunoprecipitated with 50 μ g of Ci-Bra antibody (red arrow).

Primers: *Ci-tune* Forward: 5'-GTGTTGCGTACACACTCAAAGTCAG-3'

Ci-tune Reverse: 5'-GCAGGGCAGTTCTGATAAACACGTTGT-3

Lane 3: negative control: PCR product of *Ciona* chromatin immunoprecipitated with 50 μ g of pre-immune serum. Lane 4: 134-bp PCR product obtained by amplifying 10 μ L of a 50% dilution (~200 ng) of the WCE input sample (red arrow).

In (E), ChIP was performed on two biological replicates and qPCR was performed on 10 ng of each sample, in triplicate, using SYBR green in a Prism 7700 Real-Time qPCR thermocycler (ABI, Carlsbad, CA, USA). To obtain standard curves, we used samples containing 20, 2, 0.2 and 0.02 ng/ μ L of *Ciona* genomic DNA, each in duplicate. The % input and standard deviation were calculated from the average of triplicate immunoprecipitated/input WCE scores. One representative biological dataset is shown in the figure. The *p*-value was calculated using a two-tailed Student's *t* test.

TABLE 1

ChIP buffers composition.

IP buffer:
150 mM NaCl
50 mM Tris pH 7.5
5 mM EDTA
0.5% NP-40
1% Triton X-100
RIPA buffer:
50 mM HEPES pH 7.5
0.5 M LiCl
1 mM EDTA
1% NP-40
0.5% sodium deoxycholic acid
Bicarbonate/SDS buffer:
0.1 M NaHCO ₃
1% SDS
TE Buffer:
10 mM Tris-Cl, pH8.0
1 mM EDTA
Tris/Acetic acid/EDTA (TAE) Buffer (1X):
40 mM Tris
20 mM acetic acid
1 mM EDTA