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Running title: ChIPseq and RIPseq of *Legionella*

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Summary

Transcriptional and post-transcriptional regulators play a critical role in allowing a bacterium to adapt to the diverse environments and conditions it encounters. In order to characterize the role of these regulators the identification of their specific interaction partners is of utmost importance. Co-Immunoprecipitation (IP) is based on antigen/antibody complex formation to purify a protein of interest from the rest of the samples together with its interaction partner. This method allows us to study direct interaction of a regulator with its specific binding partners like protein-RNA, protein-DNA or protein-protein interactions. IP typically requires careful optimization and troubleshooting depending on the varying physicochemical characteristics of the protein of interest. In the following chapter we present a starting point and the basic guidelines to obtain the best possible results from an IP experiment with subsequent use of new generation sequencing (NGS) techniques to detect mRNA or ncRNA-targets (RIPseq) and protein-DNA interactions (ChIPseq).

1. Introduction

Bacteria have developed a variety of strategies with which they adapt their genetic expression to meet the challenges of their ever-changing surrounding environment. These include specific sigma factors, two-component systems, peptide or chemical-based quorum sensing systems, repressors, positive regulators, as well as small regulatory RNAs. Alone or in combination, these mechanisms enable bacterial cells to communicate with their environment, their hosts, and with each other, allowing the bacteria to adopt specific responses, express specific proteins (toxins, adhesins, invasins, siderophores) or develop specialized structures such as biofilms or spores to ensure survival, colonization of their ecological niches and dissemination. Expression profiles of *Legionella* lacking transcriptional regulators have been used successfully to broadly define genes impacted by the deleted regulator (*1-7*). Although providing informative data, transcriptional analysis alone cannot distinguish between direct and indirect regulatory effects or identify regulated genes that may be transcriptionally silent under the test conditions. Furthermore, in these experiments additional non-specific results like stress response or activation of subsequent regulatory cascades cannot be avoided and sometimes these secondary effects even overwhelm and hide original regulator-specific effects and only vague conclusions about primary/direct responses can be drawn from the experiments.

Co-Immunoprecipitation (IP) enables the purification of a protein based on antigen/antibody complex formation. Therefore, isolates of the protein purified from the rest of the samples can

be achieved to study direct interaction between the protein and specific binding partners like nucleotides or other proteins. The most critical factor for the success of these experiments is the quality of the antibody used, but also many other factors during the different steps from lysis to elution are influencing the outcome of the IP (*8*). ChIP coupled with next-generation sequencing (ChIP-seq) has revolutionized whole-genome mapping of DNA-binding protein sites. Although ChIP-seq rapidly gained support in eukaryotic systems (*9*), it remains underused in the mapping of bacterial transcriptional regulator-binding sites, and only three studies have been published to date (*10-12*). However, no study using *L. pneumophila* as model has been reported yet.

The following protocols should be seen only as starting points for conducting these experiments, as protocol optimization for each protein studied and each antibody used is indispensible. Here we describe two different methods i) RIPseq to characterize protein-RNA interactions and ii) ChIPseq to characterize protein-Chromatin/DNA interactions. Both of them lead to the construction of DNA-libraries optimized for Next Generation Sequencing (NGS) techniques. Alternatively, the library screening can be performed with hybridization to Microarrays (on Chip) for which additional steps for DNA dye labeling after PCR amplification are necessary.

2. Materials

2.1. Strains and growth media

1. *Legionella pneumophila* e.g. strains Paris, JR32 or Philadelphia-1

2. AYE medium. For 1 liter dissolve 12 g yeast extract and 10 g ACES, adjust pH to 6.9 with 1 M KOH. Add 10 mL of cysteine 40 g/L and 10 mL of iron pyrophosphate 30 g/L. Fill volume to 1 L with distilled water and filter sterilize.

3. CYE plates. For 1 liter dissolve 10 g yeast extract and 10 g ACES, adjust pH to 6.9 with 1M KOH, add 15 g of agar, 2 g of activated charcoal and autoclave. Add 10mL of filter sterilized cysteine 40 g/L and 10 mL of filter sterilized ferric nitrate 25 g/L. Poor plates.

