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Application Note

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A Streamlined Workflow to Perform ChIP from FFPE Samples

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Abstract

Deciphering epigenetic alterations occurring during the onset and progression of diseases is critical to characterize human malignancies and help predict which therapeutic interventions will most likely improve clinical outcomes. Because collections of malignant tissue as well as respective healthy controls exist in tissue banks, we developed a rapid and scalable extraction method to isolate chromatin from archived FFPE tissues suitable for ChIP. Here, we demonstrate how the Adaptive Focused Acoustic® (AFA®) technology combined with truXTRAC® and truChIP® effectively recovers chromatin suitable for studying histone modifications and transcription factor binding interaction events.

Introduction

Formalin Fixed and Paraffin Embedded (FFPE) tissues represent a collection of preserved solid tumors specimens, with varying tumor stages and subtypes that can be used for retrospective molecular analysis. While these collections have enormous potential for translational research applications, the preservation process presents technical challenges when performing downstream analytical applications, such as high-throughput sequencing.

Understanding the cancer epigenome is critical to distinguish the pathomechanism of primary and secondary tumors. Moreover, this insight can provide investigators information needed to improve the stratification and classification of the tumors, thereby improving the selection of therapeutic treatments. In order to utilize the existing repertoire of FFPE samples for analysis of the chromatin and transcription factor landscape, there is a need for a rapid and reproducible extraction method that allows one to perform ChIP as downstream application. Recently, Cejas[1] and colleagues have successfully performed histone ChIP from paraffin embedded tissues, however, the paraffin removal and tissue rehydration steps are time consuming. Covaris has developed truXTRAC FFPE extraction workflows to enable investigators to easily isolate high-quality DNA and RNA from FFPE tissues while maintaining sufficient quality for Next Generation Sequencing (NGS)[2,3]. In addition, truChIP Chromatin Shearing Kits are recognized as the gold standard for the preparation of chromatin from mammalian cells and fresh tissues. As a result, we evaluated the combination of the two workflows and developed a simple, fast, and scalable chromatin extraction method from FFPE tissues to enable ChIP-based applications to be performed as a downstream application.

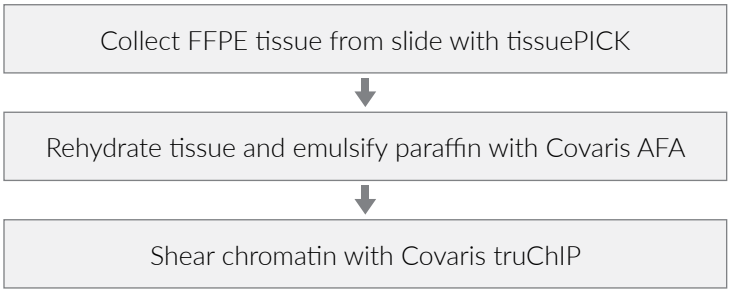
About Covaris truXTRAC and truChIP Workflow Solutions

The Covaris truXTRAC FFPE products provide the highest quality extraction of DNA, RNA, and total nucleic acid from FFPE tissues using Focused-ultrasonicators for next generation sequencing (NGS). The focused AFA energy actively removes paraffin compared to classic passive methods (*e.g.*, organic solvent based), which enables tissue rehydration and paraffin removal to occur at the same time.

The Covaris truChIP Chromatin products allow for the efficient and reproducible preparation of chromatin from all cultured mammalian cells and fresh tissue using the AFA technology. The workflow ensures isolated soluble chromatin is gently sheared to the correct size for sequencing while preserving protein-DNA complexes to provide a truly accurate representation of the *in vivo* biology.

