

New Approaches to Enhance Chromatin Immunoprecipitation Efficiency

Kits versus in house protocols

University of Debrecen, Medical and Health Science Center
Department of Biochemistry and Molecular Biology
Nagyerdei krt 98
Debrecen 4032
Hungary

Authors: Balint L. Balint, Zoltan Simandi, Szilard Poliska, Ophir Keret and Laszlo Nagy
Corresponding author: lbalint@med.unideb.hu

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Introduction

Chromatin immunoprecipitation (ChIP) is an extremely powerful technique that allows the study of *in vivo* protein-DNA interactions. Contrary to other techniques used to monitor these types of interactions, ChIP gives information about the "time and space" occupancy of a DNA element by a native protein in its native chromatin environment¹.

The first in depth description was published already in 1997³ by Valerio Orlando, but its first occurrence in PubMed with the name "chromatin immunoprecipitation" was only in 1998². Since then, this method was used in nearly 5,000 publications. By now, the ChIP technology made a fundamental contribution to the rapid growth of an entire field of research, namely the field of epigenetics.

While ChIP is an extremely powerful technique, its wide usage is still limited by several factors. Main factors that slowed down its spread in the scientific field, are the lack of clear standards, high lab-to-lab variations and its highly labour intensiveness.

The basic idea behind the chromatin immunoprecipitation was the combination of cross-linking used to study *in vivo* protein-protein interaction and immunoprecipitation used for protein purification with a method to identify the bound DNA fragments. A similar method to ChIP was developed to study *in vivo* protein-DNA interactions in *Escherichia coli* already in 1985⁵. This method used formaldehyde to fix the protein-DNA interactions, sonication for shearing of the created complexes, several rounds of hybridization-selection and detection by specific immunosera against RNA polymerase holoenzyme. In the first ChIP publications a Southern blot-like method was used⁶ and as the radioactively labeled probe the ChIP'd DNA itself. This approach was rapidly replaced by PCR and now real time PCR is used as a state of

the art method. The development of tiling arrays⁷ and of high-throughput sequencing methods⁸ as unbiased DNA detection methods are opening new perspectives for the ChIP technology. On the other hand these new unbiased DNA detection methods are relatively expensive and the results have to be validated by classical approaches, this is why, majority of ChIP experiments at present stage are gene-centered. The future of ChIP is inevitably the whole genome approach by using tiling arrays⁷ (ChIP-on-chip) or high-throughput sequencing⁸ (ChIP-Seq). On the other hand the high price of these technologies claims for high quality inputs from the ChIP side.

Several novelties can be seen in the field of ChIP technologies like carrier ChIP⁹, μ ChIP¹⁰ or Matrix ChIP¹¹. The arrival of fast ChIP¹² protocols or magnetic ChIP are for sure important steps that will give a fresh air to the field and can be considered as developments of the basic technology that can penetrate into the field of the current applications very rapidly, and can open novel fields for this application, of which the most important could be the field of diagnostics.

Methods

We decided to test in a systematic way the currently used ChIP protocol of our laboratory with the OneDay ChIP kit, a ChIP kit developed recently by Diagenode. Our laboratory is using one of the most known method that could be termed "classical ChIP" and which is based on a protocol published by Kuo and Allis¹³. This protocol is using an overnight immunoprecipitation step, followed by extensive washes with buffers containing variable concentrations of salts and a 65°C reversal of cross-links followed by proteinase K digestion and DNA purification. DNA purification can be done by phenol-chloroform extraction or by using DNA binding columns. Our "in house" protocol was described in details earlier¹⁴.

TABLE 1. Comparison of the two compared protocols.

	In house ChIP protocol	OneDay ChIP kit
Lengths of the protocol	2-3 days	1-2 days
Starting material	1 million cells	Hundred thousand cells
Immunoselection	Overnight on a rotating wheel	Low energy ultrasound bath (30 minutes) or rotating wheel (overnight)
Washing of beads	3+1 buffer system	Single wash buffer
Reversal of cross-links	4-6 hours incubation at 65°C	10 minutes boiling
DNA purification	Phenol chloroform extraction or column	DNA purification slurry

The OneDay ChIP kit of Diagenode introduces several novelties that are improving the current ChIP technology. Developers of the protocol used for this kit¹² decided to find alternative approaches to shorten and ease the long steps of the classical ChIP protocol. The two methods are compared in Table 1.