2.2 RIP library construction

1. Formaldehyde 37%

2. Disposable 50 mL polypropylene tubes

3. Rotating wheel

4. Protease inhibitor cocktail (e.g. SigmaFast, Sigma, S8830-20TAB)

5. Lysis buffer: 50 mM HEPES-KOH pH7.5, 150mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, protease inhibitor

6. Sonicator

7. Specific antibody against protein of interest or alternatively antibody against Tag (e.g. Flag- or Strep-Tag)

8. Dynabeads® Protein A or G (Invitrogen, 100-01D or 100-03D),

9. Magnet for separation

10. Wash buffer RIPseq: 10 mM Tris-HCl pH 7.5, **250 mM NaCl**, 0.5% NP40, 0.5% Na-deoxycholate, 1 mM EDTA

11. TE buffer : 10mM Tris, 1mM EDTA, pH 7.5

12. Elution buffer: 50mM Tris, 1mM EDTA, 1% SDS, pH8.0

13. Proteinase K 20µg/µl
14. DNase I recombinant, RNase free (10U/µl)

15. RNA fragmentation kit (Ambion, AM8740)

16. Alkaline Phosphatase

17. 3'-RNA adapter P-UCGUAUGCCGUCUUCYGCUUGUidT (idT = inverted deoxyT)

18. T4 Polynucleotide Kinase

19. 5’-RNA adapter GUUCAGAGUUCUACAGUCCGACGAU

20. Reverse Transcription Specific primer CAAGCAGAAGACGGCATACGA

21. Phenol solution, pH4.3

22. Chloroform:Isoamyl alcohol 24:1

23. RNase inhibitor

24. 3M Sodium Acetate, pH5.2

25. Ethanol p.a.; Etanol 75%

26. Glycogen (20mg/ml)

27. Tobacco acid pyrophosphatase 10U/µl, TAP

28. 5’-RNA adapter (25µM) GUUCAGAGUUCUACAGUCCGACGAU

29. T4 RNA Ligase 5U/µl

30. Superscript II Reverse Transcriptase Kit (Invitrogen, 18064014)

31. 10mM dNTP mix

32. Certified Low Range Ultra Agarose

33. Agarose gel electrophoresis equipment

34. 0.5M EDTA pH8.0

35. Distilled water DNase/RNase free

36. Thermocycler

37. High Fidelity DNA Polymerase

38. Primer 1 (25µM) CAAGCAGAAGACGGCATACGA

39. Primer 2 (25µM) AATGATACGGCGACCACCGACAGGTTCAG
AGTTCTACAGTCCGA

40. PCR Clean-up kit

41. Bionalyzer 2100 (Agilent);

42. DNA 1000 Chips (Agilent, 5067-1504)

43. Bradford reagent

2.2 ChIPseq library construction

1. Formaldehyde 37%

2. Disposable 50 mL polypropylene tubes

3. Protease inhibitor cocktail

4. Lysis buffer: 50 mM HEPES-KOH pH7.5, 150mM NaCl, 1 mM EDTA, 1% Triton X-100,
0.1% Na-deoxycholate, protease inhibitor

5. Sonicator

6. Specific antibody against protein of interest or alternatively antibody against Tag (e.g. Flag- or Strep-Tag)

7. Dynabeads® Protein A or G (Invitrogen, 100-01D or 100-03D),

8. Magnet for separation

9. Wash buffer ChIPseq: 10 mM Tris-HCl pH 7.5, **250 mM LiCl**, 0.5% NP40, 0.5% Na-deoxycholate, 1 mM EDTA

10. TE buffer : 10mM Tris, 1mM EDTA, pH 7.5

11. Elution buffer: 50mM Tris, 1mM EDTA, 1% SDS, pH8.0

12. Proteinase K 20µg/µl

13. RNase A, DNase free (0.35µg/µl in PBS)

14. Genomic DNA Sample Prep Kit (Illumina, FC-102-1001)

3. RIPseq and ChIp-seq library construction (common steps; Note 1)

1. Inoculate 50 mL fresh AYE media to OD_{600} = 0.1 from an overnight culture and allow bacteria to grow to an optimal OD (depending on the expression of desired protein).
2. Transfer each culture to separate disposable 50 mL polypropylene tubes and add 1.4 mL of 37% Formaldehyde (Note 2).
3. Incubate the formaldehyde-treated cells for 20 minutes at room temperature on a rotating platform, and transfer to a rotating platform overnight at 4°C.
4. Spin the 50 mL polypropylene tubes containing the formaldehyde-treated cells for 5min at 5000g (4°C), pour off the supernatant and wash the cells 3 times in cold 1X PBS (if not using the cells immediately, store cell pellet at -80°C).
5. Resuspend the cell pellets using 1ml Lysis Buffer + protease inhibitor
   And pulse-sonicate them with around 60 watt five times for 20sec
   For ChIP-seq additional DNA fragmentation:

sonication on ice for 15 min 30sec ON/ 30sec OFF (avoid raising temperature!!) or nebulization necessary