Materials and Method

truChIP FFPE Workflow



Required Material

Covaris

- Covaris Focused-ultrasonicator and accessories
- tissuePICK (PN 520163) or sectionPICK (PN 520149)
- truChIP FFPE Chromatin Shearing Kit (PN 520257) or a combination of truXTRAC FFPE DNA microTUBE Kit for Chemagen Technology (PN 520176) and truChIP Chromatin Shearing Kit (PN 520127)

Other

- PMSF (Sigma-Aldrich, P7626)
- BSA (Euromedex, 04-100-812-C)
- NaCl (Euromedex, 1112-A)
- Protease Inhibitors Cocktail (PIC) (Sigma-Aldrich, SRE0055-1BO)
- IgG antibodies (Diagenode, C15410206) or H3K4 me2 antibodies (Diagenode, C15410035)
- Protein G Magnetic beads (Cell signaling, #8740)
- CTCF antibody (Diagenode C15410210-50)
- Qubit™ dsDNA HS Assay Kit (Q32851)

Tissues

- Colorectal cancer tissues (8H, 24H, and 72H of fixation)

Buffers

- Dilution Buffer (0.01% SDS, 1.1% Triton X 100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl)
- Wash Low Buffer (0.1% SDS, 1% Triton X100, 2mM EDTA, 20 mM tris/HCl pH8.0, 150 mM NaCl)
- Wash High Buffer (0.1% SDS, 1% Triton X100, 2mM EDTA, 20 mM tris/HCl pH8.0, 450 mM NaCl)
- LiCl Wash Buffer (0.5M LiCl, 1% NP40, 1% deoxycholate, 20 mM tris/HCl pH 8.0)
- Elution Buffer (50 mM NaHCO3, 1% SDS)

Detailed Protocol

Day 1

1. Capture region of interest (ROI) on FFPE tissues slides using sectionPICK or tissuePICK (2 slides, 10 µm) as described in standard procedure. These methods are specifically designed to collect FFPE tissue from non-stained sections mounted on slides and the exact procedures are illustrated in a short / movie under http://covaris.com/products/ffpe-extraction/tissuepick-instructions-2/
2. Release the tissue into the microTUBE Screw-Cap FFPE (blue cap).
3. Add 100 µl of Tissue SDS Buffer and 0.8 µl of Proteinase K (final concentration 80 ng/µl), incubate at 40C for 10 minutes with mixing after 5 minutes of incubation.

NOTE: Depending of tissue type and quantity, we recommend testing different quantities of tissue and Proteinase K concentration, see Figure 1 for an example of titration.

4. Process the sample on a Covaris M220 using the following settings:
   - Time: 5 minutes
   - Duty Factor: 20%
   - Peak Incident Power: 75W
   - 200 Cycles per Burst
   - 20C
5. Add 1 µl of Protease Inhibitors Cocktail (PIC) and 0.2 mM of PMSF. From here proceed at 4C.
6. Transfer 90 µl of the sample into a microTUBE Snap-Cap.
7. Add 40µl of 1X D3 Chromatin Shearing Buffer and PIC at 1X final concentration.
8. Process the sample on a Covaris M220 using the following settings:
   - Time: 10, 15, 20, and 30 minutes
   - Duty Factor: 15%
   - Peak Incident Power: 75W
   - 200 Cycles per Burst
   - 7C

NOTE: Please contact Covaris at applicationsupport@covaris.com if using another instrument than Covaris M220.

9. Centrifuge at 5000 x g for 5 minutes at 4C and retain the supernatant.
10. Place the sample on ice and determine chromatin concentration using Qubit fluorometric quantification.
11. Transfer 450 ng of chromatin in two 1.5 mL tubes and adjust to a final volume of 300 µl using Dilution Buffer. Set aside 4.5 ng of chromatin in a separate tube and label it as the 1% input sample.
12. Add 1.5 µg of IgG or H3K4 me2 antibody to the chromatin prepared in step 11. Incubate overnight at 4C on an overhead rotating system.

NOTE: The quantity of chromatin needs to be optimized based on the antibody used for the ChIP and the quality of the chromatin.

13. Block beads for day 2: add 66 µl of Protein G Magnetic beads in a fresh 1.5 mL tube and wash beads three times with 200 µl of Dilution Buffer using a magnetic rack. Add 200 µl of Dilution Buffer, add 6.6 µg of BSA and incubate at 4C.

