Chromatin Assembly

(version A3)

Catalog No. 53500

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Overview

Active Motif's Chromatin Assembly Kit makes it possible to generate high-quality chromatin from supercoiled and linear DNA. The kit is based on an ATP-dependent assembly method that uses purified components. The Chromatin Assembly Kit utilizes purified recombinant *Drosophila* chromatin assembly complex ACF and human histone chaperone NAP-1 (h-NAP-1) with purified HeLa core histones for *in vitro* assembly of extended, regularly ordered, periodic arrays of nucleosomes. Active Motif's Chromatin Assembly Kit contains proven reagents that provide an easy and complete solution for chromatin generation.

product	format	catalog no.
Chromatin Assembly Kit	10 reactions	53500

Each Chromatin Assembly Kit contains reagents for 10 reactions. A reaction is defined as chromatin generated from 1 µg of DNA.

Introduction

In eukaryotes, genomic DNA is packed into chromatin to consist as chromosomes within the nucleus. The assembly of genomic DNA and histones into chromatin is a fundamental process that affects a broad range of gene regulatory processes, including DNA replication, DNA repair, gene expression and progression through the cell cycle. Research investigating DNA regulatory mechanisms would ideally be conducted with the gene sequence of interest assembled into chromatin to more closely represent the natural *in vivo* state.

Recent research has indicated that the transcriptional regulation of a promoter on naked DNA differs from that on a promoter assembled into chromatin. Thus, using unassembled DNA can generate misleading results[1-3]. This calls into question the value of using DNA constructs that are not in a chromatin-like environment for investigating the sequence of interest's role in regulating transcription. Because Active Motif's Chromatin Assembly kit enables you to closely mimic the chromatin environment, you can study regulation under conditions that are closer to real life.

The basic structural unit of chromatin is the nucleosome, which consists of 146 base pairs (bp) of DNA wrapped around a histone octamer. The histone octamer consists of two copies each of the core histone H2A-H2B dimers and H3-H4 dimers. Crystallographic studies have shown that an H3-H4 tetramer occupies the central region of the nucleosome, and the H2A-H2B dimers bind to the peripheral region. Nucleosomes are regularly spaced along the DNA in what is commonly referred to as "beads on a string".

The assembly of core histones and DNA into nucleosomes is mediated by chaperone proteins and associated assembly factors. Nearly all of these factors are core histone-binding proteins. Some of the histone chaperones, such as nucleosome assembly protein-1 (NAP-1), exhibit a preference for binding to histones H3 and H4. It has also been observed that newly synthesized histones are acetylated and then subsequently deacetylated after assembly into chromatin. The factors that mediate histone acetylation or deacetylation therefore play an important role in the chromatin assembly process.

While ATP-dependent chromatin assembly yields extended periodic arrays of nucleosome, the spacing of the nucleosomes is not driven by the DNA sequence itself. However, when the DNA sequences are assembled into chromatin in the presence of their sequence-specific regulatory factors, the resulting chromatin closely resembles the configuration of the genes *in vivo*[3-5]. Because chromatin assembled by the ATP-dependent ordering and specific factors is more like chromatin in a natural cell, it is an excellent substrate for investigating transcriptional regulation and the mechanisms that affect chromatin-dependent gene expression[1-15]. Assembled chromatin is useful for many downstream applications such as histone acetyltransferase (HAT) assays, *in vitro* chromatin immunoprecipitation (ChIP) assays and *in vitro* transcription assays[3].

Methods for Chromatin Assembly

In general, two *in vitro* methods have been developed for reconstituting or assembling chromatin. One method is ATP-independent, while the second is ATP-dependent. The ATP-independent method for reconstituting chromatin involves the DNA and core histones plus either a protein like NAP-1 or salt to act as a histone chaperone. This method results in a random arrangement of histones on the DNA that does not accurately mimic the native core nucleosome particle in the cell. These particles are often referred to as mononucleosomes because they are not regularly ordered, extended nucleosome arrays and the DNA sequence used is usually not longer than 250 bp[13]. To generate an extended array of ordered nucleosomes on a greater length of DNA sequence, the chromatin must be assembled through an ATP-dependent process.