6. Centrifuge at max speed for 20min and transfer supernatant in a new tube, quantify the total protein with Bradford and adjust it to 1mg protein/ml (Note 3)
7. Preparation of magnetic beads
   - Aliquot 100µL of Dynabeads ProteinG for each sample to be immunoprecipitated and wash them 3 times in 1XPBS + 1%BSA solution, resuspend them in 250µl PBS/BSA + add 5-10µg of antibody and incubate them over night at 4°C on a rotating platform (Note 4)
   - do the same with 200µl of beads, but incubate them over night without antibody or with an unspecific antibody)
   - Wash the beads 3 times in cold PBS/BSA and resuspend them in 100µl PBS/BSA per sample
8. Co-Immunoprecipitation
   - Add 100µl of magnetic beads (**without antibody or with an unspecific antibody**) to the protein solution (prepared in step 6) and incubate for 4h at 4°C on a rotating platform (pre-clearing of solution from unspecific protein-beads interaction); place them on a magnet and transfer the supernatant into a fresh tube
   - Divide the fraction in two equal aliquots.
     Add in one 100µl of magnetic beads **with specific antibody**. For the second part, add 100µl of magnetic beads **without antibody or with an unspecific antibody** (negative control). Incubate samples over night at 4°C on a rotating platform
9. Place samples on a magnet and wash the beads twice with cold Lysis buffer containing additional 350mM NaCl
10. Wash the beads five times in cold Wash buffer **(important for RIPseq use NaCl instead of LiCl!!!)**
11. Wash once with cold TE buffer
12. Resuspend beads in 150µl Elution buffer and incubate them for 15min at 65°C with brief vortexing every 2min
13. Place the solution on a magnet and transfer supernant into a new tube (discard the beads!)
14. Reverse cross-linking (by incubation over night at 65°C)
15. P/C/I extraction and NaAc precipitation (Note 5 and 6), resuspend pellet in 50µl distilled water

16. Add 140µl TE buffer + 3µl Glycogen + 7µl Proteinase K (20µg/µl) and incubate 2h at 37°C
17. Extract again with P/C/I and resuspend after NaAc precipitation in 44µl distilled H_2O. Continue with step 3.1 for RNA-Immunoprecipitation or with 3.2 for DNA/Chromatin-IP

3.1. RIPseq (continuation)

1. DNase I digestion: add 5µl of DNase 10xbuffer and 1µl of DNase I and incubate reaction for 30min at 25°C. Exract with P/C/I and precipitate samples with NaAc. Resuspend the dry pellet in 10µl distilled RNase-free water
2. Fragmentation: the RNA was metal-catalyzed heat fragmented to sizes of around 100-200nt using the RNA fragmentation kit
   1.1µl 10x fragmentation reagent (buffered zinc solution)
   10µl RNA
   Incubation at 70°C for 5min
   Terminate reaction by putting on ice and add 1.1µl stop solution
   After NaAc precipitation, resuspend the dry pellet in 26µl RNase-free distilled H_2O
3. RNA dephosphorylation:
   a) TAP treatment
   Denature the RNA from step 2 for 10min at 65°C
   After 1min on ice, add 3µl TAP buffer and 1µl TAP (10U) and incubate for 1h at 37°C
   b) Alkaline Phosphatase treatment
   Add 14µl distilled H2O, 5µl 10x Dephosphorylation buffer and 1µl Alkaline Phosphatase (10U) and incubate for 30min at 37°C

   Phenol/Chloroform/IAA extraction, NaAc precipitation; resuspend the dry pellet in 5.9 µl distilled water
4. 3'-RNA adapter ligation
   5.9µl RNA
   0.6µl 3'adapter (100µM)
   Incubate mix for 10min at 65°C, put on ice for 1min and add
   1µl T4 RNA ligase buffer

1µl ATP solution
0.5µl RNase inhibitor
1µl RNA ligase
Incubate for 6h at 20°C, following 4°C over night, purify with columns (e.g. NucleoSpin) and resuspend RNA after NaAc precipitation in 10µl distilled water

5. Re-phosphorylation of 5' end
10µl RNA
2µl PK buffer
1µl ATP solution
0.5µl RNase inhibitor
1µl Polynucleotide Kinase
5.5µl RNase free H2O

P/C/I extraction and precipitation with NaAc; resuspend pellet in 5.3µl distilled H_2O

6. 5'-RNA adapter ligation:
5.3µl RNA
1.2µl 5'-RNAadapter (25µM)
Incubate 10min at 65°C, on ice for 1min and add
1µl T4 RNA ligase buffer
1µl ATP solution
0.5µl RNase inhibitor
1µl RNA ligase
And incubate for 6h at 20°C, following 4°C over night