Day 2 *(All washes are performed at 4C)*

1. Wash beads three times with 300 µl of Dilution Buffer. Add 66 µl of Dilution Buffer and transfer 30 µl of blocked beads into each ChIP sample (IgG and H3K4me2), incubate for 4 hours at 4C on an overhead rotating system.
2. Wash beads two times with the Wash Low Buffer: place the samples in the magnetic rack, aspirate and discard the supernatant and wash samples by adding 1000 µl of Wash Low Buffer, incubate for 3 minutes in an overhead rotating system. Repeat Step 2 once.
3. Wash the beads with Wash High Buffer: place samples in magnetic rack, aspirate, and discard the supernatant and

wash samples by adding 1000 µl of Wash High Buffer, incubate 3 minutes in an overhead rotating system.

4. Wash with LiCl wash buffer: place the samples in the magnetic rack, aspirate, and discard the supernatant and wash samples by adding 1000 µl of LiCl Wash Buffer, incubate 3 minutes in an overhead rotating system.
5. Carefully remove entire residual buffer from the beads and add 60 µl of Elution buffer. Shake for 10 minutes on a vortexer and transfer eluates into clean tubes. Repeat Step 5 once and combine both eluates into the same tube.
6. Add 5 µl of NaCl (5M) and reverse X-link by incubating at 65C overnight.

Day 3

1. Perform DNA purification using a phenol/chloroform extraction or DNA purification kits with Columns (Active Motif, 58002) or Magnetic beads (Ipure kit, Diagenode).
2. Analyze ChIP efficiency by qPCR.

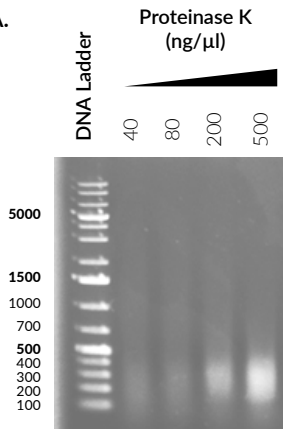
Results

Here, we aimed to develop a fast and easy-to-use extraction method for the isolation of chromatin from FFPE tissue suitable for ChIP as downstream application. Therefore, we combined Covaris truXTRAC and truChIP kits and utilized the AFA technology at two time points of the workflow.

After capturing the target area from the tissue, we rehydrated the tissue and emulsified paraffin using Covaris truXTRAC in combination with Proteinase K digest. We discovered that the titration of Proteinase K is critical to determine the optimal balance of extracting enough chromatin amount and at the same time preserving chromatin integrity suitable for ChIP as downstream application (***Figure 1***). As a result, we recommend to titrate Proteinase K concentration for the tissue of choice prior to performing experiments with precious tissues.

Titration of Proteinase K Determines Optimal Balance Between Chromatin Yield and IP Efficiency

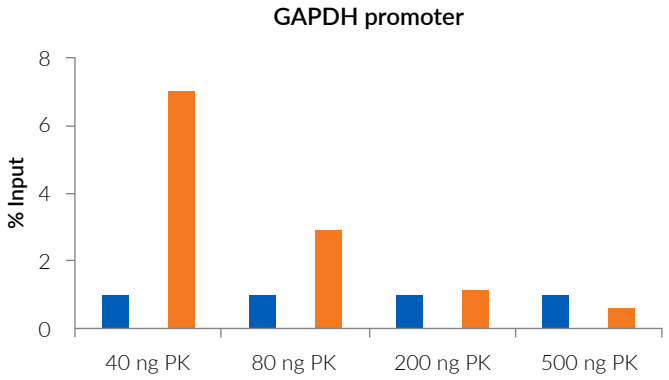
A.



B.

Proteinase K [ng/µl]	DNA [ng/µl]	total DNA [ng]
40*	6.4	832
80	8.3	1079
200	9.4	1222
500	12.1	1575

C.



D.