The ATP-dependent assembly of periodic nucleosome arrays, which are similar to those seen in native chromatin, requires the DNA sequence, core histone particles, a chaperone protein and ATP-utilizing chromatin assembly factors. ACF (ATP-utilizing chromatin assembly and remodeling factor) or RSF (remodeling and spacing factor) are two widely researched assembly factors that are used to generate extended ordered arrays of nucleosomes into chromatin *in vitro*[1, 13].

ACF and RSF differ slightly in chromatin assembly mechanisms. ACF requires a histone chaperone protein like NAP-1 and does not require the histones to be acetylated in the assembly of the core particle. In contrast, RSF does require the acetylation of histones H2A and H2B, but it does not need a protein to function as a histone chaperone[13].

It is important to verify the chromatin assembly reaction resulted in an extended and ordered array of nucleosomes spaced along the DNA. By performing a simple partial enzymatic digestion followed by a quick deproteinization treatment, agarose gel analysis of the digestion will show a ladder of DNA fragments corresponding to mono-, di-, tri- and oligonucleosomes. This indicates the nucleosomes are assembled into regularly spaced arrays (Figure 1). Assembled chromatin that yields six or more DNA fragments upon digestion is considered high-quality and suitable for further assays[13].

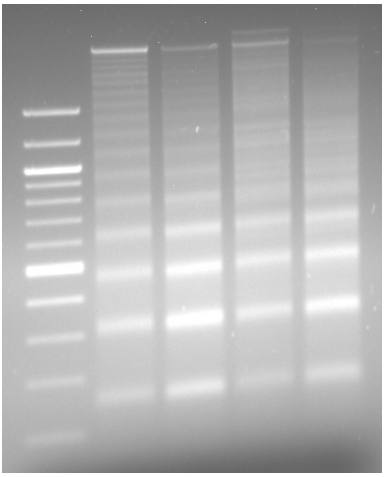

Figure 1: Partial enzymatic digestion of chromatin assembled from supercoiled circular and linear DNA.
One hundred µl reactions were performed on 1 µg of either linear or circular DNA using the Chromatin Assembly Kit protocol. Following the chromatin assembly incubation, each reaction was divided in two, and partially digested with 0.5 µl Enzymatic Shearing Cocktail for 2 and 4 minutes, respectively. Samples were subsequently deproteinated with Proteinase K, phenol/chloroform extracted and analyzed by agarose gel electrophoresis.

Lane 1: 100 bp DNA ladder.
Lane 2: Circular DNA digested with Enzymatic Shearing Cocktail for 2 minutes .
Lane 3: Circular DNA digested with Enzymatic Shearing Cocktail for 4 minutes.
Lane 4: Linear DNA digested with Enzymatic Shearing Cocktail for 2 minutes.
Lane 5: Linear DNA digested with Enzymatic Shearing Cocktail for 4 minutes.

Both the circular and linear DNA produced high-quality assembled chromatin, with at least 11 distinct bands of DNA corresponding to mononucleosomes, dinucleosomes, *etc.* visible.

Chromatin Assembly Kit Applications

The Chromatin Assembly kit may be used to study any function of DNA in its native environment. Relevant applications include:

- *In Vitro* Transcription Assays. *In vitro* studies of factors (or inhibitors) that affect the initiation of transcription by a transcription factor
- Studies of nucleosome core histone modifications such as acetylation and methylation
- *In vitro* ChIP assays. *In vitro* ChIP assays can be performed with *in vitro*-assembled chromatin to find other proteins associated with the promoter during the transcription activation process. *In vitro* ChIP assays could also be used for determining if an inhibitor can affect the process of recruitment of proteins needed to initiate the transcription process

Kit Components and Storage

Store components at the temperatures indicated below. Before beginning your first assay, the following components must be aliquoted to eliminate subsequent freeze-thaw cycles:

10X ATP Regeneration System: Prepare 10 aliquots of 11 µl each. Store at -80°C. Use a new aliquot each reaction.

Recombinant h-NAP-1 Protein: Prepare 5 aliquots of 3.5 µl each. Store at -80°C. Each vial of this component should only go through 2 freeze-thaw cycles.