Our approach was to compare the two protocols using the same chromatin samples and the same antibodies. For this comparison we used the OneDay ChIP kit and QPCR reagents of Diagenode and our own reagents¹⁴. We used a subtype of the promyelocytic leukemia cell line HL60 (CDM1 subtype) that grows in serum free media and that was extensively studied in our laboratory.

OneDay ChIP kit protocol

For the OneDay ChIP method we used the reagents provided with the kit and followed the recommendations of the supplier. Briefly: sheared chromatin from 1 million HL60 cells was used per immunoprecipitation (IP). The chromatin was diluted with 1x ChIP buffer supplemented with proteinase inhibitors and the immunoprecipitation was performed with either an anti-TBP (TBP: TATA box binding protein) antibody or IgG antibodies as a negative control (1 μ l/IP). Immunoselection was performed in a cooled ultrasound water-bath for 30 minutes. After immunoselection tubes were centrifuged for 10 minutes at full speed at 4°C on a tabletop centrifuge and supernatant mixed with washed antibody binding beads. After 1 hour incubation at 4°C on a rotating wheel, the diluted chromatin with 1 ml wash buffer and gently overlaid onto 12 ml of wash buffer supplemented with proteinase inhibitors (transferred in 15 ml conical tube). Tubes were kept on ice for 5 minutes and then centrifuged for 5 minutes 300 g at 4°C. After centrifugation 12 ml of the supernatant was removed, the remaining buffer with the beads transferred to a 1.5 ml tube and centrifuged for 2 minutes 500 g at 4°C. Beads were mixed with 100 μ l DNA purification slurry, boiled for 10 minutes and mixed with the Proteinase K solution provided with the kit. After 30 minutes incubation in a thermo-mixer at 55°C, tubes were boiled again for 10 minutes and spinned. A fraction of 70 μ l of supernatant was removed, and the beads supplemented with 130

μ l of DNase free water, vortexed, spinned and 130 μ l of solution removed and mixed with the first aliquot. Regarding the input sample, 10% of the diluted chromatin was ethanol precipitated, and the pellet dried by leaving tubes with open caps. The dried pellet was resuspended in nuclease free water and purified with the same procedure as described for the ChIP samples. The DNA solution was quantified with a NanoDrop spectrophotometer and 5 μ l was used for subsequent QPCR reactions.

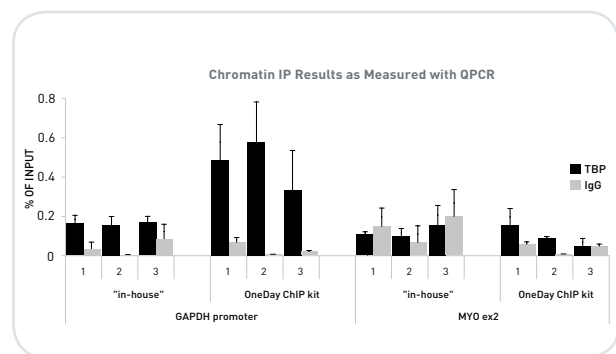


Figure 1. QPCR analysis of ChIP performed with in house protocol or OneDay ChIP kit. Copy numbers of TBP bound GAPDH core promoter fragments and myoglobin exon 2 fragments and their corresponding IgG values normalized to input. Error bars represent the standard deviations of QPCR measurements.

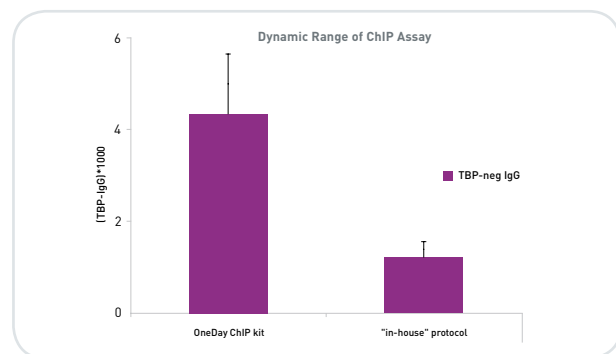


Figure 2. Increase in the dynamic range of the ChIP assay. Values represented are the mean of the three replicate chromatin samples as measured with a QPCR assay for binding of TBP to the GAPDH core promoter. Represented values were calculated with the formula: (TBP-IgG)*1000. Error bars represent the standard deviations of different chromatin samples.

