Protocol

Calling Card Analysis in Budding Yeast

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Calling card analysis is a high-throughput method for identifying the genomic binding sites of multiple transcription factors in a single experiment in budding yeast. By tagging a DNA-binding protein with a targeting domain that directs the insertion of the Ty5 retrotransposon, the genomic binding sites for that transcription factor are marked. The transposition locations are then identified en masse by Illumina sequencing. The calling card protocol allows for simultaneous analysis of multiple transcription factors. By cloning barcodes into the Ty5 transposon, it is possible to pair a unique barcode with every transcription factor in the experiment. The method presented here uses expression of transcription factors from their native loci; however, it can also be altered to measure binding sites of transcription factors overexpressed from a plasmid.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Agarose gels (0.7%) and running buffer

Barcoded Ty5 plasmid (pRM1001-series; 200 ng/μL)

These plasmids, which are available from the corresponding author, each contain an 8-bp barcode within the transposon that will be matched to each tagged strain.

Betaine (5 M)

BSA (20 mg/mL; molecular biology grade)

Carrier (Sheared Salmon Sperm) DNA Solution (10 mg/mL; Invitrogen)

Chloroform

dH₂O

dNTP mix (10 mM each of dATP, dCTP, dGTP, dTTP)

Ethanol (70%, 100%)

Galactose – Ura agar plates <R>

Genomic DNA from yeast strain yRM1009

This strain is available from the corresponding author.

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Glucose – His agar plates <R>

Glucose – His 5-fluoroorotic acid (5-FOA) agar plates <R>

Glucose – Ura medium <R>

Glycerol (20%; optional; see Step 16)

HindPII restriction enzyme and appropriate buffer

HpaII restriction enzyme and appropriate buffer

Lithium acetate (1 M)

Phenol:chloroform:isoamyl alcohol (PCA) (25:24:1)

Phusion DNA polymerase

Phusion HF buffer (5×)

Polyethylene glycol 3500 (PEG 3500; 50% w/v)

Primers (all sequences are listed 5' to 3')

“Forward” cloning primer: (N)₄₀AGAGTGTGCGCATAGTGATAC

This primer includes 40 bp upstream of the stop codon of the ORF of the DNA-binding protein of interest (without including the stop codon) plus 20 bp to amplify the tagging domain.

“Reverse” cloning primer: (N)₄₀CGCACTTAACTTCGCATCTG

This primer includes the reverse complement of the 40 bp downstream from the ORF of the DNA-binding protein of interest (without including the stop codon) plus 20 bp to amplify the tagging domain.

“Forward” inverse PCR primer: AATGATACGGCGACCACCGAGATCTACACTCTTCCCTA
CACGACGCTCTTCCGATCTAATTCACCTACGTCAACA

“Reverse” inverse PCR primer: CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCATTCC
TGCTGAACCGCTCTTCCGATC

RedTaq DNA polymerase and associated buffer

RNaseA (20 mg/mL)

Sodium acetate (3 M, pH 5.2)

TaqI restriction enzyme and appropriate buffer

T4 DNA ligase and associated buffer

Yeast calling card lysis buffer <R>

Yeast strain of desired background

Strain must be deficient for SIR4, HIS3, and URA3 and sensitive to nourseothricin (NAT) antibiotic.

YPD <R> (liquid medium and agar plates)

YPD agar plates with and without 100 µg/mL NAT are needed.

Equipment

Agarose gel apparatus

Centrifuge

Conical centrifuge tubes (15-mL)

Glass beads (0.2 mm diameter)

Glass culture tubes (5-mL; disposable)

Glass yeast spreader

Heat blocks

Incubator (30°C)

Microcentrifuge

Microcentrifuge tubes (1.5-mL)

Microcentrifuge tube rotator

PCR (polymerase chain reaction) tubes

QIAquick PCR purification kit (Qiagen)

Sequencing machine (Illumina GA, Illumina Miseq, or Illumina HiSeq with paired-end capabilities)

D. Mayhew and R.D. Mitra

Spectrophotometer for DNA quantification (Nanodrop [Thermo Scientific] or Qubit fluorometer [Invitrogen])
 Thermocycler
 Vacuum concentrator (e.g., Speedvac)
 Velvet replica plating pads and base
 Vortexer
 Water bath

METHOD

Cloning Strain

This stage involves cloning the Ty5 integrase-interacting domain of Sir4 to the carboxyl terminus of the desired TF. If the desired tagged strains have already been created, proceed to Step 11. See Figure 1 for an overview of the entire protocol.

