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Fine Mapping of Genomic Targets of Nuclear Proteins in Cultured Cells

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ABSTRACT

The aim of this technique is to analyze interactions of proteins or protein complexes with chromosomal DNA.

The following protocol has been established for the cultured cell line Schneider 2 (S2) from Drosophila melanogaster. If other tissue is used, certain steps of the protocol may need to be optimized, although the basic rationale would be the same. The main difference is likely to be in the cross-linking step. Cells growing in suspension can be easily cross-linked by adding a concentrated stock of the cross-linking buffer directly to the growth medium (see below). The same procedure is used for yeast (Strahl-Bohlsinger et al. 1997). Adherent mammalian cells are also cross-linked by adding a concentrated stock of the cross-linking buffer to the medium. After cross-linking and quenching with glycine, the cells are scraped and washed off the culture dishes with PBS and pooled. Then cells are lysed according to the protocol below (see Fig. 2). When using more compact material, such as embryos or imaginal discs, cross-linking conditions are more vigorous and might include treatments with detergents or polar solvents, which allow the formaldehyde to better penetrate the sample. For additional protocols, see Cao et al. (2002; imaginal discs from D. melanogaster), Orlando et al. (1998; embryos from D. melanogaster), and Chua et al. (2004; tobacco shoots). For further specialized protocols, see also the Web sites listed after the reference section.

MATERIALS

Buffers, Solutions, and Reagents

Glycine (powder)

PBS (phosphate-buffered saline [pH 7.4]) (stable at room temperature)

Fixation solution (prepare fresh before use)
11% formaldehyde (from a 37% stock equilibrated with methanol)
100 mM NaCl
1 mM EDTA
0.5 mM EGTA
50 mM HEPES (pH 8)
100 mM PMSF (phenylmethylsulfonyl fluoride) (in isopropanol; stable at room temperature)

Cell lysis buffer (stable at room temperature; add PMSF and proteinase inhibitors and put on ice before use)
5 mM PIPES (pH 8)
85 mM KCl
0.5% NP-40
1 mM PMSF proteinase inhibitors (leupeptin, aprotinin, pepstatin; each final concentration 2 μg/ml)

Nuclear lysis buffer (stable at room temperature; add PMSF and proteinase inhibitors and put on ice before use)
50 mM Tris-HCl (pH 8)
10 mM EDTA
0.8 % SDS (sodium dodecyl sulfate)

1 mM PMSF protease inhibitors (leupeptin, aprotinin, pepstatin; each final concentration 2 μg/ml)

Protein A/G agarose beads

Antibody

Dilution buffer (stable at room temperature; add PMSF and proteinase inhibitors and put on ice before use)
10 mM Tris-HCl (pH 8.0)
0.5 mM EGTA
1% Triton X-100
140 mM NaCl
1 mM PMSF
protease inhibitors (leupeptin, aprotinin, pepstatin; each final concentration 2 μg/ml)

RIPA buffer (stable at room temperature; add PMSF before use)
10 mM Tris-HCl (pH 8.0)
1 mM EDTA
0.5 mM EGTA
1% Triton X-100
0.1% sodium deoxycholate
0.1% SDS
140 mM NaCl
1 mM PMSF

LiCl buffer (stable at room temperature)
0.25 M LiCl

0.5% NP-40

0.5% sodium deoxycholate

1 mM EDTA

10 mM Tris-HCl (pH 8.0)

TE (stable at room temperature)

1 mM EDTA

10 mM Tris-HCl (pH 8.0)

Protein A/G agarose beads (50%), pre-swollen and blocked

SDS, 10%

[α-32P]dCTP (specific activity 3000 Ci/mmole)

Proteinase K (stock 20 mg/ml, store at -20°C)

RNase, DNase-free (stock 10 mg/ml, store at -20°C)

Phenol/chloroform/isoamyl alcohol (25:24:1, store at 4°C)

Chloroform/isoamyl alcohol (24:1, prepare fresh before use)

Sodium acetate, 3 M (pH 5.2)

Glycogen (5 mg/ml, store at -20°C)

Ethanol, 100% and 70%

Gel loading solution (in water; store at 4°C)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol

Ligase buffer

12.5 mM MgCl2

25 mM dithiothreitol (DTT)

1.25 mM ATP

50 mM Tris-HCl (pH 7.6)