Results

To analyse the specific enrichment of the immunoprecipitated transcription factor (in our case TBP) we used QPCR assays covering the core promoter of the GAPDH gene and the exon 2 region of the myoglobin gene. We expected enrichment on the GAPDH core promoter, without enrichment on myoglobin exon 2, since GAPDH is constitutively expressed in myeloid leukemia cell, while myoglobin is not. As shown on Figure 1 all the DNA samples produced with the OneDay ChIP kit were showing significantly higher values on the positive signals than samples obtained with the "in house" protocol. More importantly the dynamic range of the measurements, namely the difference between the positive signal and the background represented by IgG values increased by 3.5 fold, from a factor of 1.21 in the case of the "in house" protocol to a factor of 4.34 in the case of OneDay ChIP kit, as shown in Figure 2. The ratio of TBP bound fragments to the IgG signals, also named "fold enrichment" shows a similar trend. In all cases the fold enrichment of the TBP if compared to IgG was higher in the samples prepared with the OneDay ChIP kit than using the "in house" protocol (**Figure 3**). These trends can be explained if we analyse the signal values of TBP and IgG samples for the two methods applied. The ratio of the signal values of the OneDay ChIP kit relative to the "in house" protocol increased almost three fold in the case of TBP signal on GAPDH core promoter without a change in the case of TBP on myoglobin exon 2. Similarly, in the case of IgG there was no significant increase in the signal values on the GAPDH core promoter, while the data obtained by the "in house" protocol were showing a higher signal for IgG on myoglobin exon 2. These comparisons are represented on Figure 4. Finally the specificity of the ChIP assay, represented by the ratio of the positive control "bound region" and the negative control "unbound region", in our case GAPDH core promoter and myoglobin exon 2 respectively as seen on Figure 5, was markedly enhanced using the OneDay kit, compared to our in house protocol.

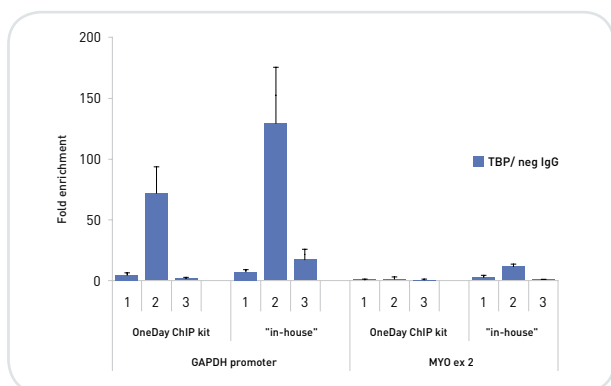


Figure 3. Fold enrichment of TBP compared to IgG values with the two investigated protocols on the two genomic regions. Error bars represent the standard deviations of the normalized QPCR measurements.

Conclusions

In our experiment we compared two ChIP protocols on the same chromatin samples and using the same antibodies, PCR reagents and equipment. We found that the OneDay ChIP kit compared to the "in house" protocol had an increase dynamic range by 3.5 fold and in QPCR signal values by 2.9 fold. These better parameters of the OneDay ChIP kit were reflected in better fold enrichments of TBP compared to IgG and an increase in specificity (ratio of GAPDH binding to myoglobin exon 2 binding for TBP).

In general we can say that the OneDay ChIP kit performed better in all parameters investigated, it was faster and easier to perform. After this set of experiment we introduced the One Day Kit in our laboratory. In Table 3 we report the general feedback of our colleagues structured in the so called de Bono's "Six Hats" format¹⁵.

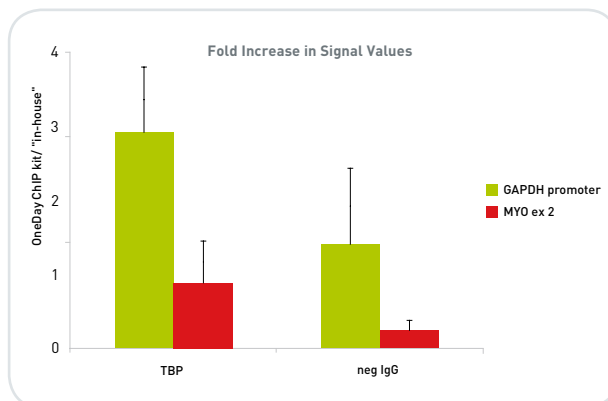


Figure 4. Fold increase in the signal values in the One Day Kit Samples compared to classical ChIP. Error bars represent the standard deviations of different chromatin samples.