7. Reverse Transcription using RT Specific primer
10µl RNA +0.5µl RT Specific primer (100µM)
Denature at 65°C for 10min, on ice 1min and add
6µl first strand buffer
1.5µl DTT (100mM)
1.5µl dNTP
1µl RNase inhibitor
8µl RNase-free H2O

1.5µl Superscript II RT

Incubate 1h at 42°C and purify on columns (e.g. NucleoSpin), recover with 30µl of distilled water

8. PCR amplification (see also Note 7)

30µl template cDNA
10µl Phusion HF buffer
8µl H_2O
0.5µl Primer 1 (25µM)
0.5µl Primer 2 (25µM)
0.5µl dNTP (25mM)
0.5µl Phusion DNA Polymerase

98°C 1min

98°C 10sec
60°C 30sec
72°C 30sec
15 cycles

72°C 10min
4°C

9. Size fractionation

Run samples of step 8 on a low range ultra 2% agarose gel. Cut the zones between 100-170nt and 170-250nt and purify independently with columns (e.g. Nucleospin).
Combine the samples of the two "zones", precipitate and resuspend pellet in 30µl-distilled water
Purify with columns (e.g. Nucleospin), precipitate and resuspend pellets in 10µl-distilled water

10. Check quality and quantity with the Bioanalyzer (DNA 1000) (Note 8)
11. Ready for Sequencing with Illumina

3.2. ChIP-seq library construction (continuation)

1. RNase digestion: add add 56µl of RNase A (final concentration 0.2 µg/µl) and incubate reaction for 2h at 37°C. Extract with P/C/I and precipitate samples with NaAc. Resuspend the dry pellet in 30µl distilled RNase-free water
2. blunt end-DNA by incubation with T4 DNA Polymerase
   30µl DNA sample
   45µl water
   10µl T4 DNA Ligase Buffer (with 10mM ATP)
   4µl dNTP (10mM)
   5µl T4 DNA Polymerase
   1µl Klenow Enzyme
   5µl T4 Polynuleotide Kinase
   Incubate 30min at 20°C and purify with columns (e.g. NucleoSpin)

3. Adenylation of 3' ends
   32µl DNA sample
   5µl Klenow buffer
   10µl dATP (1mM)
   3µl Klenow Exo-
   Incubate 30min at 37°C, purification with columns (e.g. NucleoSpin)

4. Adapter Ligation
   18µl DNA sample
   25µl Ligase Buffer
   2µl PE Adapter Oligo Mix (Illumina)
   5µl DNA Ligase
   Incubate 15min at 20°C, purification and resuspend in 30µl distilled water

5. PCR amplification (as decribed in 3.1. step 8)
6. Size fractionation on a low range ultra 2% agarose gel (see also 3.1. step 9)
7. Check quality and quantity with the Bioanalyzer (DNA 1000) (Note 8)

4. Notes

1. Keep aliquots of the different stages and test them by western blot to monitor the efficiency of your lysis, binding, washing and eluation steps.
2. Formaldehyde step is optional, but usually necessary as the protein-nucleotide interactions are weak and unstable resulting in too small quantities. Alternative to wild type cultures, cultures overexpressing the protein of interest can be used to increase quantity.
3. For IP experiments an amount of at least 3-5mg of total protein is recommended (depending on abundance of the protein of interest).
4. Specific antibodies or antibodies against Tags can be used similarly; test quality of antibodies in western blot experiments before use.
5. Phenol/Chloroform/IAA (P/C/I) extraction: Add 50% of Phenol to the sample and mix vigorously by vortexing. Add the same amount of Chloroform/Isoamyl alcohol and mix well. Centrifuge at max speed for 5 min, transfer the supernatant to a new tube and add 1 vol of Chloroform/Isoamyl alcohol, mix vigorously, centrifuge and transfer supernatant into a new tube.
6. NaAc precipitation: Add 10% 3M sodium acetate (pH5.2), 2% glycogen and 2.5vol of ethanol p.a. to the sample and store it min 2h (better over night) at-20°C. Centrifuge at 4°C for 15min at max speed and discard supernatant. Wash RNA pellet with 500µl ice-cold 75% ethanol centrifuge again for 5min, discard supernatant and let the pellet dry for 10min at RT. Resuspend the dry pellet RNase-free distilled H_2O.
7. Number of cycles during PCR amplification can differ between experiments depending on the amount of cDNA obtained in previous step. Typically are 12 to maximal 17 cycles. More cycles are not recommended as additional PCR steps may introduce a significant amplification bias in cDNA representation.
8. DNA amounts are typically in nM range; anyhow, for the hybridization on the Cluster Station concentrations of 1–10 pM are recommended.

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