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Transcription Factor Binding Studies with IP-Star® Compact Automated System

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High sensitivity automated ChIP experiments to study the transcriptional regulation of the PTK2 gene by the AML1-ETO transcription factor and siRNA technology.

Introduction

Professor Olaf Heidenreich from the Northern Institute of Cancer Research at the University of Newcastle has focused his research on studying the role of oncogenic fusion genes such as AML1/MTG8 or MLL/AF4 in leukemic persistence.

The translocation (8;21) is the most prominent in childhood acute myeloid leukemia. It results in the fusion gene AML1-ETO which acts as a transcriptional regulator with different properties than the wild type RUNX1. To uncover the genes that are directly regulated by AML1-ETO, Dr Heidenreich's lab performed ChIP sequencing in the t(8;21) rearranged cell line Kasumi-1[1].

They included a sample in which AML1-ETO expression was diminished by the use of RNA interference. Comparison of cells treated without siRNA (Mock), a mismatch control siRNA (siAGF6) and with a siRNA that specifically targets the fusion site in the AML1-ETO mRNA (siAGF1) led to the identification of high confidence ChIP sequencing peaks. Additionally, DNAse hypersensitivity sites were assessed. In combination with a transcriptome analysis in the same experimental setting, ~600 genes that are directly regulated by AML1-ETO were identified. One of the genes that directly regulated by AML1-ETO is PTK2.

ChIP experiments require long and multi-step protocols, and the risk of introducing inconsistency is very high. The goal of this experiment was to confirm the findings from the ChIP sequencing by using an automated ChIP technology in order to reduce all inconsistency introduced by the pipetting variations and human variability. We confirmed two AML1-ETO binding sites in the PTK2 promoter by using the following combination of tools: the Bioruptor UCD-300-TO with associated reagents for chromatin preparation and the SX-8G IP-Star® Compact Automated System with associated reagents for ChIP and an ETO antibody for the immunoprecipitation step.

We chose five AML1-ETO binding sites in a putative PTK2 promoter region that were analyzed by qRT-PCR following ChIP. Beside the primers for the AML1-ETO binding sites in the putative PTK2 promoter region, a sequence in the PTK2 coding region that is not bound by AML1-ETO served as a control.

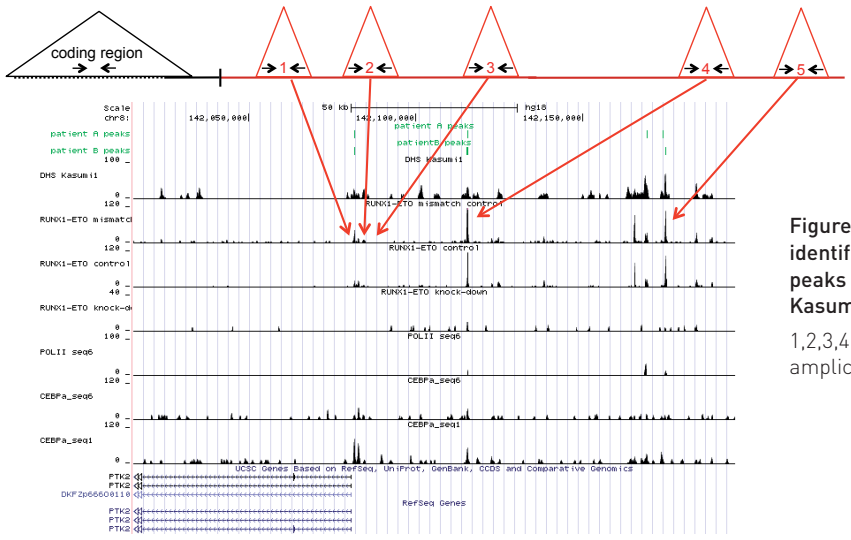

Figure 1. Five AML1-ETO binding sites were identified as high confidence ChIP sequencing peaks in the t(8;21) rearranged cell line Kasumi-1.

1,2,3,4 and 5 indicate the position of ChIP amplicons within the promoter region

Workflow and Results

Below is a description of using Diagenode's SX-8G IP-Star® Compact system to study transcriptional gene regulation by automated chromatin immunoprecipitation experiments.