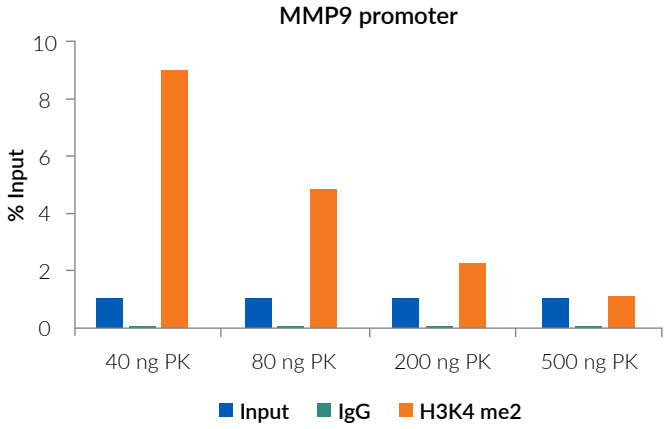


Figure 1. ***A.*** Chromatin was extracted from FFPE tissue sections after paraffin removal and sonication and 15 µl were resolved on a 1.2 % agarose gel. ***B.*** Chromatin extracted from two 10 µm slices of FFPE tissue sections using different concentration of proteinase K (*3 slides were used for the point 40 ng/µl of proteinase K) was quantified using Qubit. ***C.*** and ***D.*** Chromatin immunoprecipitation (ChIP) for H3K4me2 was performed from FFPE tissue extracted as in A. and B. In short 450 ng of chromatin was used; samples were pre-treated with increased concentration of proteinase K before paraffin removal to dissociate the tissue. H3K4me2 enrichment was accessed at the promoters of two active genes, *i.e.* GAPDH (C.) and MMP9 (D.).

Having determined 80 ng/µl of Proteinase K for 10 minutes as a suitable working condition for histone ChIP after chromatin extraction from FFPE tissue, we next wanted to know whether the same settings would be suitable for transcription factors, such as CTCF. Strikingly, ChIP for CTCF from FFPE extracted material using truChIP FFPE worked with comparable efficiency as a ChIP from fresh frozen tissue performed in parallel (***Figure 2***) suggesting similar chromatin integrity after extraction from FFPE tissue.

CTCF ChIP on FFPE Extracted Material Shows Comparable Enrichment as on Fresh Frozen Tissue

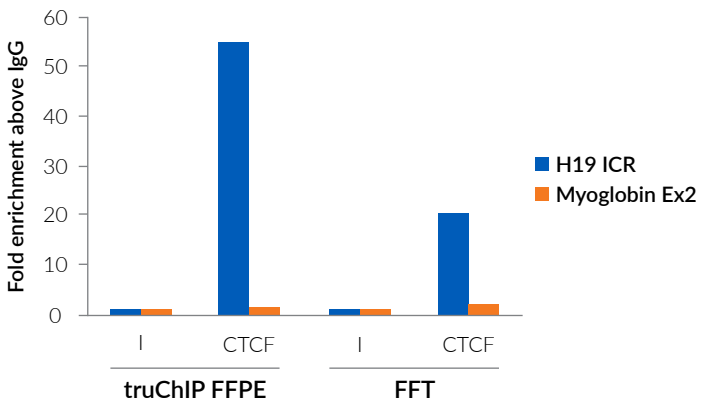
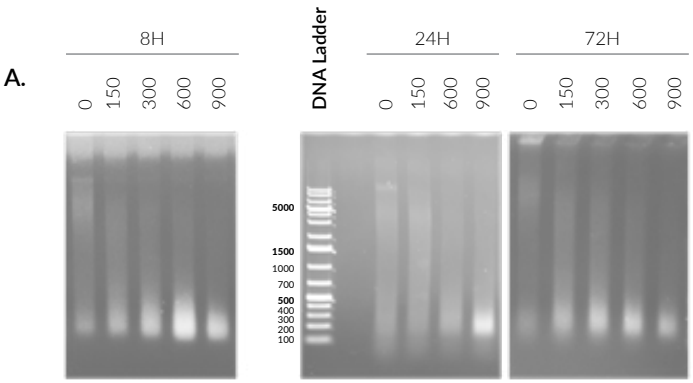
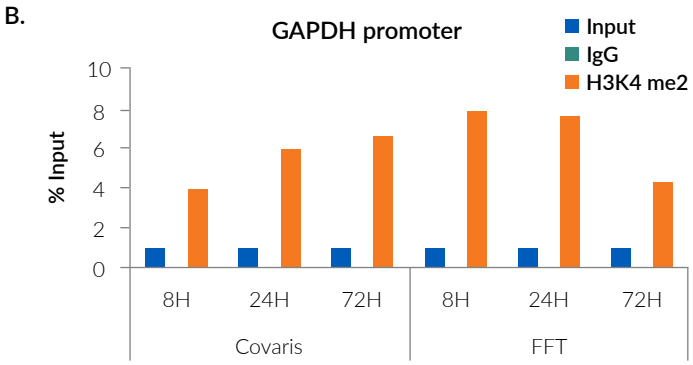


