Instruction Manual

High Resolution Library Preparation kit

Cat. No. C05010023

Version 1 / 23.06.2014

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Introduction

Association between proteins and DNA is a major mechanism in many vital cellular functions such as gene transcription and epigenetic silencing. It is crucial to understand these interactions and the mechanisms by which they control and guide gene regulation pathways and cellular proliferation.

Chromatin immunoprecipitation (ChIP) is a technique to analyse the association of proteins with specific genomic regions in intact cells. ChIP can be used to study changes in epigenetic signatures, chromatin remodelling and transcription regulator recruitment to specific genomic sites. The different steps of the ChIP assay are cell fixation (crosslinking), chromatin shearing, immunoprecipitation, reverse crosslinking followed by DNA purification and analysis of the immunoprecipitated (IP'd) DNA. Enrichment of specific sequences in the IP'd DNA indicates that these sequences were associated with the protein of interest in vivo. Analysis of specific regions can be performed by quantitative polymerase chain reaction (qPCR). However, in recent years, ChIP combined with high-throughput Next-Generation Sequencing (ChIP-seq) has become the gold standard for whole-genome mapping of protein-DNA interactions. ChIP-seq on transcription factor has notably given insights on their binding sites. The High Resolution Library Preparation kit was developped to increase the resolution of the ChIP-seq data. This new technique for ChIP-seq library preparation reduces ChIP-seq peaks width resulting in a better determination of DNA motif bound by trancription factors. This new Diagenode kit allows to generate high resolution ChIP-seq data on Illumina Sequencing plateforms.

High Resolution ChIP-seq workflow

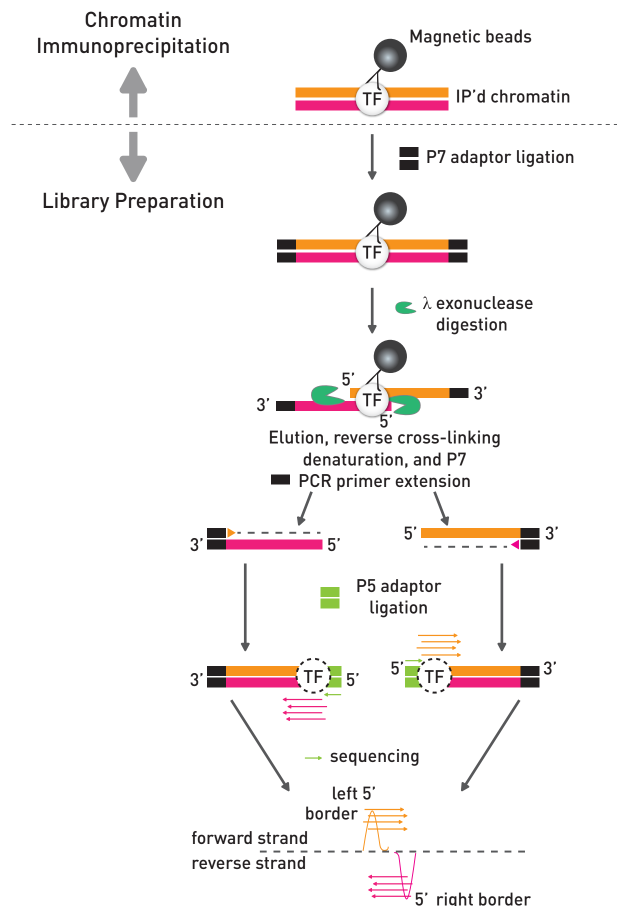

Figure 1: After ChIP, a P7-adaptor is ligated to the ChIP'd DNA. Then a 5-to-3 lambda exonuclease digests up to the transcription factor binding site and selectively eliminates the P7 adaptor sequence attached at the 5 end of each strand. After crosslink reversal and elution the single-stranded DNA is made double-stranded by P7 PCR primer extension.

Afterwards a P5 adaptor is ligated to the exonuclease-treated end, and the resulting library anlyzed by Next-Generation Sequencing

By mapping the 5 ends of the resulting sequencing tags to the reference genome the bound motif can be determined accurately.

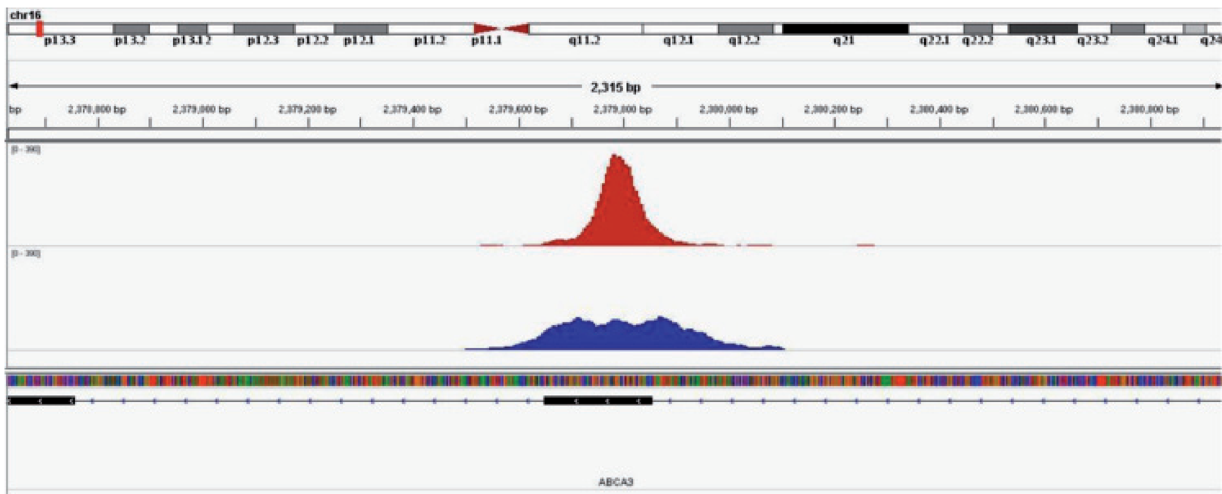

Figure 2: Two ChIP-seq experiments were performed using an antibody against ER alpha on the same samples. The libraries for one of the experiments (top lane, red) were produced using the High Resolution Library Preparation Kit, the other (bottom lane, blue) is a standard ChIP-seq experiment. The figure shows the enrichments near the known ER alpha target gene ABCA3 as a bar chart of read coverage. The scale of both profiles is identical. Due to the tighter read distribution, the High Resolution Library Preparation Kit enables the accurate identification of the actual binding site, which is on the exon. In contrast, the standard ChIP-seq produced a less significant, more dispersed enrichment pattern.