Hybridization buffer

7% SDS

1 mM EDTA

1% bovine serum albumin (BSA)

0.5 M NaHPO4 (pH 7.2), this is 0.25 M Na2HPO4 with the pH adjusted to 7.2 with ortho-phosphoric acid (see Orlando et al. 1997)

Wash buffer

5% SDS 1 mM EDTA 0.5% BSA 40 mM NaHPO4 (pH 7.2)

Cells

Chosen tissue or cell line; in this case, Drosophila Schneider SL2 tissue culture cells, with appropriate growth facilities and media

Special Equipment

Sonicator

Hybridization oven, preset to 65°C

Heating blocks, preset to 50°C, 65°C

Glass beads (150-200 μm, acid-washed)

Falcon tubes, 15-ml and 50-ml

Shaker, at 4°C

Rotator, at 4°C

Oven, preset to 80°Cv Centrifuge, precooled to 4°C

Kits and Computer Software

Random-primed DNA synthesis kit

PCR product purification kit

DNA quantification software

Additional Reagents

This protocol requires equipment and reagents for restriction enzyme digestion (HindIII), agarose gel electrophoresis (including ethidium bromide and UV transilluminator), and PCR.

METHOD

Chromatin Preparation

Grow 100 ml of Drosophila Schneider SL2 tissue culture cells in an appropriate medium (e.g., Schneider's Drosophila medium supplemented with 12.5% fetal bovine serum or in serum-free insect culture medium), in cell culture bottles, to a density of 3 x 106 to 6 x 106 per ml.

Add the fixation solution (1/10th of volume of cells; e.g., 11 ml into 100 ml of medium-the final formaldehyde concentration should be 1%) directly to the flask and mix. Incubate fixation reaction for 10 min at 4°C on a shaker.

Stop the fixation by adding glycine powder to a final concentration of 125 mM. Mix well. Transfer cells to a 50-ml Falcon tube and collect by centrifuging at 800g for 5 min at 4°C. Wash the cells once with ice-cold PBS.

Resuspend the cell pellet in 15 ml of ice-cold cell lysis buffer, pipetting up and down until all the cells have been resuspended. Stand them on ice for 10 min. Collect the nuclei by centrifuging at 2000g for 5 min at 4°C. Carefully discard the supernatant and resuspend the pellet in 2 ml of ice-cold nuclear lysis buffer by pipetting up and down. Transfer the suspension to a 15-ml Falcon tube that has been cut down to the 10-ml mark to allow the sonicator tip to reach the suspension. Leave on ice for 10 min.

Add ~0.5 ml of glass beads to the cell suspension. Store on ice or sonicate immediately.

Sonicate the sample with six 30-sec pulses (output near microtip limit), using a high-power sonicator (e.g., Sanyo Soniprep 150, exponential microprobe, 10 amplitude microns). Keep the tube cool by holding it in a beaker containing an ice/water mix.

The sonicator tip should be immersed roughly 1/4 into the liquid. Avoid foaming. If foaming occurs, centrifuge the tube briefly to reduce the foam layer. Leave on ice for some minutes and sonicate again. For initial trial experiments, take an aliquot from the chromatin suspension after each sonication pulse (e.g., 10 µl). Increase the sample volume to 100 µl with TE and process as described in step 8.

Transfer the sonicated suspension to two 15-ml Falcon tubes (leaving most of the glass beads behind) and centrifuge for 10 min at 12,000-14,000g at 4°C. Dilute the supernatant with dilution buffer to a final volume of 8 ml (i.e., 4 times dilution). Rotate the tubes on a wheel for 10 min at 4°C. Take a 50-µl aliquot to check the average size of the DNA fragments (steps 8, 9, and 10). From the remaining sample, prepare 600-ml aliquots and store at -80°C, or use the chromatin directly for immunoprecipitation.

To the 50-µl aliquot taken in step 7, add 50 µl of TE. Incubate overnight at 65°C (if not using safelock-tubes, seal tubes with Parafilm). Add proteinase K to 500 mg/ml and SDS to 0.5% (w/v). Incubate at 50°C for 3 hr. Centrifuge briefly.