Figure 2. ChIP for CTCF was performed from FFPE extracted tissue using truChIP FFPE workflow as well as from fresh frozen tissue (FFT). In each ChIP, 850 ng of chromatin was precipitated using CTCF antibody or IgG as a negative control. Fold enrichment above IgG control was determined. Enrichment was accessed at the H19 imprinting control region (ICR) in comparison to a negative control region within Exon 2 of the Myoglobin gene.

Next, we wanted to determine how the ChIP performance after chromatin extraction from FFPE tissue with truChIP FFPE method compares to a published method (***Figure 4***). A direct comparison of both methods revealed very comparable ChIP enrichments independent of the time of fixation (***Figure 3***). The results suggest that the truChIP FFPE method is a suitable and provides an easier alternative to existing protocols.

ChIP Enrichment Upon truChIP FFPE Tissue Extraction is Comparable with the Published Protocol





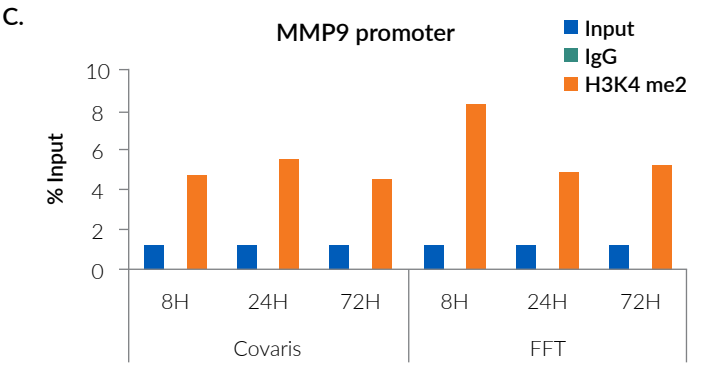


Figure 3. ***A.*** A shearing time course for 0, 150, 300, 600, and 900 seconds was performed on FFPE extracted tissues that were formalin fixed for 8h, 24h, and 72h and purified chromatin was resolved on an agarose gel. ***B. and C.***: H3K4me2 ChIP was performed for FFPE extracted tissues that were formalin fixed for 8h, 24h, and 72h using the truChIP FFPE workflow (sonication time 15 minutes) in comparison to the FIT-Seq protocol adopted from Cejas and colleagues. H3K4me2 enrichment was measured at the promoters of two active genes, i.e. GAPDH (B.) and MMP9 (C.).

Discussion

There is emerging evidence that epigenetic characterization provides a powerful tool to better understand the pathomechanisms of diseases including cancer, helps to stratify risk and treatment groups, and further holds huge potential to improve therapeutic intervention. Given the comprehensive tissue collections of FFPE specimens, tissue banks provide an ideal source to study chromatin and transcription factor landscape in well-described clinical samples including follow-up studies that would allow to follow epigenetic changes during the course of disease. However, so far chromatin extraction from FFPE samples has been challenging and existing protocols are not straight forward for processing multiple samples from one patient cohort at the time.