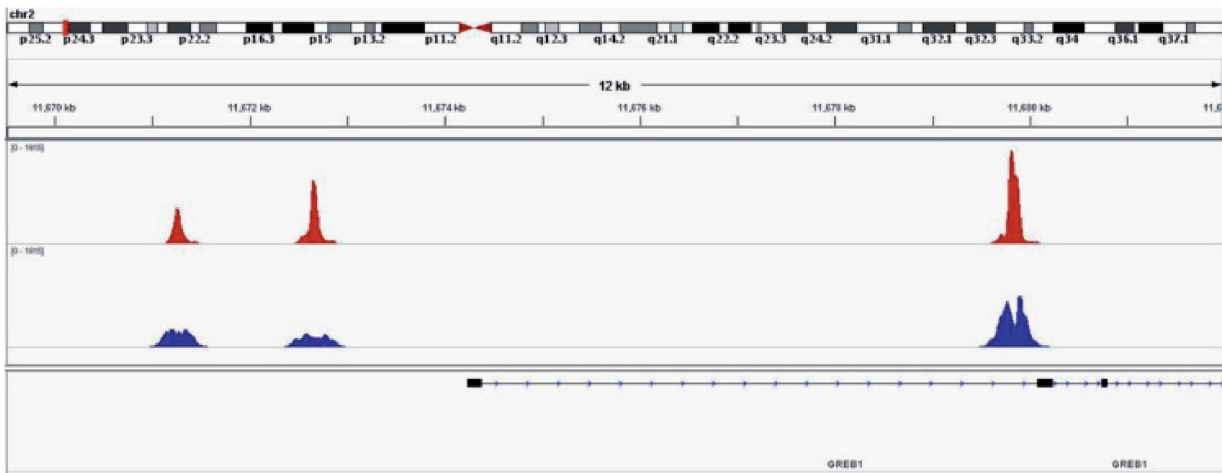

Figure 3: Two ChIP-seq experiments were performed using an antibody against ER alpha on the same samples. The libraries for one of the experiments (top lane, red) were produced using the High Resolution Library Preparation Kit, the other (bottom lane, blue) is a standard ChIP-seq experiment. The figure shows the enrichments near the known ER alpha target gene GREB1 as a bar chart of read coverage. The scale of both profiles is identical. It is evident that with the High Resolution Library Preparation Kit the read distribution is significantly tighter around the binding sites, giving a higher enrichment and less background.

Kit Materials

The content of the kit is sufficient to perform 24 ChIP assays, 6 chromatin extractions and 6 chromatin shearing optimization reactions. Store the components at the indicated temperature upon receipt (Table 1).

Table 1: Components supplied with the High Resolution Library Preparation kit

Description	Quantity	Storage
Wash Buffer 1	370 ml	4°C
Wash Buffer 2	240 ml	4°C
End Repair Buffer	1710 µl	**-20°C**
Ligation Buffer 1	1910 µl	**-20°C**
Nick Repair Buffer	1900 µl	**-20°C**
Nuclease 2 Buffer	1910 µl	**-20°C**
Elution Buffer	3900 µl	4°C
Proteinase K	100 µl	**-20°C**
P7 primer	450 µl	**-20°C**
dNTP	20 µl	**-20°C**
P5 adaptor	470 µl	**-20°C**
Resuspension Buffer	1250 µl	4°C
Precipitant eP1	310µl	4°C
Co-Precipitant eCP1	40 µl	**-20°C**
Ultra Pure Water	400 µl	4°C
Indexing Reagent 2	22 µl	**-20°C**
Indexing Reagent 4	22 µl	**-20°C**
Indexing Reagent 5	22 µl	**-20°C**
Indexing Reagent 6	22 µl	**-20°C**
Indexing Reagent 7	22 µl	**-20°C**
Indexing Reagent 12	22 µl	**-20°C**
Indexing Reagent 16	22 µl	**-20°C**
Indexing Reagent 19	22 µl	**-20°C**

Required Materials Not Provided

- T4 DNA polymerase (3U/µl) (NEB M0203L)
- Klenow fragment DNA pol (5U/µl) (Enzymatics P7060L)
- T4 PNK (10U/µl) (Enzymatics Y9040L)
- T4 DNA ligase (2000 U/µl) (NEB M0202M)
- Phi29 polymerase 10U/µl (NEB M0269L)
- Lambda exonuclease 5U/µl (NEB M0262L)
- Lambda exonuclease buffer 10x (NEB B0262S)
- RecJf exonuclease 30U/µl (NEB M0264L)
- Phi29 commercial reaction buffer 10x (NEB B0269S)
- T4 DNA ligase buffer 10x (NEB B0202S)
- NEBNext High-Fidelity 2X PCR Master Mix (NEB M0541L)
- Gloves to wear at all steps
- Ethanol
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- AMPure beads
- Agarose
- Sybrsafe
- Glycerol
- TE
- Xylene Cyanol FF (Sigma, Cat N° X4126)
- QIAgen MinElute gel extraction kit
- Quant-IT dsDNA HS assay kit (Invitrogen)

Equipment and accessories:

- Magnetic rack for 2 ml tubes
- DiaMag02 magnetic rack (Cat N° kch-816-001)
- Refrigerated centrifuge for 1.5 ml tubes
- Thermomixer for 2 ml tubes
- Vortex
- Qubit system
- qPCR cycler
- System for agarose gel migration and transilluminator

Kit method overview and time table

Table 3: High Resolution Library Preparation kit protocol overview

Step		Time needed	Day
1	End repair	1h	1
2	Adapter 1 ligation	1h15	1
3	Nick repair	30 min	1
4	Nuclease digestions	1h30	1
5	Elution and DNA purification	4h to ON	1-2
6	Second Strand Synthesis	2h15	2
7	Adapter 2 ligation	2h	2
8	Library amplification	1h45	2
9	Size selection	2h	2 or 3

Index Sequences

Index	Sequence	Index	Sequence
2	CGATGT	7	CAGATC
4	TGACCA	12	CTTGTA
5	ACAGTG	16	CCGTCC
6	GCCAAT	19	GTGAAA

Notes Before Starting

Starting material

This protocol is for use at the end of a ChIP protocol, with ChIP'd DNA still linked to magnetic beads. So after the incubation of beads with antibody and chromatin, you can start following the High Resolution Library Preparation kit. The experimental reaction conditions should be the same as for a classical ChIP-seq experiment.