1. Amplify the Sir4-NatR sequence from genomic DNA of strain yRM1009. Add the following PCR mix components and mix well:

1× Phusion HF buffer
 0.5 μ M “forward” cloning PCR primer
 0.5 μ M “reverse” cloning PCR primer
 0.2 mM of each dNTP
 1 unit Phusion DNA Polymerase
 100 ng genomic DNA of yRM1009
 dH₂O to 50 μ L

2. Perform the following cycling program:

No. of cycles	Temperature	Time
1	98°C	30 sec
30	98°C	10 sec
	58°C	30 sec
	72°C	2 min
	72°C	5 min
1	12°C	Hold

3. Run 5 μ L of the PCR product on 0.7% agarose gel using standard techniques to confirm success of PCR.

A single product at 1.8 kb is expected.

4. Inoculate the yeast strain of the desired background into 5 mL of YPD in a disposable 5-mL glass culture tube. Grow to saturation overnight at 30°C with shaking. Dilute 50 μ L of the saturated starter culture into 5 mL YPD in a disposable 5-mL glass culture tube. Grow at 30°C with shaking to an OD₆₀₀ between 0.8 and 1.0 (~ 6 h).
5. Pellet the cells by centrifuging the culture tube at 3000 rpm for 2 min at room temperature. Remove the supernatant. Resuspend the cells in 1000 μ L of dH₂O and transfer the cells to a microcentrifuge tube. Pellet the cells by centrifuging the tube at 10,000 rpm for 2 min at room temperature. Remove the supernatant.
6. Add the following components of the transformation mix with the remaining PCR product from Step 3 directly on top of the pelleted cells to the following final concentrations and to a total volume of 360 μ L:

FIGURE 1. Overview of the calling card protocol. (A) Barcoded Ty5 transposons are separately transformed into yeast strains with TFs tagged with Sir4. (B) The strains are pooled, expression of Ty5 is induced, and the subsequent growth medium selects for genomic insertions. (C) Genomic DNA is digested with restriction endonucleases. (D) Digested fragments are self-ligated. (E) Inverse PCR adds Illumina sequencing adaptors.

Component	Amount (per reaction)	Final concentration
PEG 3500 (50% w/v)	240 μ L	33.3%
Lithium acetate (1 M)	36 μ L	0.1 M
Carrier DNA solution	10 μ L	250 ng/ μ L
PCR product plus dH ₂ O	74 μ L	

- Vortex the mixture and incubate in a water bath for 40 min at 42°C.
- Centrifuge the tube at 10,000 rpm for 2 min at room temperature and remove supernatant. Resuspend the cell pellet in 1 mL of YPD and incubate with rotation for 60 min at 30°C.

9. Centrifuge the tube at 10,000 rpm for 2 min at room temperature and remove YPD. Resuspend the cell pellet in 50 μ L dH₂O and plate on a YPD agar plate containing 100 μ g/mL NAT and incubate for 2 d at 30°C.
10. Select NAT resistant colonies and Sanger sequence the DNA bordering the tagged ORF to verify a correct clone.

Select at least three clones for each cloned strain.

Plasmid Transformation and Induction

This stage requires four different growth steps on separate days with 1 h of hands-on time each day.

11. Select a unique barcoded Ty5 donor plasmid for each transcription factor–tagged strain to be examined.
12. Separately inoculate each tagged strain from step 10 into 5 mL of YPD in a disposable 5-mL glass culture tube. Grow to saturation overnight at 30°C with shaking. Dilute 50 μ L of the saturated starter culture into 5 mL YPD in a disposable 5-mL glass culture tube. Grow at 30°C with shaking to an OD₆₀₀ between 0.8 and 1.0 (~ 6 h).
13. Pellet the cells by centrifuging the culture tube at 3000 rpm for 2 min at room temperature. Remove the supernatant and wash cells as in Step 5.
14. For each tagged strain, add the following components of the transformation mix with a barcoded Ty5 plasmid to the following final concentrations and to a total volume of 360 μ L:

Component	Amount (per reaction)	Final concentration
PEG 3500 (50% w/v)	240 μ L	33.3%
Lithium acetate (1.0 M)	36 μ L	0.1 M
Carrier (Sheared Sperm DNA Solution)	10 μ L	250 ng/ μ L
Ty5 plasmid (200 ng/ μ L)	1 μ L	0.6 ng/ μ L
dH ₂ O	73 μ L	

15. Vortex the mixture and incubate in a water bath for 40 min at 42°C.
16. Centrifuge tube at 10,000 rpm for 2 min at room temperature and remove the transformation mix. Resuspend the cell pellet in 50 μ L of dH₂O and plate on a Glucose – His plate. Incubate at 30°C for 2 d.