Here, we describe a novel, fast and easy procedure to extract chromatin from FFPE tissue in a suitable quality for ChIP analysis. In order to dissociate the tissue to allow chromatin release, treatment with Proteinase K is required. However, the concentration needs to be titrated and determined empirically in order to preserve intact chromatin for the downstream ChIP application (***Figure 1***).

Our procedure enables extraction of chromatin from FFPE tissue which is suitable for histone (***Figure 1***) as well as transcription factor (***Figure 2***) ChIP. Furthermore, for the transcription factor CTCF we obtain a comparable enrichment at the H19 imprinting control region when performing ChIP from FFPE extracted samples as when using fresh frozen material, suggesting a suitable integrity of the chromatin sample (***Figure2***).

Furthermore, the efficiency of ChIP was not changing drastically when comparing different periods of formalin fixation ranging from 8h to 72h (***Figure 3***), suggesting that most samples available in tumor banks should be suitable for such extraction and might be compared to each other even if fixed for slightly different periods. Strikingly, ChIP enrichment nicely compares to enrichments observed when following the more tedious FiT protocol published by Cejas and coworkers (Figure 3).

Streamlined Workflow Compared to the Published FFT Protocol

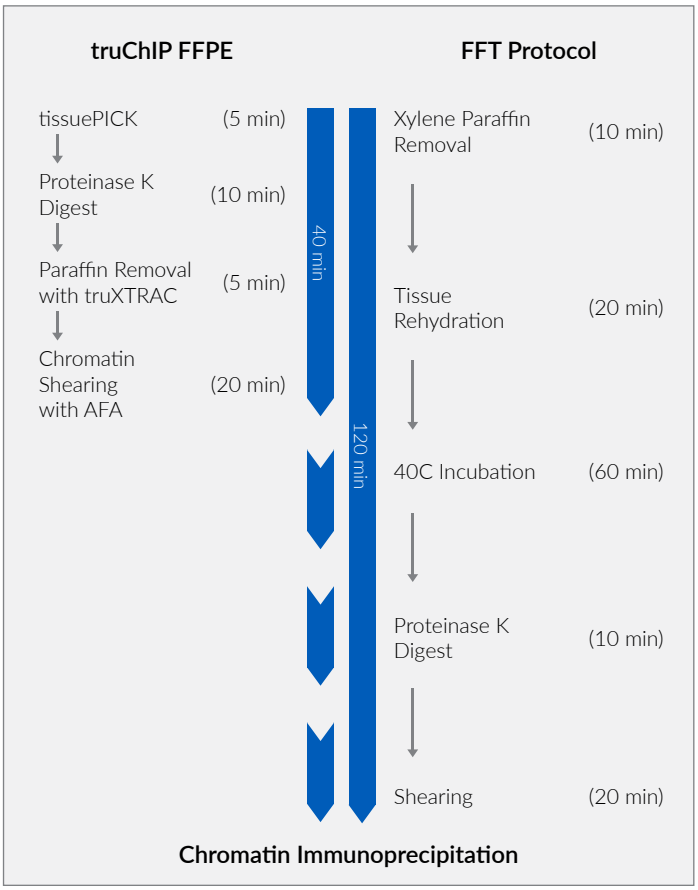


Figure 4. Optimized FFPE sample preparation for ChIP is fast and simple
The optimized truChIP FFPE workflow preparation requires only 1/3 of the time that published protocols need, as AFA technology allows simultaneous paraffin removal and tissue rehydration and therefore presents a robust and easy-to-use workflow.

Conclusion

The main challenge to monitor changes in chromatin and transcription factor landscape during onset and progression of diseases is availability of suitable material. Here, we provide a fast and easy protocol to extract chromatin from FFPE tissues with suitable quality for histone and transcription factor ChIP. This allows utilization of existing tissue collections and will broaden our understanding of epigenetic alterations in diseases and lay foundation for the identification of novel targets for therapeutic interventions.

References

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