Reactions on beads

During the bead washes with Wash Buffer eW2, it is important to leave the tubes on the magnet. After the last wash with Wash Buffer eW2, spin quickly the beads, put them on the magnetic rack and remove any residual Wash Buffer eW2. Then resuspend the pellet of beads directly in the enzymatic reaction mix. Make sure the beads do not dry during the procedure as this will result in reduced performance. Don't let the beads in the Wash Buffer eW2 for too long, so prepare the enzymatic mix before washing the beads.

In addition during all the washes, make sure not to loose beads as this will reduce the amount of library generated. Be very carreful during the second wash with Wash Buffer eW2. In addition, to avoid sample lost, it is recommended to add Wash Buffer eW1 directly in the enzymatic mix during the first wash with Wash Buffer eW1. It is important to vortex each reaction mix in order to have an homogeneous mix before addition to the beads. In addition, vortex the beads after the addition of the enzymatic mix. However make sure that there are no beads in the cap before starting the incubation. In addition, it is recommended to work with 2 ml tubes for enzymatic reactions to facilitate the mixing of the beads during the different incubation periods.

Purification using AMPure beads

AMPure XP purification is the prefered method for library purification after the different enzymatic reactions out of beads. It is recommended to add AMPure beads using a 1,8 ratio. In addition, always use freshly prepared ethanol 80%.

Quantification and pool of the libraries

Determine the concentration of the libraries generated with a highly sensitive method such as the 'Quant-IT dsDNA HS assay kit' on the Qubit system from Invitrogen. In most cases it is sufficient to use only 1 μl of the library for quantification. Individual libraries can be pooled at desired molar ratio to allow multiplex sequencing of the pooled libraires. Libraries that are being pooled must have been prepared with different Indexing Reagents. When using fewer than 12 indices, follow Illumina Multiplexing Sample Preparation recommandations as summurized in the table below:

Plexity	Option	Index
2	1	AD006 and AD012
	2	AD005 and AD019
3	1	AD002 and AD007 and AD019
	2	AD005 and AD006 and AD015
	3	2-plex options with any other adapter
4	1	AD005 and AD006 and AD012 and AD019
	2	AD002 and AD004 and AD007 and AD016
	3	3-plex options with any other adapter

Sequencing

The High resolution ChIP-seq Library Preparation kit generates librairies which are ready for cluster amplification and sequencing on the Illumina Genome Analyzer, HiSeq and MiSeq plateforms using standard Illumina clustering and sequencing reagents and protocols for multiplexed libraries.

Short protocol

STEP 1. End repair

1. Spin the tubes containing the beads-antibody-chromatin mix and transfer the samples to new 2 ml tubes
2. Wash 6 times the beads with Wash Buffer eW1
3. Wash 2 times the beads with Wash Buffer eW2 (with the tubes on the magnetic rack)
4. Prepare the End Repair pre-mix following the table below and mix by vortexing

Component	Volume for 1 reaction
End Repair Buffer	89 µl
T4 DNA polymerase (3U/µl)	5 µl
Klenow fragment DNA pol (5U/µl)	1 µl
T4 PNK (10U/µl)	5 µl

5. Add 100 µl of the End Repair pre-mix to the beads and mix by vortexing.
6. Incubate at 30°C for 30 min in a thermomixer (900 rpm)
7. Wash 2 times the beads with Wash Buffer eW1
8. Wash 2 times the beads with Wash Buffer eW2 (with the tubes on the magnetic rack)

STEP 2. Adapter 1 ligation

9. Prepare the adapter ligation pre-mix and mix by vortexing

Component	Volume for 1 reaction
Ligation buffer 1	99 µl
T4 DNA ligase(2000 U/µl)	1 µl

10. Add 100 µl of the adapter ligation pre-mix to the beads and mix by vortexing.
11. Incubate at 25°C for 60 min in a thermomixer (900 rpm)
12. Wash 2 times the beads with Wash Buffer eW1
13. Wash 2 times the beads with Wash Buffer eW2 (with the tubes on the magnetic rack)

STEP 3. Nick repair

14. Prepare the Nick repair pre- mix and mix by vortexing

Component	Volume for 1 reaction
Nick repair buffer	98.5 µl
Phi29 polymerase (10U/µl)	1.5 µl

15. Add 100 µl of the nick repair pre-mix to the beads and mix by vortexing.
16. Incubate at 30°C for 20 min in a thermomixer (900 rpm)
17. Wash 2 times the beads with Wash Buffer eW1
18. Wash 2 times the beads with Wash Buffer eW2 (with the tubes on the magnetic rack)

STEP 4. Nuclease digestions

19. Prepare the Nuclease 1 pre-mix and mix by vortexing

Component	Volume for 1 reaction
Lambda exonuclease buffer (10X)	10 µl
Lambda exonuclease (5U/µl)	2 µl
Water	88 µl

20. Add 100 µl of the nuclease 1 pre-mix to the beads and mix by vortexing.
21. Incubate at 37°C for 30 min in a thermomixer (900 rpm)
22. Wash 2 times the beads with Wash Buffer eW1
23. Wash 2 times the beads with Wash Buffer eW2 (with the tubes on the magnetic rack)
24. Prepare the Nuclease 2 pre-mix and mix by vortexing

Component	Volume for 1 reaction
Nuclease 2 Buffer	99 µl
RecJ exonuclease (30U/µl)	1 µl

25. Add 100 µl of the Nuclease 2 pre-mix to the beads and mix by vortexing.
26. Incubate at 37°C for 30 min in a thermomixer (900 rpm)
27. Wash 2 times the beads with Wash Buffer eW1
28. Wash 2 times the beads with Wash Buffer eW2 (with the tubes on the magnetic rack)

STEP 4. Elution and DNA purification

29. Add 200 µl of Elution Buffer and 5 µl of proteinase K to the beads. Incubate for 4 hours to ON in a thermomixer at 1300 rpm and 65°C.
30. Place IP tubes in the magnetic rack. Keep the bead captured and transfer the supernatant to a new tube. Add 200 µl of TE.
31. Add 400 µl of phenol/chloroform/isoamyl alchohol to each sample and mix by vortexing.
32. Centrifuge samples for 10 minutes at 14,000 x g at RT.
33. Transfer the upper aqueous phase into new 1.5 ml tubes.
34. Precipitate the DNA by adding 16 µl of precipitant eP1, 2 µl of co-precipitant eCP1 and 800 µl of cold 100% ethanol to the sample. Vortex and incubate at -80°C for 30 min.
35. Centrifuge for 25 minutes at 14,000 x g at 4°C. Remove the supernatant and add 800 µl of ice-cold 70 % ethanol to the pellet.
36. Centrifuge for 10 minutes at 14,000 x g at 4°C. Remove the supernatant, leave tubes opened at RT to evaporate the remaining ethanol.
37. Resuspend the pellet in 20 µl of Ultra Pure Water (pre-heated at 55°C).