Transformed yeast can be frozen in 20% glycerol at –80°C at this step to avoid repeating the previous steps in future experiments.

17. Inoculate a single colony from each barcoded-plasmid transformed strain in 5 mL of liquid Glucose – Ura medium in a disposable 5-mL glass culture tube. Grow to saturation overnight at 30°C with shaking. Pellet cells by centrifuging the culture tube at 3000 rpm at room temperature for 2 min. Remove medium.
18. Resuspend the cells in each tube in 1 mL dH₂O. Combine all transformed strains into a single tube and mix well. Plate 100 μ L of this mixture onto each Galactose – Ura agar plate and incubate at room temperature for 2 d.

Yeast should form a confluent lawn.

The Ty5 transposon is induced by galactose, so this is the step at which transcription factor binding is measured. If additional growth conditions are desired in the experiment, these should be supplied at this step. The number of Galactose – Uracil plates should scale with the number transcription factors being studied. As a rule of thumb, use two 10-cm plates for each strain in the experiment (enough for 5×10^3 insertions), but DNA-binding proteins with a very large number of targets may require more plates to gather enough insertions to map all of their binding sites.

19. Replica plate cells to YPD agar plates by pressing the yeast-side of the Galactose – Ura agar plate to a clean velvet, removing, and then pressing a new YPD agar plate against the velvet. Use a clean velvet for each pair of plates. Incubate the plates for 1 d at 30°C.

Yeast should form a confluent lawn.

20. Replica plate cells from YPD plates to Glucose – His 5-FOA agar plates and incubate for 2 d at 30°C.

This step selects for genomic insertions of Ty5 and counter-selects against cells still containing the plasmid. Each 10-cm plate should have $\sim 2 \times 10^3$ colonies by the second day of growth.

See Troubleshooting.

DNA Extraction

Requires a total of 2 h.

21. Pipette 1 mL of liquid YPD onto each Glu – His 5-FOA plate and scrape the yeast with a glass yeast spreader to put the cells into solution. Pipette the solution into a 15-mL conical tube. Combine cells from different plates into a single conical tube. Mix by gently inverting the tube.
22. Transfer 500 μ L of the liquid mixture to a 1.5-mL microcentrifuge tube. Centrifuge at 13,000 rpm for 10 min at room temperature in a microcentrifuge and pour off the liquid.
23. To the pelleted cells add:
 - 400 μ L Yeast calling card lysis buffer
 - 400 μ L Glass beads (measure using a microcentrifuge tube)
 - 400 μ L Phenol:chloroform:isoamyl alcohol (25:24:1)
24. Vortex for 10 min at room temperature.
25. Centrifuge at 13,000 rpm for 10 min at room temperature.
26. Transfer the aqueous supernatant to a new microcentrifuge tube.
27. Add 400 μ L of phenol:chloroform:isoamyl alcohol (25:24:1). Repeat Steps 24–26.
28. Add 400 μ L of chloroform. Repeat Steps 24–26.
29. Precipitate DNA by adding 40 μ L of 3 M sodium acetate and 1000 μ L of ethanol (100%) at room temperature. Vortex and incubate for 60 min at -70°C .
30. Centrifuge at 13,000 rpm for 10 min at room temperature. Carefully remove the supernatant.
31. Carefully wash the pellet with 500 μ L of ethanol (70%) at room temperature.
32. Centrifuge at 13,000 rpm for 1 min at room temperature.
33. Remove ethanol supernatant with a micropipette and dry the pellet in a vacuum concentrator until all liquid is removed (~ 10 min). Reconstitute the pellet in 50 μ L dH₂O and 2 μ L RNaseA and incubate for 30 min at 37°C.

At this step, genomic DNA can be frozen at -20°C and stored long-term before subsequent steps.

Genomic Digestions

Requires a total of 3.5 h.