Recombinant ACF Complex: Prepare 5 aliquots of 6 µl each. Store at -80°C. Each vial of this component should only go through 2 freeze-thaw cycles.

Creatine Kinase: Prepare 5 aliquots of 2 µl each. Store at -80°C. Each vial of this component should only go through 2 freeze-thaw cycles. Discard unused portion after second freeze-thaw.

HeLa Core Histones: Prepare 4 aliquots of 6 µl each in the siliconized microcentrifuge tubes provided. **Do not** use uncoated tubes. Leave the remaining in the supplied tube. Store at -80°C. Each vial of this component may go through 3 freeze-thaw cycles.

Reagent	Quantity	Storage / Stability
Recombinant h-NAP-1 Protein (5.0 mg/ml)	18 µl	-80°C for 6 months
HeLa Core Histones (1.5 mg/ml)	24 µl	-80°C for 6 months
Recombinant ACF Complex (0.25 mg/ml)	30 µl	-80ºC for 6 months
High Salt Buffer	100 µl	-20ºC for 6 months
Low Salt Buffer	700 µl	-20ºC for 6 months
10X ATP Regeneration System	110 µl	-80ºC for 6 months
Creatine Kinase	10 µl	-80ºC for 6 months
Supercoiled DNA (0.1 mg/ml)	20 µl	-20ºC for 6 months
0.1 M $CaCl_2$	30 µl	Room temp to -20ºC for 6 months
Enzymatic Shearing Cocktail	50 µl	-20ºC for 6 months
4X Enzymatic Stop Solution	400 µl	Room temp to -20ºC for 6 months
Proteinase K	80 µl	-20ºC for 6 months
Glycogen	10 µl	-20ºC for 6 months
5X Orange G Dye	500 µl	-20ºC for 6 months
Siliconized microcentrifuge tubes (1.5 ml)	25	Room temperature

The Chromatin Assembly Kit is for research use only. Not for use in diagnostic procedures.

Additional materials required

- Water bath
- Sterile distilled water (dH_2O)
- Microcentrifuge Tubes
- 20% SDS
- Phenol/chloroform
- 5M NH_4OAc
- 100% and 70% ethanol
- TE (10 mM Tris, 1 mM EDTA pH 8.0)
- Reagents for purifying high-quality supercoiled DNA (*e.g.* Qiagen Plasmid Maxi Kit, Catalog No. 12163).
- 100 bp DNA ladder (*e.g.* New England Biolabs, Catalog No. N3231S)
- Standard Tris/Glycine agarose gel supplies
  5X Tris Glycine Buffer: 107 g Tris base, 576 g Glycine and dH_20 to 4 L.

Component Aliquoting Recommendations

Before beginning the first assay, the following components should be thawed completely, mixed by gentle vortexing and aliquoted as described below. Because the HeLa Core Histones are very sticky, it is very important that you aliquot them into the provided siliconized tubes.

10X ATP Regeneration System

This component should be aliquoted and used fresh each time. We recommend that you make 10 aliquots of 11 µl each. Store all aliquots at -80°C.

Recombinant h-NAP-1 Protein

Prepare 5 aliquots of 3.5 µl each. Store at -80°C. This component should only go through 2 freeze-thaw cycles.

Recombinant ACF Complex

Prepare 5 aliquots of 6 µl each. Store at -80°C. This component should only go through 2 freeze-thaw cycles.

Creatine Kinase

Prepare 5 aliquots of 2 µl each. Store at -80°C. This component should only go through 2 freeze-thaw cycles.

HeLa Core Histones

Prepare 4 aliquots of 6 µl each in the provided siliconized microcentrifuge tubes. Because core histones frequently stick to the walls of microcentrifuge tubes, especially in small volumes such as these, it is imperative to aliquot into the provided siliconized microcentrifuge tubes. Store at -80°C. This component may go through 3 freeze-thaw cycles.

Component Preparation Recommendations

Prior to starting the assay please prepare the following:

Preparation of Sample DNA

Prepare high quality DNA at 0.1 µg/µl.