Step 6: Second strand synthesis

38. Transfer the samples into PCR tubes and incubate in a thermocycler at 95°C for 5 min.

39. Prepare the Second Strand Synthesis pre-mix and mix by vortexing

Component	Volume for 1 reaction
P7 primer	23 µl
Phi29 reaction buffer (10x)	5 µl

40. Add 28 µl of Second Strand Synthesis pre-mix to the 20 µl of sample. Mix by vortexing and incubate in a thermocycler:

1 cycle	65°C	5 min
1 cycle	30°C	2 min

41. Add 1 µl of Phi29 Polymerase and 1 µl of dNTP per tube. Vortex and incubate in a thermal cycler:

1 cycle	30°C	20 min
1 cycle	65°C	10 min

42. Purify the samples using AMPure beads with 1,8 volume of beads and elute in 20 µl of RSB.

Step 7: Adapter 2 ligation

43. Prepare the ligation 2 pre-mix and mix by vortexing

Component	Volume for 1 reaction
P5 adapter	24 µl
T4 DNA ligase buffer (10x)	5 µl
T4 DNA ligase (2000U/µl)	1 µl

44. Add 30 µl of the ligation 2 pre-mix to the 20 µl of sample, mix by vortexing, and incubate in a thermal cycler:

1 cycle	25°C	60 min
1 cycle	65°C	10 min

45. Purify the samples using AMPure beads with 1,8 volume of beads and elute in 20 µl of RSB.

Step 8: Library Amplification

46. Add 5 µl of Indexing Reagent and 25 µl of NEBNext High-Fidelity 2X PCR Master Mix to each tube, mix by vortexing and incubate in a thermal cycler using the following conditions:

1 cycle	98°C	30 sec
18 cycles	98°C	10 sec
	65°C	30 sec
	72°C	30 sec
1 cycle	72°C	5 min
1 cycle	4°C	Hold until next step

47. Purify the sample using AMPure beads with 1,8 volume of beads and elute in 25 µl of RSB.

Step 9: Size selection on agarose gel

48. Add 10 µl of 50 % glycerol containing Xylene cyanol to the samples and run them on a 2 % agarose gel containg Sybrsafe. Cut out bands between 200 and 350 bp

49. Purify using the QIAgen Min elute gel extraction kit following the manufacturer's recommendations.

Detailed protocol

STEP 1. End repair

1. After the ChIP reaction, briefly spin the tubes containing the beads associated with the antibody and chromatin and transfer the sample into a new 2 ml tubes.
2. Place the tubes in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW1. To wash the beads, add 1,2 ml of Wash Buffer eW1, remove the tubes from the magnet and shake the tubes to resuspend the beads.
3. Repeat the wash as described above five time with Wash Buffer eW1.
4. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW2. To wash the beads, add 1 ml of Wash Buffer eW2. With the tubes on the magnet, gently shake the tubes.
5. Repeat the wash as described above once with Wash Buffer eW1.
6. After the last wash with wash Buffer eW2, briefly spin the tubes, place them on the magnetic rack and remove residual Wash Buffer eW2.
7. In a separate tube, prepare the End Repair pre-mix following the table below and mix by vortexing

Component	Volume for 1 reaction
End Repair Buffer	89 µl
T4 DNA polymerase (3U/µl)	5 µl
Klenow fragment DNA pol (5U/µl)	1 µl
T4 PNK (10U/µl)	5 µl

8. Add 100 µl of the End Repair pre-mix from Step 1 point 7 to the beads and mix by vortexing to fully resuspend the beads.
9. Incubate at 30°C for 30 min in a thermomixer (900 rpm)
10. Briefly spin the tubes and add 1,2 ml of Wash Buffer eW1. Remove the tubes from the magnet and shake the tube to resuspend the beads.
11. Spin the tubes briefly and place them in the magnetic rack. Wait for one minute and remove the supernatant. Add 1,2 ml of Wash Buffer eW1, remove the tubes from the magnet and shake the tubes to resuspend the beads.
12. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW2. To wash the beads, add 1 ml of Wash Buffer eW2. With the tubes on the magnet, gently shake the tubes.
13. Repeat the wash as described above once with Wash Buffer eW2.
14. After the last wash with wash Buffer eW2, briefly spin the tubes, place them on the magnetic rack and remove residual Wash Buffer eW2.

STEP 2. Adapter 1 ligation

15. In a separate tube, prepare the Adapter Ligation pre-mix and mix by vortexing

Component	Volume for 1 reaction
Ligation Buffer 1	99 µl
T4 DNA ligase(2000 U/µl)	1 µl

16. Add 100 µl of the Adapter Ligation pre-mix from Step 2 point 15 to the beads and mix by vortexing to fully resuspend the beads.
17. Incubate at 25°C for 60 min in a thermomixer (900 rpm)
18. Briefly spin the tubes and add 1,2 ml of Wash Buffer eW1. Remove the tubes from the magnet and shake the tube to resuspend the beads.
19. Spin the tubes briefly and place them in the magnetic rack. Wait for one minute and remove the supernatant. Add 1,2 ml of Wash Buffer eW1, remove the tubes from the magnet and shake the tubes to resuspend the beads.
20. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW2. To wash the beads, add 1 ml of Wash Buffer eW2. With the tubes on the magnet, gently shake the tubes.
21. Repeat the wash as described above once with Wash Buffer eW2.
22. After the last wash with wash Buffer eW2, briefly spin the tubes, place them on the magnetic rack and remove residual Wash Buffer eW2.

STEP 3. Nick repair

23. In a separate tube, prepare the Nick Repair pre-mix and mix by vortexing

Component	Volume for 1 reaction
Nick Repair Buffer	98.5 µl
Phi29 polymerase (10U/µl)	1.5 µl

24. Add 100 µl of the Nick Repair pre-mix from Step 3 point 23 to the beads and mix by vortexing to fully resuspend the beads.

25. Incubate at 30°C for 20 min in a thermomixer (900 rpm)

26. Briefly spin the tubes and add 1,2 ml of Wash Buffer eW1. Remove the tubes from the magnet and shake the tube to resuspend the beads.

27. Spin the tubes briefly and place them in the magnetic rack. Wait for one minute and remove the supernatant. Add 1,2 ml of Wash Buffer eW1, remove the tubes from the magnet and shake the tubes to resuspend the beads.

28. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW2. To wash the beads, add 1 ml of Wash Buffer eW2. With the tubes on the magnet, gently shake the tubes.

29. Repeat the wash as described above once with Wash Buffer eW2.

30. After the last wash with wash Buffer eW2, briefly spin the tubes, place them on the magnetic rack and remove residual Wash Buffer eW2.

STEP 4. Nuclease digestions

31. In a separate tube, prepare the Nuclease 1 pre-mix and mix by vortexing

Component	Volume for 1 reaction
Lambda exonuclease buffer (10X)	10 µl
Lambda exonuclease (5U/µl)	2 µl
Water	88 µl

32. Add 100 µl of the Nuclease 1 pre-mix from Step 4 point 31 to the beads and mix by vortexing to fully resuspend the beads.
33. Incubate at 37°C for 30 min in a thermomixer (900 rpm)
34. Briefly spin the tubes and add 1,2 ml of Wash Buffer eW1. Remove the tubes from the magnet and shake the tube to resuspend the beads.
35. Spin the tubes briefly and place them in the magnetic rack. Wait for one minute and remove the supernatant. Add 1,2 ml of Wash Buffer eW1, remove the tubes from the magnet and shake the tubes to resuspend the beads.
36. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW2. To wash the beads, add 1 ml of Wash Buffer eW2. With the tubes on the magnet, gently shake the tubes.
37. Repeat the wash as described above once with Wash Buffer eW2.
38. After the last wash with Wash Buffer eW2, briefly spin the tubes, place them on the magnetic rack and remove residual Wash Buffer eW2.
39. In a separate tube, prepare the Nuclease 2 pre-mix and mix by vortexing

Component	Volume for 1 reaction
Nuclease 2 Buffer	99 µl
RecJ exonuclease (30U/µl)	1 µl

40. Add 100 µl of the Nuclease 2 pre-mix from Step 4 point 39 to the beads and mix by vortexing to fully resuspend the beads.
41. Incubate at 37°C for 30 min in a thermomixer (900 rpm)
42. Briefly spin the tubes and add 1,2 ml of Wash Buffer eW1. Remove the tubes from the magnet and shake the tube to resuspend the beads.
43. Spin the tubes briefly and place them in the magnetic rack. Wait for one minute and remove the supernatant. Add 1,2 ml of Wash Buffer eW1, remove the tubes from the magnet and shake the tubes to resuspend the beads.
44. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer eW2. To wash the beads, add 1 ml of Wash Buffer eW2. With the tubes on the magnet, gently shake the tubes.
45. Repeat the wash as described above once with Wash Buffer eW2.
46. After the last wash with wash Buffer eW2, briefly spin the tubes, place them on the magnetic rack and remove residual Wash Buffer eW2.

STEP 5. Elution and DNA purification

47. Add 200 µl of Elution Buffer to the beads. Then add 5 µl of proteinase K to each tube. Mix and incubate for 4 hours to ON in a thermomixer at 1300 rpm and 65°C.
48. Briefly spin the tubes and place them in the magnetic rack. Transfer the supernatant to a new tube. Add 200 µl of TE.
49. Add 400 µl of phenol/chloroform/isoamyl alchohol to each sample and mix by vortexing.
50. Centrifuge samples for 10 minutes at 14,000 x g (13,000 rpm) at room temperature.
51. Transfer the upper aqueous phase into new 1.5 ml tubes.

52. Precipitate the DNA by adding 16 µl of precipitant eP1, 2 µl of co-precipitant eCP1, and 800 µl of cold 100% ethanol to the sample. Vortex and incubate at -80°C for 30 min.
53. Centrifuge for 25 minutes at 14,000 x g (13,000 rpm) at 4°C. Carefully remove the supernatant and add 800 µl of ice-cold 70 % ethanol to the pellet.
54. Centrifuge for 10 minutes at 14,000 x g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened at RT to evaporate the remaining ethanol.
55. Resuspend the pellet in 20 µl of Ultra Pure Water (pre-heated at 55°C). Resuspend by pipetting up and down.

STEP 6. Second strand synthesis

56. Transfer the samples into PCR tubes.
57. Incubate in a thermal cycler at 95°C for 5 min.
58. After the incubation, transfer immediatly the tubes on ice.
59. In a separate tube, prepare the Second Strand Synthesis pre-mix and mix by vortexing

Component	Volume for 1 reaction
P7 primer	23 µl
Phi29 reaction buffer (10x)	5 µl

60. Add 28 µl of Second Strand Synthesis pre-mix to the DNA sample from step 58. Mix by vortexing and incubate in a thermocycler using the following conditions:

1 cycle	65°C	5 min
1 cycle	30°C	2 min

61. Add 1 µl of Phi29 Polymerase (10 U/µl) and 1 µl of dNTP in each tube. Mix by vortexing. Briefly centrifuge the tubes and incubate in a thermal cycler using the following conditions:

1 cycle	30°C	20 min
1 cycle	65°C	10 min

62. Briefly spin the tubes and add 90 µl of AMPure beads. Vortex to mix the beads with the sample.
63. Incubate at RT for 15 min.
64. Place the tube on a magnetic rack until the liquid appears clear.
65. Remove the supernatant and with the tubes on the magnetic rack add 180 µl of freshly prepared ethanol 80%.
66. Incubate for 30 sec and remove te supernatant.
67. Repeat the wash with ethanol as described above.
68. After the second wash with ethanol, spin briefly the tubes and place them on the magnetic rack. Remove the residual trace of ethanol.
69. Remove the tubes from the magentic rack and leave at RT for 15 min to dry the beads.
70. Resuspend the dried beads in 20 µl of Resuspension Buffer (preheated to RT) by vortexing.
71. Incubate at RT for 2 min
72. Briefly centrifuge the tubes and place them on the magnetic rack. Transfer the supernatant into new PCR tubes.

STEP 7. Adapter 2 ligation

73. In a separate tube, prepare the Ligation pre-2 mix and mix by vortexing

Component	Volume for 1 reaction
P5 adapter	24 µl
T4 DNA ligase buffer (10x)	5 µl
T4 DNA ligase (2000U/µl)	1 µl

74. Add 30 µl of the Ligation 2 pre-mix from Step 7 point 73 to the 20 µl of sample and mix by vortexing.
75. Briefly spin the tubes and incubate in a thermal cycler using the following conditions:

1 cycle	25°C	60 min
1 cycle	65°C	10 min

76. Briefly spin the tubes and add 90 µl of AMPure beads. Vortex to mix the beads with the sample.
77. Incubate at RT for 15 min.
78. Place the tube on a magnetic rack until the liquid appears clear.
79. Remove the supernatant and with the tubes on the magnetic rack add 180 µl of freshly prepared ethanol 80%.
80. Incubate for 30 sec and remove te supernatant.
81. Repeat the wash with ethanol as described above.
82. After the second wash with ethanol, spin briefly the tubes and place them on the magnetic rack. Remove the residual trace of ethanol.
83. Remove the tubes from the magentic rack and leave at RT for 15 min to dry the beads.
84. Resuspend the dried beads in 20 µl of Resuspension Buffer (preheated to RT) by vortexing.
85. Incubate at RT for 2 min
86. Briefly centrifuge the tubes and place them on the magnetic rack. Transfer the supernatant into new PCR tubes.