34. Measure DNA concentration with a spectrophotometer.
35. Put 4 μ g of DNA into each of three PCR tubes. Perform the following three digestions in a total volume of 50 μ L each with the final concentrations and conditions as listed:
 - i. TaqI digestion
 - 4 Units TaqI enzyme
 - 1 $\times$ TaqI-compliant buffer
 - 1 $\times$ BSA
 - dH₂O to 50 μ L
 - Incubate for 3 h at 65°C followed by a heat inactivation for 20 min at 80°C.

D. Mayhew and R.D. Mitra

ii. HindPII digestion

4 Units HindPII enzyme

1× HindPII-compliant buffer

dH₂O to 50 µL

Incubate for 3 h at 37°C followed by a heat inactivation for 20 min at 65°C.

iii. HpaII digestion

4 units HpaII enzyme

1× HpaII-compliant buffer

dH₂O to 50 µL

Incubate for 3 h at 37°C followed by a heat inactivation for 20 min at 65°C.

36. Purify digestions using the QIAquick PCR purification kit, following the manufacturer's protocol. Elute DNA in 50 µL of dH₂O.

Self-Ligation

Requires a total of 14 h or overnight.

37. To each of the eluates from Step 36 add the reagents below to the following final concentrations:

18 units T4 ligase

1× T4 ligation buffer

dH₂O to 400 µL

38. Incubate for at least 12 h at 14°C.

Overnight incubation is preferable.

39. Precipitate DNA by following Steps 29–32.

40. Remove ethanol and dry the pellet in a vacuum concentrator. Resuspend the pellet in 50 µL dH₂O and vortex.

Requires a total of 4 h.

41. Add the following PCR mix components to half (25 µL) of each of the reconstituted DNA samples from Step 40 to yield the indicated concentrations:

1× RedTaq PCR Buffer

0.5 µM “forward” inverse PCR primer

0.5 µM “reverse” inverse PCR primer

0.2 mM of each dNTP

0.5 M betaine

4 units RedTaq Enzyme

dH₂O to 50 µL

42. Perform the following cycling program:

No. of cycles	Temperature	Time
1	93°C	2 min
30	93°C	30 sec
	60°C	6 min
1	12°C	Hold

43. Run 5 μL of each PCR product on a 0.7% agarose gel using standard techniques to confirm the success of PCR.
Expect products ranging from 200 bp to 2 kb.
See Troubleshooting.
44. Purify PCR products using QIAquick PCR purification kit, following the manufacturers's protocol. Elute DNA in 50 μL of dH_2O and quantify the DNA concentration using Nanodrop/Qubit.
45. Combine equimolar quantities of the 3 PCR products and dilute to 10 nM in dH_2O for Illumina sequencing.
Assume an average PCR product size of 200 bp, which makes 10 nM equal to 1.32 ng/ μL . At least 20 μL of the final solution is needed for Illumina sequencing.
46. Sequence the DNA on a paired-end Illumina sequencing run according to the manufacturer's instructions.
47. Analyze data by demultiplexing reads using the 8-bp barcode (the first eight bases on the second read), removing the first 17 bp of the read (corresponds to the Ty5 transposon), and aligning the remaining bases of the read to the reference genome of the background yeast strain.
See Troubleshooting.

TROUBLESHOOTING

Problem (Step 20 or 47): A high background of yeast on the Glucose – Histidine + 5-FOA selection step or a high percentage of plasmid reads in the sequencing is observed.

Solution: The 5-FOA selection step is not killing all of the yeast still containing the Ty5 plasmid. An additional round of replica plating on Glucose – Histidine + 5-FOA plates will reduce plasmid in the extracted DNA.

Problem (Step 43): A thick band is observed at 100 bp with a smear of genomic DNA above this product.

Solution: Primer–dimers have formed during the inverse PCR. Perform a QIAquick gel extraction (QIAGEN) to separate the larger products from the primer–dimer size fragments before sequencing.

DISCUSSION

Chromatin immunoprecipitation read out by either microarrays (ChIP-chip) (Horak and Snyder 2002) or next-generation sequencing (ChIP-seq) (Johnson et al. 2007) has been an invaluable technique for identifying the binding sites of transcription factors (TFs) in yeast. For studies requiring large numbers of TFs, the nature of ChIP-based methods requires increased time and work for each additional TF in the experiment. Large sequencing capacities of next-generation sequencers allow for ChIP-seq libraries of many TFs to be barcoded and sequenced in parallel (Lefrançois et al. 2009); however, the immunoprecipitation for each TF is performed independently. This limitation makes large-scale ChIP experiments laborious. The ability of the calling card technique to multiplex many TFs through most of the steps in this protocol makes scaling up these experiments more feasible.