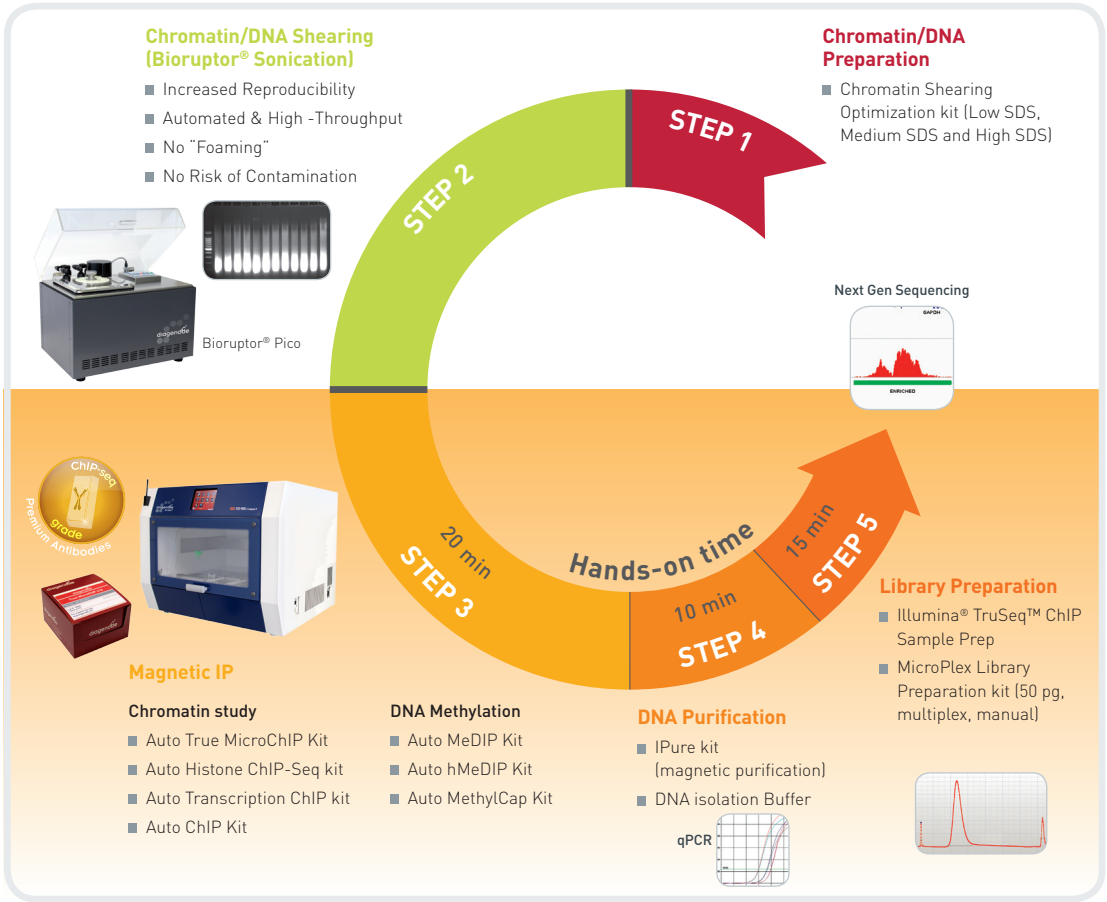

Figure 2. Diagenode provides a full suite of product solutions for ChIP experiments.
For Step 1, we offer products to isolate nuclei and chromatin. Step 2 describes reproducible sample shearing with the Bioruptor® product line. In Step 3 and Step 4, the Diagenode IP-Star® Compact provides error-free, walk-away automation for all your immunoprecipitation and antibody capture needs.

Step 1: Generation of a AML1-ETO knockdown cell line

SKNO-1 cells were electroporated with a siRNA that specifically targets the fusion site in the AML1-ETO mRNA (siAGF1), a mismatch control siRNA (siAGF6) at 350V for 10ms and with no siRNA (Mock). 3 days post electroporation cells were harvested and lysed in RLT buffer containing 1% β-mercaptoethanol. Following mRNA and protein purification, cDNA was transcribed and GAPDH and AML1-ETO levels were assessed by qRT-PCR. In addition, Western blot was performed and AML1-ETO protein was detected using GAPDH protein as a loading control.