STEP 8. Library Amplification

87. Add 5 µl of Indexing Reagent and 25 µl of NEBNext High-Fidelity 2X PCR Master Mix to each tube.
88. Vortex the tubes. Briefly spin the tubes and incubate in a thermal cycler using the following conditions:

1 cycle	98°C	30 sec
18 cycles	98°C	10 sec
	65°C	30 sec
	72°C	30 sec
1 cycle	72°C	5 min
1 cycle	4°C	Hold until next step

89. Briefly spin the tubes and add 90 µl of AMPure beads. Vortex to mix the beads with the sample.
90. Incubate at RT for 15 min.
91. Place the tube on a magnetic rack until the liquid appears clear.
92. Remove the supernatant and with the tubes on the magnetic rack add 180 µl of freshly prepared ethanol 80%.
93. Incubate for 30 sec and remove te supernatant.
94. Repeat the wash with ethanol as described above.
95. After the second wash with ethanol, spin briefly the tubes and place them on the magnetic rack. Remove the residual trace of ethanol.
96. Remove the tubes from the magentic rack and leave at RT for 15 min to dry the beads.
97. Resuspend the dried beads in 25 µl of Resuspension Buffer (preheated to RT) by vortexing.
98. Incubate at RT for 2 min

99. Briefly centrifuge the tubes and place them on the magnetic rack. Transfer the supernatant into new 1,5 ml tubes.

STEP 8. Size selection on agarose gel

100. Poor 2 % agarose gel containg Sybrsafe
101. Prepare High Resolution Library samples for migration on agarose gel by adding 10 µl of 50 % glycerol containing Xylene cyanol to each sample.
102. Prepare ladder for migration by additing 3 µl of 50% glycerol containing Xylene cyanol to 8 µl of ladder.
103. Load samples and the ladder on the gel. Run at 120V for 45 min
104. Cut out bands between 200 to 350 bp.
105. Purify using the QIAgen MinElute Gel Extraction kit following the manufacturer's recommendations. However, to dissolve the gel we recommend to use an incubation at RT instead of 50°C. In addition, note that as described in the Qiagen manual, it is recommended to let the column stand 2-5 min after addition of Buffer PE.

ChIP-seq data analysis recommendations

To find the captured regions of the genome after the sequencing you must perform a) a reference alignment followed by b) a peak calling, then c) further data analysis (annotation, visualization etc.) to help you find what you are looking for. There are abundant software tools for each task that use different approaches to the same problem; choose your preferred one considering your dataset and scientific goals. The workflows for different sequencers basically differ only in the alignment step, since every sequencer has its own characteristic read set (short or long, fixed or variable length, nucleotide or colour code etc.).

a) The built-in aligners with default settings worked very well for our ChIP-seq experiments (e.g. ELAND for Illumina®, TMAP for PGM). If you cannot access them, open source tools are also available; we have positive experience with BWA: http://bio-bwa.sourceforge.net. If you use a multipurpose aligner, do not forget to use settings appropriate to your dataset; please consult with the manual of your software.

b) The purpose of the peak calling is to find the enriched regions in the alignment. Take extreme care when you choose and set up your peak caller, since the outcome can vary widely depending on the used software and its settings. We advise you to read the comparative literature and the software manuals to fully understand how a certain program works. One of the key features of your data is the expected length of the enrichment regions. Transcription factors tend to produce short and sharp peaks, while histone marks create broad islands of enrichment. A remarkable tool for sharp peak detection is MACS, while SICER is dedicated to histone marks, and tools like ZINBA can be used for both with decent outcomes. Versions MACS 2 and higher are reported to be better suited for histone marks than previous versions.

The availability of the mentioned softwares:

- MACS: https://github.com/taoliu/MACS
- SICER: http://home.gwu.edu/~wpeng/Software.htm
- ZINBA: http://code.google.com/p/zinba

While MACS is a prominent tool for shorter peaks, sometimes it has difficulties with broader regions, therefore we recommend you to set a wider local peak background and lower the qvalue cutoff if necessary for histone marks. In some cases turning off the local lambda calculation provides a better coverage of broad enrichment islands, though this can result in more false positive peaks detected. Please refer to the MACS manual if you are not sure how to tweak the parameters.

Also as a general recommendation when using peak callers pay particular attention to the stringency, which can be usually set by a p-value, q-value, e-value, FDR or similar probability descriptor, based on the statistical model. Use a stringent setting for short and high enrichments (eg. a good transcription factor dataset) and a lenient setting for noisy data or low, wide, dispersed enrichment patterns (produced by many histone marks).

c) Having your peaks you can start decrypting the epigenetic code.

The visual inspection is a common first step, especially if the aim of your experiment was to see if certain genes have certain histone modifications/transcription factors attached, or you want to check some positive/negative control sites for enrichment. Choose the appropriate viewer software according to the output format of your peak caller and your preferences.

Annotation is always very useful, since you can identify biological features that are relevant to your peaks, or check if you have the peaks at the expected loci, like H3K4me3 enrichments in the promoter regions of active genes. You can expand the annotation with a gene ontology/pathway analysis of the peak associated genes, thus discovering how your transcription factor/histone modification is involved in the cell's or the whole organism's life.

Motif search is almost an obligatory analysis for the sequence specific transcription factors, but you may find common motifs among histone modification sites as well, so you can check for example if you indeed have promoter specific motifs in your theoretically promoter specific enrichments.

A lot of programs, including peak callers themselves output descriptive statistics of the peaks, measuring for example their enrichment ratios, significances, width, heights, reads in peaks. This characterization helps you better understand your data, which is essential for most applications; a typical example is the comparison of performance of different sample preparation protocols or different sequencer setups.

The final recommended analysis type is the comparative analysis. We encourage scientists to use replicates in their experiments; removing peaks that are not common could effectively reduce false positives. You can also use a validated reference set of peaks for comparisons, but that is rarely available. Additionally, if you have other biologically relevant data from your samples, it is wise to compare and integrate them. For example, an RNA-seq dataset is a great source of validation for histone marks that are supposed to regulate gene expression.