Add one volume of phenol-chloroform-isoamyl alcohol, vortex for 2 min, and centrifuge at 12,000-14,000g for 8 min. Transfer the aqueous supernatant to a new

tube. Add one volume of chloroform-isoamyl alcohol, vortex for 2 min, and centrifuge at 12,000-14,000g for 8 min. To the second aqueous supernatant, add 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol and mix well. Leave at -20°C for at least 30 min. Centrifuge at 12,000-14,000g for 15 min at 4°C. Carefully discard the supernatant and wash the pellet in 800 µl of 70% ethanol. Centrifuge again and allow the pellet to air-dry for 5-10 min. Dissolve the pellet in 10 µl of TE.

Add to each sample 0.5 µg of DNase-free RNase, and incubate for 30 min at 37°C. Add 3 µl of gel loading solution. Run the sample on a 0.8% agarose gel (~15-20 cm long for best separation). When the bromophenol blue dye has migrated along 2/3 of the gel, stain gel with 0.5 µg/ml ethidium bromide and view on a UV transilluminator. If the average length of the DNA is not short enough (there should be a smear of the molecular weight of ~300-1000 bp), the stored aliquots can be resonicated (step 6).

Immunoprecipitation and Reversal of Cross-links

For each immunoprecipitation (IP), the mock control and the input control, take 300 µl of chromatin (obtained in step 7) and add an equal volume of RIPA buffer. Add 20 µl of protein A/G agarose beads using a cutoff (wide aperture) pipette tip. Incubate for 1-2 hr at 4°C for preclearing, and centrifuge in a microfuge at 13,000 rpm for 10 min at 4°C.

Transfer the resulting supernatant to a new tube and add the appropriate amount of antibody (usually 1 µg of an affinity-purified antibody; dilutions of 1:100 to 1:500). Use the same amount of precleared chromatin in the controls, without the addition of antibody (for mock and input control), or with preimmune serum or an appropriate nonspecific antibody. For details and discussions on antibody production, affinity purification, types of antibodies (polyclonals, monoclonals), and subclasses of

immunoglobulins, see Harlow and Lane (1988). Incubate the samples from 2-3 hr to overnight at 4°C on a rotator.

Centrifuge the samples in a microcentrifuge at 13,000g for 10 min at 4°C. Transfer the IPs to new tubes. Add 20 µl of the 50% protein A/G agarose bead solution and incubate for a further 2-4 hr. Pellet the beads with a short centrifugation (20 sec at maximum speed) in a benchtop centrifuge. Transfer the supernatant of the no-antibody control to a new tube and leave on ice. This material will serve as total input control. Discard the other supernatants. Wash the beads five times with 600 µl of RIPA buffer, once with 600 µl of LiCl buffer, and once with 600 µl of TE (pH 8.0), collecting the beads between washes with brief centrifugations. Finally, resuspend the beads in 100 µl of TE.

Add 1 mg of DNase-free RNase (also to the input control) and incubate samples overnight at 65°C. The next day, adjust samples to 0.5% SDS and 0.5 mg/ml proteinase K and incubate for a further 3 hr at 50°C. Phenol-chloroform-extract the samples as described in step 9. Back-extract the phenol phase by adding an equal volume of TE (pH 8.0) and vortex. Combine the aqueous phases and perform one more chloroform extraction. Precipitate the DNA by adding glycogen to 100 µg/ml as carrier, 1/10 volume of 3 M sodium acetate (pH 5.2), and 2.5 volumes of 100% ethanol. Incubate at -20°C for 2 hr to overnight. Collect the DNA by centrifuging at 12,000-14,000g for 15 min at 4°C, and wash the pellet in 800 µl of 70% ethanol. Repeat centrifugation and discard the supernatant. Allow the pellet to air-dry for 5-10 min. Redissolve the precipitated DNA in 30 µl of TE (PCR analysis) or 9 µl of water (Southern analysis) and store at 4°C (to avoid DNA precipitation, do not freeze).

PCR Analysis

15. Perform the test, negative control, and the input-control (dilutions of 1/10, 1/100,

and 1/1000 of the input) PCRs in 25-µl volumes, using the optimum magnesium concentration for each primer pair. Start by using 1 ml of the immunoprecipitated DNA as a template (in 1x reaction buffer, 0.25 µM NTPs, 1 mM primer, 0.5 units of Taq polymerase).

Number of Cycles Denaturation Annealing Polymerization

1 94°C for 2 min

35 94°C for 1 min 60-65°C for 1 min 72°C for 1 min

1 72°C for 6 min

Adjust the annealing temperature and number of cycles for each primer pair until no signal is detected for the negative control-IP DNA. Signals obtained from the test reactions under these conditions can be considered significant.

