qPCR of Yeast ChIP DNA

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[Abstract] This protocol is adapted from Chris Yellman's personal notes for qPCR of yeast ChIP DNA to verify binding sites already identified by microarray/sequencing analysis. In addition, qPCR is a quick way to assay the quality of a ChIP sample (provided you have a few well-characterized binding sites) before submitting it for sequencing/microarray analysis. This protocol is designed for use with a Roche LightCycler.

Materials and Reagents

1. PCR primers
   a. Prepare 10 µM primer stocks in water from 100 µM stocks in TE.
   b. Pick several positive control primer pairs and at least one negative site. Primer design criteria are described in the protocol.
2. LightCycler 480 SYBR Green I Master mix (Roche Diagnostics, catalog number: 04707 516001)
   The mix contains Taq, dNTPs and the intercalating SYBR Green dye.
3. Yeast genomic DNA for a positive control dilution series
   You can use a glass bead lysis yeast genomic DNA preparation that has been RNase treated, Qiagen cleaned and stored in TE, or purchase directly from Invitrogen.
4. 10 µl capacity multi-well pipetter
   384-well reaction plate with transparent adhesive plastic cover

Equipment

1. Covered container
2. Microcentrifuge tube
3. 384-well reaction plate

Procedure

1. Design PCR primers

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Primer 3 program (http://frodo.wi.mit.edu/primer3/) can be used to design primers. The following primer design criteria have worked for me: amplified regions of 200-250 bp in length, melting temperatures of ~59-61 °C and primer length 20. Other design criteria were chosen by Primer 3 at the default values.

2. Prepare a positive control DNA dilution series

   To determine the amplification efficiency of each primer pair to be used, prepare a DNA dilution series. An 8-fold dilution series provides a convenient reference. Dilute the DNA in pure water from 8x diluted out to 8^7 (2,097,152x) diluted. Include a no DNA sample.

3. Dilute the ChIP DNA samples in water

   The ChIP DNA must be diluted to an appropriate concentration. Each qPCR reaction will use 2 µl of dilute template.

   This amount is 1/40 of a ChIP from ~4.5 x 10^9 cells and it will suffice for 10 qPCR reactions with 1 µl left over. The ChIP DNA analyzed in each qPCR react ion represents the amount recovered from ~1 x 10^7 cells.

   Note: It's possible that this is too little ChIP DNA and the amount should be increased two-fold.

4. Prepare the reaction mixtures (reaction volume of 10 µl)

   Prepare for triplicate (if the data are to be published) or duplicate analysis of each sample. I usually prepare enough volume for one extra reaction per 8-10 reactions, storing everything on ice and in a covered container (dark) during preparation.

   Each 10 µl reaction contains the following:

   a. 2 µl pure water
   b. 5 µl SYBR Green I Master
   c. 1 µl of PCR primers (0.5 µl of each primer in a pair from 10 µM stock)
   d. 2 µl DNA template

   First dilute the SYBR Green I Master mix with 2 µl of pure water + 5 µl of SYBR Green per reaction. Distribute the desired volume of dilute SYBR Green master mix into a microcentrifuge tube for each primer pair. Add the PCR primers (remember to include the extra reaction volume) to prepare primer pair master mixes.

5. Set up the qPCR reactions and add templates

   Distribute 8 µl of the desired master reaction mixture to each well of a 384-well reaction plate. Add 2 µl of template DNA to each well and water to the negative controls and cover the plate with clear adhesive plastic film. Keep in the dark until running the PCR.

6. Run the qPCR thermocycler program

   In addition to the qPCR sample reactions, set up melting curve analysis for each primer pair used.

7. qPCR data analysis

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Verify that the melting curve analysis returns a single peak for each primer pair. Determine relative quantification by the $2^{-\Delta\Delta}$Cp method (2nd derivative maximum) and calculate the mean $^{\Delta\Delta}$Cp value for each set of duplicate or triplicate samples. For each target tested, calculate the standard deviation of the mean $^{\Delta\Delta}$Cp across a panel of negative controls. Determine the statistical significance of the data assuming a normal distribution. Note: One modification to relative quantification by the $2^{-\Delta\Delta}$Cp method would be to apply an efficiency-corrected equation (Pfaffl, 2001).

Notes

Quantitative PCR (qPCR) uses fluorescence to detect PCR product accumulation. The crossing point (Cp) or threshold cycle (Ct) is the point at which fluorescence rises appreciably above background. Other methods of analysis use the linear range of the amplification curve. The Cp value is used for the relative quantitation of qPCR. The qPCR machine detects the fluorescence and software calculates Cp values from the intensity of the fluorescence. I have used the method of maximum second derivative of the amplification curve as a standard to compare samples. This is the point on the curve with the maximum positive change in curvature. It is the point at which the signal becomes detectable and enters the linear range of amplification.

The SYBR Green method uses a dye in the PCR reaction which binds to newly synthesized double-stranded DNA and emits fluorescence The SYBR Green dye intercalates with doublestranded DNA, cansing the SYBR Green to fluoresce.

Real-time PCR, also known as kinetic PCR, qPCR, qRT-PCR and RT-qPCR, is a quantitative PCR method for the determination of copy number of PCR templates such as DNA or cDNA in a PCR reaction. There are two types of real-time PCR: probe-based and intercalator-based. Both methods require a special thennocycler equipped with a sensitive camera that monitors the fluorescence in each well of the multi-well plate at frequent intervals during the PCR Reaction. Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers asregular PCR does, an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. The TaqMan method is more accurate and reliable than the SYBR Green method, but also more expensive.

References

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