Recommended free tools for the peak analysis:

- IGV (visualization): www.broadinstitute.org/igv
- UCSC Genome Browser (visualization): http://genome.ucsc.edu
- HOMER (motif search, annotation, gene ontology, comparison, statistics): http://homer.salk.edu/homer
- PinkThing (annotation, conservation, comparison, gene ontology, statistics): http://pinkthing.cmbi.ru.nl
- GREAT (annotation, statistics): http://great.stanford.edu

When analysing ChIP-seq, please always keep an eye on sequencing quality and the performance of the software tools used for analysis. For example with a low quality sequencing with a lot of read errors you will have a hard time finding the peaks you are looking for, despite your excellent IP'd DNA. To control the quality use the vendor supplied software and metrics, like the ones available in the Illumina® pipeline. Open source tools can also be used, e.g. the FastQC by Babraham Institute: http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc. Some dedicated softwares exist for the quality control of the ChIP as well, like CHANCE: http://song.igb.illinois.edu/chance.html.

Throughout this chapter we recommended some free tools, because they are accessible for everyone and we have tested most of them. Please note that there are commercial softwares for the same purposes as well, most of them capable of performing several tasks, or even a complete ChIP-seq workflow. Here are a few examples that we know of (but we have not tested them):

- CLC Genomics Workbench: http://clcbio.com
- Partek Genomics Suite: www.partek.com/partekgs
- NextGENe: www.softgenetics.com/NextGENe.html
- Strand NGS: www.strand-ngs.com
- Geneious: www.geneious.com
- GenoMiner: http://astridbio.com/genominer-genome-analyzer
- GenoMatix: www.genomatix.de

Troubleshooting guide

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak.	Make sure you perform the fixation step for the correct period of time, at the right temperature and with the correct formaldehyde concentration. e.g: incubate for 8 minutes at room temperature with 1 % formaldehyde final concentration (weight/ volume). Also, use high quality, fresh formaldehyde.
	Cross-linking is too strong.	
	Proteins have unique ways of interacting with the DNA. Some proteins are not directly bound to the DNA but interact with other DNA-associated proteins.	Very short or very long cross-linking time can lead to DNA loss and/or elevated background, therefore the optimal cross-linking time should be found empirically as maximal specificity and efficiency of ChIP.
	Both cross-linking time and formaldehyde concentration are critical.	Cross-linking can affect both efficiency of chromatin shearing and efficiency of specific antigen immunoprecipitation. Shorter cross-linking times (5 to 10 minutes) and/or lower formaldehyde concentrations (1%, weight/ volume) may improve shearing efficiency while, for some proteins especially those that do not directly bind DNA, this might reduce the efficiency of cross-linking and thus the yield of precipitated chromatin.
	The optimal duration of cross-linking varies between cell type and protein of interest.	It is possible to optimize the fixation step by testing different incubation times: such as 10, 20 and 30 minutes. Do not cross-link for longer than 30 minutes as cross-links of more than 30 minutes can not be efficiently sheared.
	Efficient fixation of a protein to chromatin in vivo is a crucial step for ChIP. The extent of cross-linking is probably the most important parameter.	Two major problems concerning the subsequent immunoprecipitation step should be taken into account: 1/ an excess of cross-linking can result in the loss of material or reduced antigen availability in chromatin, or both. 2/ the relative sensitivity of the antigen epitopes to formaldehyde. It is essential to perform the cross-linking step with care.
	It is essential to quench the formaldehyde.	Use glycine to stop the fixation: quench formaldehyde with 125 mM glycine for 5 minutes at room temperature (add 57 µl of 1.25M glycine per 513.5 µl of sample, see STEP 2). Alternatively, wash the fixed cells properly and make sure you get rid of ALL the formaldehyde.
Cell lysis	Temperature is critical.	Perform cell lysis at 4°C (cold room) or on ice. Keep the samples ice-cold at all times during the cell lysis and use ice-cold buffers see STEP 3.
	Protein degradation during lysis can occur.	Add the protease inhibitors to the lysis buffer immediately before use.
Cell type	Kit protocol validation.	HeLa, NCCIT 293T, Chondrocytes, P19, ASC (adipose stem cells) and Keratinocytes have been used to validate the Magnetic ChIP protocol.
Cell number necessary per ChIP	The amount of cells required for a ChIP experiment is determined by cell type, protein of interest and antibodies used.	You can use from 1,000,000 to 10,000,000 cells per IP.

Chromatin shearing	Optimal shearing conditions are important for ChIP efficiency.	Shearing conditions for each cell type must be optimized from cell collection, fixation to shearing method (settings of the sonicator apparatus).
	Critical points for shearing optimization.	1) Not to start with a too large amount of cells (1x 10e6 cells or less is ok) 2) Keep samples cold (4°C) 3) High % SDS favours better sonication but inhibits immunoselection (optimal range: 0,1 to 1%). Dilutions must be adapted accordingly prior to immunoselection; the final SDS concentration should not be higher than 0.15 to 0.20% (e.g. If the shearing buffer contains 0.75% SDS, the sheared chromatin is diluted 3.5 to 4.0 fold in the [P.I.-ChIP buffer 1x])
	Shear the samples of chromatin using the Bioruptor® from Diagenode (cat. No. UCD-200, UCD-300, UCD-400).	Maintain temperature of the samples close to 0°C. The chromatin shearing needs to be optimized for each cell type. A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.
Sheared chromatin analysis	Purify the DNA from the sheared chromatin as described in the kit protocol to analyse the shearing.	Extract total DNA from an aliquot of sheared chromatin and run on 1% agarose gel (stain with EtBr). In order to analyse the sheared chromatin on gel, take DNA purified from the sheared chromatin input -prepared at STEP 3 . Some unsheared chromatin can be analysed on gel as well (purify it as done with the input sample (see "6. Additional protocols" section). Chromatin eqvivalent to 100,000 cells, one million cells or more can for sure be visualized on a gel.
	Do not load too much DNA on a gel.	Loading of large quantities of DNA on agarose gel can lead to poor quality pictures, which do not reflect the real DNA fragmentation. The DNA amount to load depends on the size of the well and on the gel size.
	Agarose concentration.	Do not use more than 1-1.5% agarose gel and run slowly (Volt/cm and time depend on the gel size).
	Running buffer concentration.	1x TAE or TBE is preferred to 0.5x TAE, which can lead to smears on agarose gel.
Sheared chromatin amounts	How much sheared chromatin do I need to prepare?	Most of the sheared chromatin is to be used in the ChIP experiment, but remember that some of the sheared chromatin is needed as control as it corresponds to the input sample for the ChIP experiment and it can also be checked on agarose gel.
	Dilute the sheared chromatin in ChIP buffer for Immuno-selection incubation.	The sheared chromatin is diluted in complete Buffer A prior to the immunoselection incubation (see STEP 3: Add 870 µl of complete Buffer A to the 130 µl of sheared chromatin). Dilute the sheared chromatin at least 7 fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Antibody binding beads	Beads are in suspension.	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Bead centrifugation.	Don't spin the beads at high speed. Use gentle centrifugation (500 x g for 2-3 minutes) as described in the manual protocol. g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of rotation in mm. (http://www.msu.edu/~venkata1/gforce.htm). It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage.	Store at 4°C. Do not freeze.
	Antibody binding capacity.	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Protease inhibitors	Storage.	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C, and thawed before use.
Other enzyme inhibitors	Specific enzyme inhibitors are not included in the kit, such as phosphatase inhibitors.	Add phosphatase inhibitors or others to Buffers A and B, if necessary, depending on your research field and protein(s) of interest to be ChIP'd Add NaBu for histone ChIPs.