Note 1: The quality of the DNA is important for the efficiency of the chromatin assembly reaction. The quality of the DNA may be checked on a TAE agarose gel. In general, high-quality DNA can be prepared by standard CsCl gradient methods or a commercial kit may be used *e.g.* Qiagen Plasmid Maxi Kit, Catalog No. 12163.

Note 2: Ensure that the DNA is not contaminated with RNA. Histones can interact with RNA, which will cause variable and inconsistent results.

Preparation of Complete 10X ATP Regeneration System

Prepare a sufficient quantity of Complete 10X ATP Regeneration System by adding 0.3 µl of Creatine Kinase to 10 µl of 10X ATP Regeneration System. Use immediately. Discard any remaining Complete 10X ATP Regeneration System that you have made and make up fresh each time.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

One reaction of the protocol below will generate 100 µl of assembled chromatin from 1 µg input DNA. However, the partial enzymatic digestion protocol used to verify chromatin assembly will use a minimum of 50 µl per reaction in order to visualize the material on a gel. Thus, depending on what you plan to do with the assembled chromatin, you may wish to perform two or more reactions of the same material and then check only one of them. In addition, the assembled chromatin is known to be stable at 4°C for 2 days only, and it should not be frozen. Therefore, you should plan your assembly reactions, and how/when you plan to use them, carefully.

A. Chromatin Assembly Reaction

1. Set water bath to 27°C.
2. Add the following reagents in the order shown in the table below. If desired, siliconized tubes can be used; however, uncoated microcentrifuge tubes can be used.

   Note: Keep reaction reagents on ice during reaction set-up and return to appropriate -80°C or -20°C storage immediately after addition.

Component	Positive Control	Negative Control	Sample
Recombinant h-NAP-1	1.4 µl	–	1.4 µl
HeLa Core Histones	1.8 µl	–	1.8 µl
High Salt Buffer	10 µl	10 µl	10 µl
Low Salt Buffer	–	3.2 µl	–

3. Gently vortex the samples. Centrifuge to collect material at the bottom of the microcentrifuge tube. Incubate on ice for 15 minutes.
4. Add 64.3 µl of Low Salt Buffer to each reaction.
5. Add the following reagents in the order outlined in the table below.

Component	Positive Control	Negative Control	Sample
Recombinant ACF Complex	2.5 µl	–	2.5 µl
Complete 10X ATP Regeneration System	10 µl	10 µl	10 µl
Supercoiled DNA (control)	10 µl	10 µl	–
Sample DNA	–	–	1 µg
Low Salt Buffer	–	2.5 µl	–
dH_2O	–	–	to 100 µl
Total Volume	100 µl	100 µl	100 µl

6. Gently vortex the samples. Briefly centrifuge to collect material at the bottom of the microcentrifuge tube. Incubate at 27°C for 4 hours. After incubation, the sample may be stored at 4°C for up to 2 days. Chromatin should not be frozen at this point.

   Note: Though reaction conditions have been optimized for chromatin assembly from circular plasmid DNA, this protocol will also work for linear DNA (see Figure 1).

The assembled chromatin prepared above can be used directly in downstream assays. Certain applications, such as electron microscopy studies, may require further purification, which can be accomplished by sucrose gradient sedimentation or gel filtration[1].

The following steps are optional. They are included so that you can check for successful chromatin assembly.

B. Analysis of Assembled Chromatin by Partial Digestion

Partial digestion with the Enzymatic Shearing Cocktail will reveal periodic spacing of assembled nucleosomes. After the chromatin is digested, it is deproteinated. The resulting DNA fragments are resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. High-quality chromatin should yield 6 or more distinct bands.

1. Add 3.0 µl of 0.1 M $CaCl_2$ (3 mM final concentration) to each 100 µl reaction of assembled chromatin. Mix well.
2. Digest each reaction with 1 µl of Enzymatic Shearing Cocktail for 2 minutes at room temperature.

   Note: We recommend that two time points be tested for each enzymatic digestion e.g., split the 100 µl reaction into two tubes and digest each aliquot with 0.5 µl of Enzymatic Shearing Cocktail for 2 and 4 minutes respectively. Splitting the reaction below 50 µl will make reaction efficiency difficult to visualize by agarose gel electrophoresis.