After the amplification, add 6 µl of gel loading solution to each PCR, load half of the reaction onto a 1.5% agarose gel, and visualize amplified DNA with ethidium bromide. To increase signal intensities, the amount of template for the PCR (of all the samples, including the negative controls) can be increased.

Quantify the resulting bands (e.g., with the QuantityOne software by Bio-Rad) and plot them as percentage of the input (the total of chromatin-DNA used for one IP, from step 13).

Southern Analysis

Prepare the oligonucleotide linker by annealing two oligonucleotides, a 24-mer of sequence 5′AGAAGCTTGAATTCGAGCAGTCAG-3′, and a 20-mer of sequence 5′CTGCTCGAA TTCAAGCTTCT (when ordering the synthetic oligonucleotides,

take care that only the 24-mer is phosphorylated at the 5′ end). Mix equimolar amounts of these oligonucleotides in TE (e.g., 10 µl of a 100 µM stock of both oligonucleotides in 100 µl of TE), boil for 5 min, and allow the reaction to cool slowly to room temperature.

Resuspend the immunopurified-chromatin DNA in 9 µl of ligase buffer containing the linker adapter at a final concentration of 0.8 µM. Add 4 units of T4 DNA ligase (Roche) and incubate at 4°C for 24 hr.

Use the ligated mixture directly as a template in a 100-ml PCR using 1 unit of Taq polymerase, 1x corresponding buffer, and 2 mM Mg2+. The primer used is the 20-mer oligonucleotide described above, added to a final concentration of 1 mM. Amplification is performed as follows.

Number of Cycles Denaturation Annealing Polymerization
1 94°C for 2 min
35 94°C for 1 min 55°C for 1 min 72°C for 1 min
1 72°C for 6 min

Extract the samples once with phenol-chloroform-isoamyl alcohol and once with chloroform-isoamyl alcohol; ethanol-precipitate as described in step 9. Remove linkers by digesting the DNA with HindIII and separate them from the amplified DNA by gel filtration (e.g., with the QiaQuick PCR purification kit, QIAGEN). An aliquot with linkers may be stored at -20°C as reservoir.

Label the amplified DNA with [α-32P]dCTP (specific activity 3000 Ci/mmole) using a random-primed DNA synthesis kit according to the instructions of the manufacturer.

Separate the digested target DNA (e.g., bacterial clones, lambda clones) on an agarose gel and transfer the DNA to nylon membranes using standard techniques (Sambrook and Russell 2001). Bake the membrane at 80°C for 2 hr. Hybridization is best performed in glass bottles and a hybridization oven. Prehybridize the membrane for 3 hr at 65°C in 10 ml of hybridization buffer. Add the heat-denatured probe, from the previous step, directly to the hybridization solution, and incubate the filter overnight at 65°C.

Wash filters once with wash buffer for 10 min at 65°C, and at least four times, for 5 min each, at 65°C in the same buffer, but containing 1% SDS. After washing, seal the filters into plastic bags and expose to X-ray film or, for a more sensitive and quantitative analysis of the hybridization signals, analyze using a phosphorimager.

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Strahl-Bolsinger S., Hecht A., Luo K., and Grunstein M. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. 11: 83-93.

WWW RESOURCES

http://inside.wi.mit.edu/young/pub/locationanalysis.html

Genome-wide location analysis, Young Lab, Whitehead Institute

http://genomics.ucdavis.edu/farnham/protocol.html

Protocols (ChIP-CpG island microarray binding analysis; chromatin immunoprecipitation assay (ChIPs); ChIPs in tissues; ChIPs cloning protocol; preparation of total RNA from tissue; preparation of target cRNA for Affymetrix GeneChip analysis)

http://www.fhcrc.org/labs/hahn/methods/mol_bio_meth/hahnlab_ChIP_method.html

Yeast chromatin immunoprecipitation (ChIP), Hahn Lab, Fred Hutchinson Cancer Research Center

http://www.igh.cnrs.fr/equip/cavalli/link.labgoodies.html

Protocols, Cavalli Lab, Institut de Génetique Humaine

http://www.protocol-online.org/cgi-bin/prot/search.cgi?query=ChIP&submit2=Search

Protocol Online, ChIP