Negative ChIP control(s)	Use non-immune IgG in the IP incubation mix.	Use the non-immune IgG fraction from the same species the antibodies were produced in.
	Do not add antibody to the IP.	Incubation with beads, which were not coated with antibodies antibodies could also be used as a negative ChIP control as well as non-immune IgG. At STEP 4, the IP incubation mix includes sheared chromatin and beads but no antibody.
	Use antibody and specifically blocked antibody in parallel.	Use one antibody in ChIP and, and the same antibody that is blocked with specific peptide. To specifically blocked one antibody: pre-incubate the antibody with saturating amounts of its epitope specific peptide for about 30 minutes at room temperature before use in the IP incubation mix. Use in ChIP, the blocked antibody as a negative control in parallel with the unblocked antibody.
Antibody in IP	How many negative controls are necessary?	If multiple antibodies - of the same specie - are to be used with the same chromatin preparation then a single negative ChIP control is sufficient for all of the antibodies used.
	Why is my antibody not working in ChIP?	Antibody-antigen recognition can be significantly affected by the cross-linking step resulting in loss of epitope accessibility and/or recognition.
	Which antibody should I use in ChIP?	Use ChIP-grade antibodies. If not available, it is recommended to use several antibodies directed against different epitopes of the same protein. Verify that the antibodies can work directly in IP on fresh cell extracts. Also, when testing new antibodies, include known ChIP-grade antibodies as positive control for your ChIP assay.
	How do I choose an antibody for ChIP?	Be aware of the possible cross-reactivity of antibodies. Verify by Western blot analysis the antibody specificity. Antigen affinity purification can be used to increase titer and specificity of polyclonal antibodies.
	Are my antibodies going to bind the protein A or protein G?	There is a significant difference in affinity of different types of immunoglobulins to protein A or G. Thererfore, in function of the antibody used for your ChIP, it is recommended to choose either protein A or protein G coated beads.

Species	Immunglobulli Isotype	Protein A	Protein G
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++
	IgGM	Use anti Human IgM	
	IgGF	-	+
	IgGA	-	+
Mouse	IgG1	+	+++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+
	IgGM	Use anti Mouse IgM	
Rat	IgG1	-	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Chicken	All Isotypes	-	++
Cow	All Isotypes	++	+++
Goat	All Isotypes	-	++
Guinea Pig	All Isotypes	+++	++
Hamster	All Isotypes	+	++
Horse	All Isotypes	++	+++
Pig	All Isotypes	+	++
Rabbit	All Isotypes	+++	++
Sheep	All Isotypes	-	++

Immuno-selection incubation	What is the best incubation time for immunoselection using the ultrasonic water bath?	To incubate the sheared chromatin with antibodies for 15 to 30 minutes works for many antibodies, however, the kinetics for reaching equilibrium of epitope-antibody binding may differ for each antibody and target. Optimization might improve the results (e.g. the incubation time may need to be increased for some antibodies).
	How does the immunoselection using the ultrasonic water bath work?	The rate-limiting step in many immunoassays is associated with the slow kinetics of binding of macro-molecular antigen to antibody. It was demonstrated that the use of ultrasonic energy to enhance mass transport across liquid/solid interfaces can dramatically accelerate antigen binding to antibodies.
	What are the water bath specifications?	Model MT-3510. Capacity: 5.5 liters. Size (LxWxH): 29x15x15 cm. Frequency: 42 kHz. Max power requirement: 130 W. RF-Power: 130 W
	Can I use the kit w/o an ultrasonic water bath?	Yes, then a long incubation at 4ºC should be used. Depending on the antibody and target to be ChIP'd, the times of incubation range from 2 to 16 hours and should be determined empirically for each antibody.
Polymerase Chain Reaction	Primer design.	Primer length: 18 to 24 nucleotides/ Primer Tm: 60°C (+/- 3.0ºC)/ % GC: 50% (+/- 4%)
	Controls: negative and positive.	Negative PCR controls: PCR with DNA from samples IP'd with non-immune antibodies (negative IgG). Alternatively, PCR using DNA from ChIP samples and primers specific for a DNA region to which, your antigen of interest is not binding. Positive PCR control: PCR using input DNA.
	No PCR signal.	Include a positive PCR control as a control for your PCR mix (your primers, dNTP and Master Mix) using the Input DNA or a DNA sample of the same origin.
	High Ct values.	Use more input chromatin.
	CtNegCtl and CtTarget.	The ratio between target IP and negative control IP depends on the antibody used.
	Background is high.	Verify that you perform properly the following steps: Keep the antibody binding beads and DNA purifying slurry in suspension while adding to tubes. Check by eye that equal pellets of beads and slurry are present in each tube. Washes (step 5) are critical.
	Using end-point PCR rather than quantitative PCR.	If gel electrophoresis is used to estimate intensities of PCR products, then relative occupancy of a factor at a locus is the ratio of the intensity of the target IP band to the negative control IP band.
Freezing	Samples can be frozen at several steps of the protocol.	Pellets of formaldehyde fixed cells can be stored at - 80oC for at least a year. Sheared chromatin can be stored at - 80ºC for months, depending on the protein of interest to be ChIP'd. Purified DNA from ChIP and input samples can be stored at -20ºC for months.
	Avo id multiple freeze/thawing.	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)

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The developement of the kit has partly been funded by FP7-collaborative project. (N° 221952)

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