The number of TFs that can be included in this protocol is a function of the downstream sequencing capacity. To ensure adequate coverage of all of the insertions with each barcode, $1\text{--}2 \times 10^6$ reads are required for each barcode/TF included in the multiplex. Experiments with large numbers of TFs may make the growth and selection steps on solid plates unsuitable. For these larger experi-

ments the protocol can be altered to move these steps (18–20) to liquid cultures. For more information on the data analysis or altering the protocol to overexpress the TF-Sir4 fusion from a plasmid, please see Wang et al. (2011).

A limitation of calling cards is that there are currently fewer existing yeast strains with TFs tagged for the method compared with ChIP-ready tagged strains. The study of some TFs or background strains will necessitate cloning them as described in the first steps of this protocol. The method is easy to use and provides high signal-to-noise mapping of DNA-binding with an accuracy and resolution comparable with ChIP-seq (Wang et al. 2011). This protocol should be adaptable to many experiments for mapping binding sites of DNA-binding proteins, especially when multiple proteins need to be studied.

RECIPES

Drop-Out Mix

Reagent	Amount to add
Adenine	0.5 g
Alanine	2.0 g
Arginine	2.0 g
Asparagine	2.0 g
Aspartic acid	2.0 g
Cysteine	2.0 g
Glutamine	2.0 g
Glutamic acid	2.0 g
Glycine	2.0 g
Histidine	2.0 g
Inositol	2.0 g
Isoleucine	2.0 g
Leucine	10.0 g
Lysine	2.0 g
Methionine	2.0 g
<i>para</i> -Aminobenzoic acid	0.2 g
Phenylalanine	2.0 g
Proline	2.0 g
Serine	2.0 g
Threonine	2.0 g
Tryptophan	2.0 g
Tyrosine	2.0 g
Uracil	2.0 g
Valine	2.0 g

Combine the appropriate ingredients, in 1 L of medium minus the relevant supplements, and mix in a sealed container. Turn the container end-over-end for at least 15 min; add several clean marbles to help mix the solids.

Galactose – Ura Agar Plates

1.7 g	Yeast nitrogen base without amino acids and ammonium sulfate
1 g	Dropout mix (without uracil) <R>
5 g	Ammonium sulfate
20 g	Agar

Add H₂O to 900 mL, stir well, and autoclave. Add 100 mL of 20% galactose (sterile) and stir. Pour into sterile Petri dishes and allow to solidify.

Glucose – His Agar Plates

1.7 g	Yeast nitrogen base without amino acids and ammonium sulfate
1 g	Drop-out mix (without histidine) <R>
5 g	Ammonium sulfate
20 g	Agar

Add H₂O to 900 mL, stir well, and autoclave. Add 100 mL of 20% glucose (sterile) and stir. Pour into sterile Petri dishes and allow to solidify.

Glucose – His 5-Fluoroorotic Acid (5-FOA) Agar Plates

1.7 g	Yeast nitrogen base without amino acids and ammonium sulfate
1 g	Drop-out mix (without histidine) <R>
5 g	Ammonium sulfate
20 g	Agar

Add H₂O to 900 mL, stir well, and autoclave. Add 100 mL of 20% glucose (sterile) and 1 g 5-FOA, and stir. Pour into sterile Petri dishes and allow to solidify.

Glucose – Ura Medium

1.7 g	Yeast nitrogen base without amino acids and ammonium sulfate
1 g	Drop-out mix (without uracil) <R>
5 g	Ammonium sulfate

Add H₂O to 900 mL, stir well, and autoclave. Add 100 mL of 20% glucose (sterile) and stir. Store for up to 3 mo at 4°C.

Yeast Calling Card Lysis Buffer

2%	Triton X-100
1%	SDS
100 mM	NaCl
10 mM	Tris–HCl (pH 8.0)
1 mM	EDTA (pH 8.0)

Store for up to 12 mo at 4°C.

YPD

Peptone,	20 g
Glucose,	20 g
Yeast extract,	10 g
H ₂ O to	1000 mL

YPD (YEPD medium) is a complex medium for routine growth of yeast.

To prepare plates, add 20 g of Bacto Agar (2%) before autoclaving.

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