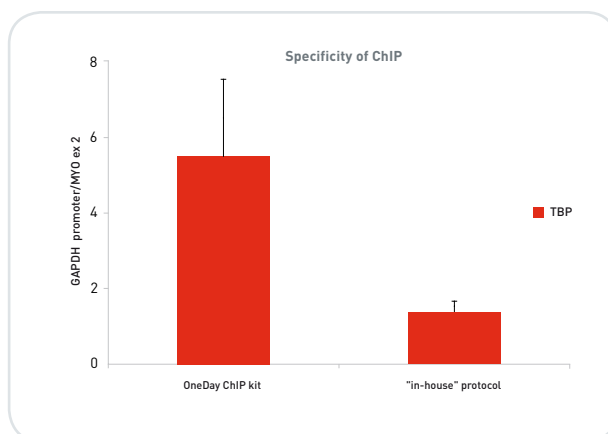


Figure 5. Specificity of Chromatin IP represented as the fold enrichment of TBP binding to the positive control GAPDH core promoter relative to the negative control myoglobin exon 2 region. Error bars represent the standard deviations of different chromatin samples.

TABLE 2. De Bono's Sixhats format comparison of the two ChIP protocols

	'In house' ChIP protocol	OneDay ChIP kit
Facts	<ul style="list-style-type: none"> • results in 2-3 days • low amount of DNA recovered (nanograms) • at least one million cells per IP • several buffers need to be prepared, chilled and used • long and labor intensive washing steps, if sample number is high (higher than 24) • additional DNA purification kit has to be used (or phenol chloroform extraction) 	<ul style="list-style-type: none"> • results in 1-2 days • significant amount of DNA recovered (tens of nanograms) • one tenth of the cells per IP needed, if compared to "in house" protocol • single wash buffer is used, supplied as a concentrate • few washing steps • all reagents available for the experiment including DNA purification
Feelings	<ul style="list-style-type: none"> • "the experiment is too long I am not prepared well enough to start it" • "other people got good results with this protocol" • "difficult to plan for three days in advance" • extremely boring washing steps if sample number is high (higher than 24) • lots of steps = lots of chances that something may go wrong • longer time needed to have the results and to repeat the experiment 	<ul style="list-style-type: none"> • "new protocol is usually improved compared to the old ones" • "I have one day = I finish the experiment" • "I can make significant progress even in half a day work" • "fewer steps = fewer chances for something to go wrong" • fully analyzed results in two days, so I can repeat my experiment in the same week
Precaution	<ul style="list-style-type: none"> • Beginners complain of poor results with this protocol • no customer support available • in some cases DNA is lost during the purification step, or during the storage • difficult to deal with high number of samples • long protocol • reagent price per first experiment is higher • siliconized tubes needed 	<ul style="list-style-type: none"> • shortcuts might be problematic if not fully tested • I do not know the composition of buffers • reagent price per IP is higher • few publications with this protocol • Kit manual is breaking the procedure in too many steps
Benefits	<ul style="list-style-type: none"> • system with all buffer composition known • easy to change the protocol • state of the art, a lot of publications with this protocol • Widely accepted in the field • Reagent price per individual sample in long term is lower 	<ul style="list-style-type: none"> • good results even for a beginner • better results, and higher signal levels • very fast • Better signal to noise ratio • Easy to perform • In case of problems I can ask for help in troubleshooting • One can deal with the protocol at high sample numbers also • Reagent price in case of few experiments is lower.
New ideas	<ul style="list-style-type: none"> • Washing steps could be adapted to affinity of the antibodies • washing column can be used to ease the washing steps • magnetic Protein A/G can ease the washing step 	<ul style="list-style-type: none"> • Sonication IP step has to be standardized or omitted • magnetic Protein A/G could be used to go high-throughput
Workflow	<ul style="list-style-type: none"> • In general this protocol is well known and used worldwide, with a lot of publications • Courses are given with this protocol 	<ul style="list-style-type: none"> • In general the protocol provides higher DNA recovery, and better signals in shorter time • The IP step should be monitored carefully • This protocol can replace the classical ChIP protocol

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