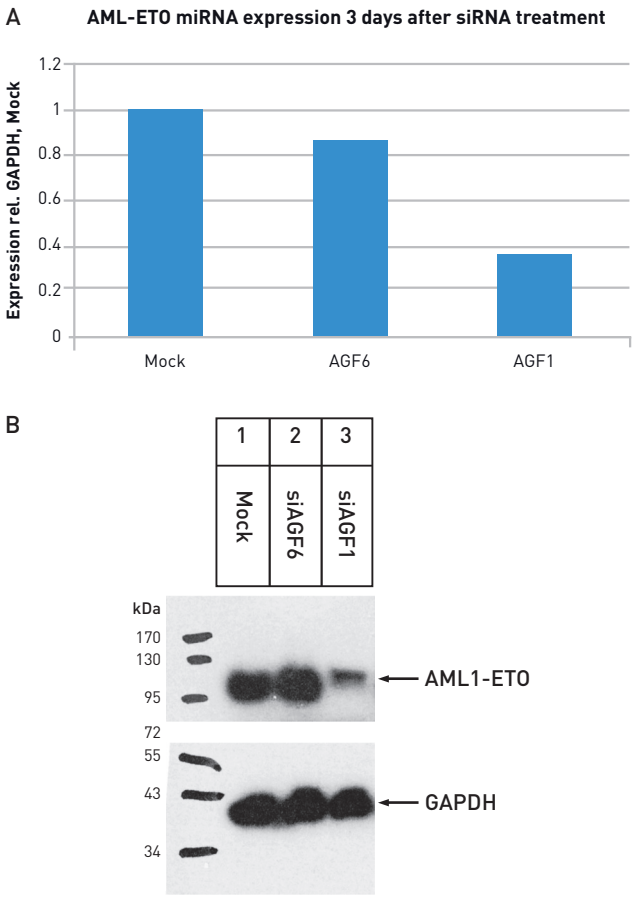

Figure 3. qRT-PCR and Western blot 3 days prior and post to siRNA electroporation.

mRNA levels (A) and Western blot (B) showing reduction of AML1-ETO protein expression after sRNA treatment.

Step 2: Chromatin preparation and fragmentation:

Chromatin from cells treated without siRNA (Mock), with a mismatch control (siAGF6) and with a siRNA that specifically targets the fusion site in AML1-ETO (siAGF1) was collected. Cells were lysed in Lysis Buffer containing 1% Triton X-100 and 0.25% SDS and sonicated with the Bioruptor UCD-300. An aliquot was reverse cross-linked and treated with Proteinase K and 2µg DNA was analyzed on a 2% agarose gel.

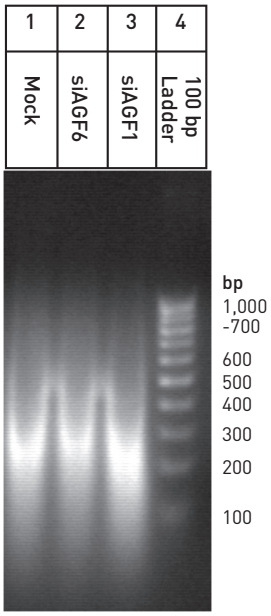

Figure 4. Chromatin shearing results in SKNO1 cell line using the Bioruptor UCD-300.

Shearing protocol consist in High Power settings, 10 min total sonication time with 30 s "ON" and "OFF" cycles. Chromatin fragments of 100-400bp were generated for the ChIP experiments.

Step 3: Automated chromatin immunoprecipitation

ChIP experiments were automated in the SX-8G IP-Star® Compact in order to analyze the association of AML1-ETO on the PTK2 promoter. We used chromatin from SKNO-1 cells treated with a siRNA that specifically targets the fusion site in AML1-ETO (siAGF1), a mismatch control (siAGF6) and cells without siRNA treatment (Mock). Sheared chromatin corresponding to 300,000 cells of SKNO1 was used per IP reaction. An ETO antibody (C-20) X (Santa Cruz) was used together with the Auto ChIP Kit (Diagenode, AB-Auto02-A100). We ran automated protocols in the IP-Star® Compact programmed as follows: a 2 hour step for antibody coating and an overnight immunoprecipitation step. The automated protocols include four automated washing steps and an elution step in DIB buffer after proteinase K digestion. Real-time PCR analysis was done selecting primers for peak 3 and peak 4 genomic regions in the PTK2 promoter.