3. Add 34 µl (or 17 µl for half volume reaction) of 4X Enzymatic Stop Solution. Mix well. Incubate on ice for 10 min. The reactions may be stored at -20°C at this point.

   Note: The quantity of enzyme and time of incubation may need to be optimized for the sample DNA. The conditions in this protocol are optimized for the included positive control DNA, which is supercoiled circular DNA.

C. Deproteinization with Proteinase K

1. Add 20% SDS to a final concentration of 0.5% (3.45 µl per 100 µl or 1.72 µl per half volume reaction).
2. Add 1 µl (or 0.5 µl per half volume reaction) of Proteinase K.
3. Incubate at 55°C for 15-30 minutes.
4. Add 1 µl Glycogen.
5. Add sterile dH_2O to final volume of 200 µl.
6. Perform a phenol/chloroform extraction once, *i.e.* add 200 µl of phenol/chlorofom, vortex samples and spin for 5 minutes at room temperature.
7. Remove the top 200 µl (supernatant) and mix with 200 µl of 5 M NH_4OAc and 1 ml 100% EtOH.
8. Spin for 15 minutes at 4°C. Discard the supernatant. (A little white pellet is visible at this point. Take care not to disturb the pellet, leave 10-20 µl close to the pellet).
9. Wash DNA pellet by adding 500 µl 70% EtOH, and centrifuge at 4°C for 5 minutes. Remove as much of the EtOH as possible.
10. Air dry for approximately 5 minutes.
11. Resuspend pellet in 8 µl dH_2O (or TE). Add 2 µl 5X Orange G dye.

    Note: The Orange G dye can be replaced by another dye. However, some dyes e.g. Bromophenol Blue migrate with bands at 100 bp.

12. The sample reactions can be stored at -20°C or analyzed immediately by agarose gel electrophoresis.

D. Agarose Gel Electrophoresis

1. Perform agarose gel electrophoresis (1.5% agarose gel in cold 1X Tris-Glycine buffer in a 4°C cold room). Run the samples and a 100 bp ladder at 150V for approximately 1 hour and 20 minutes. Add ethidium bromide to the gel and the buffer.

    Note: It is possible to run a 1.5% TAE agarose gel in cold 1X TAE buffer. However, the band resolution will be less than when using Tris-Glycine gels and buffers.

Downstream Applications

The following assays are just a few examples of how to further research chromatin-dependent processes with your assembled chromatin.

In vitro ChIP Assay

Perform an *in vitro* ChIP assay to verify transcription factor binding to the assembled chromatin and to investigate cofactor or inhibitor influence on binding[3, 14].

In vitro Transcription Assay

The transcription process is suppressed, in general, by chromatin but not by free DNA. An *in vitro* transcription assay can be used to determine what factor is involved in transcription activation from the assembled chromatin[3, 14].

In vitro HAT Assay

With the assembled chromatin, a HAT assay can be performed to study the acetylation of histones in their native environment, making it is possible to analyze which factors are involved in or are necessary for histone acetylation[3, 14, 15].

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Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
Less than 6 nucleosome bands on agarose gel without a similar smear of DNA to what is seen with the negative control.	The chromatin assembly reaction worked well but the chromatin was subsequently over-digested.	The enzymatic digestion may need to be optimized *e.g.* try less enzyme or a shorter time for the enzymatic digestion.
Less than 6 nucleosome bands on agarose gel with visible DNA of high molecular weight.	The chromatin assembly reaction worked well but the chromatin was subsequently under-digested.	The enzymatic digestion may need to be optimized *e.g.* try adding more enzyme or digesting for a longer time.
Less than 6 nucleosome bands on agarose gel with a smear of DNA similar to what is seen with the negative control.	Low efficiency of the assembly reaction due to too much or too little DNA.	Adjust the DNA concentration. Perform a titration of DNA to histone ratios ranging around 1:1[1, 13].
	Low efficiency of the assembly reaction due to poor quality DNA.	Repeat DNA purification with CsCl gradient purification or commercially available DNA purification kit *e.g.* Qiagen Plasmid Maxi Kit, Catalog No. 12163.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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