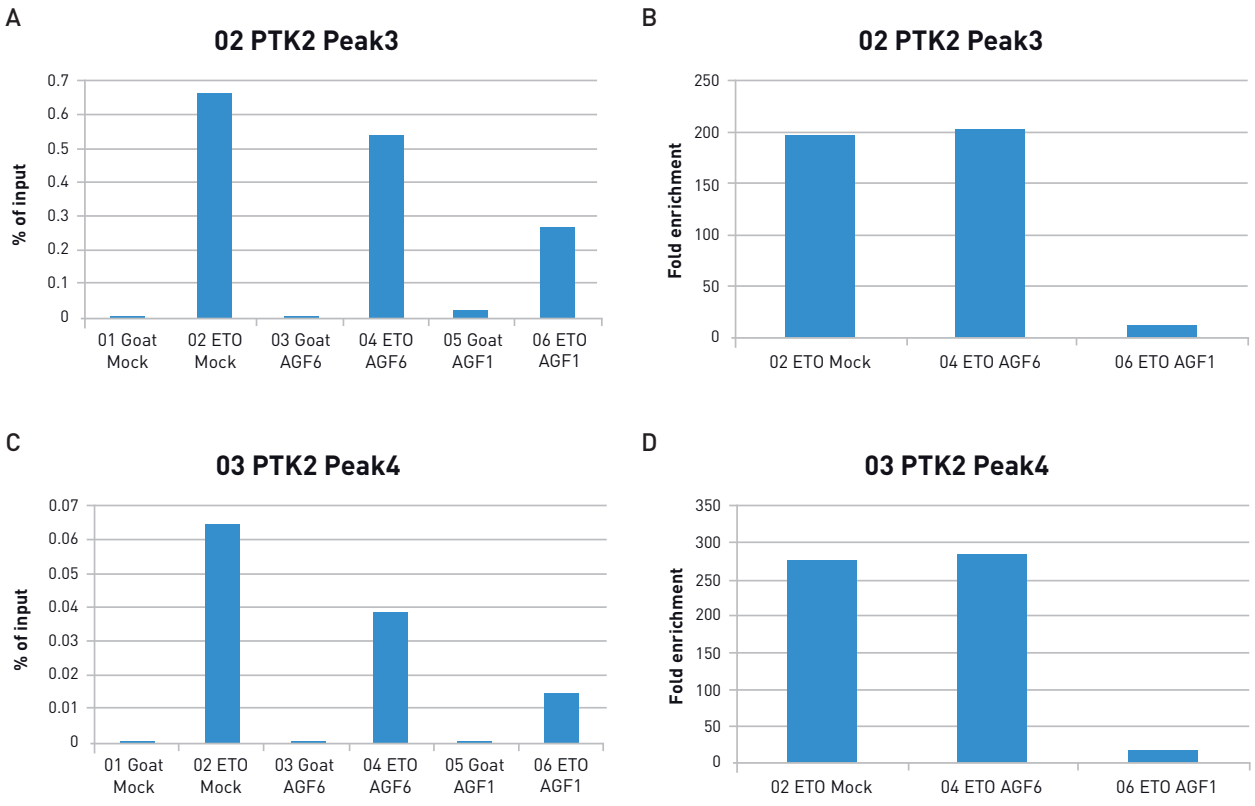

Figure 5. ChIP data from AML1-ETO antibody in two different genomic regions on the PTK2 promoter region.

ChIP analysis of ET0 and control IgG antibody in SKNO-1 cells treated with a siRNA that specifically targets the fusion site in AML1-ETO (siAGF1), a mismatch control (siAGF6) and cells without siRNA treatment (Mock). In the left diagrams (A and C), results are shown in % of input. On the right panel, results are shown as fold enrichment versus the control antibody for the three different siRNA conditions.

In both analyzed regions, peak 3 and peak 4, the Mock and siAGF6 showed enrichment of 200- 300 fold versus the control IgG. When AML1-ETO expression is diminished by the use of RNA interference enrichments that are drastically reduced to 13 fold (peak3) and 31 fold (peak 4). This indicates that AML1-ETO is specifically binding to these sequences in the PTK2 promoter region.

Conclusion

Here we showed that AML1-ETO specifically binds to sequences in a putative PTK2 promoter region (PTK2 peak 3 and peak 4). The strong fold enrichment was dramatically reduced if the AML1-ETO expression was diminished by the use of RNA interference as it was shown by real time qPCR and Western blot analysis.

Small interfering RNAs (siRNAs) are one of the most efficient tools for loss-of-function studies by specifically targeting the gene of interest resulting in a reduction at the protein expression level and are therefore used to dissect biological processes. These data clearly prove the power of using RNA interference in combination with automated ChIP technology to verify the binding of a transcription factor into specific sequences. The use of an automated system such as Diagenode's SX-8G IP-Star® Compact system is of great relevance, as the accuracy of the automated platform allows quantifying differences of protein abundance with high confidence upon cell treatments. Diagenode' IP-Star Compact is an automated system with included ChIP protocols, ideal for detecting protein binding at specific genomic regions with high precision and reproducibility while eliminating cross-contamination, sample carryover and false positives.

Automated ChIP experiments in the IP-Star® Compact Automated System can be used to detect changes in transcription factor binding after treatment with small interference RNAs. Compared to conventionnal ChIP, the use of the SX-8G IP-Star® Compact in combination with the Auto ChIP kit saves working time and improves the reproducibility of ChIP experiments.

Acknowledgements:

We thank S.Prall, C. Bonifer and O. Heidenreich for providing this data.

1 Depletion of RUNX1/ETO in t(8;21) AML cells leads to genome-wide changes in chromatin structure and transcription factor binding. Leukemia. 2012 Aug;26(8):1829-41