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EPIGENETIC ANALYSIS OF SV40 MINICHROMOSOMES

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Abstract

Simian Virus 40 (SV40) is one of the best characterized members of the polyomavirus family of small DNA tumor viruses. It has a small genome of 5243 base pairs and utilizes cellular proteins for its molecular biology with the exception of the T-antigen protein, which is coded by the virus and is involved in regulating transcription and directing replication. Importantly, SV40 exists as chromatin in both the virus particle and intracellular minichromosome. These facts combined with high yields of virus and minichromosomes following infection, and ease of manipulation, have made SV40 an extremely useful model to study all aspects of eukaryotic molecular biology. This protocol consists of two parts. The first part describes the procedures for working with SV40 and preparing SV40 chromatin from infected cells and virus particles. The second part describes how the SV40 chromatin can be used to study epigenetic regulation.

Keywords

Simian Virus 40 (SV40); minichromosomes; viral epigenetics; chromatin immunoprecipitation; next generation sequencing

INTRODUCTION

Simian Virus 40 (SV40), a member of the polyomavirus family of small DNA tumor viruses, has a closed-circular double-strand DNA genome that is 5243 base pairs (bp) in length in the wild-type laboratory strain (Tooze and Acheson 1981). Like cellular DNA, SV40 DNA is organized with cellular proteins into typical chromatin structure and is subject to typical epigenetic regulation (Coca-Prados and Hsu 1979, Cereghini and Yaniv 1984, Kube and Milavetz 1996, Milavetz, Kallestad et al. 2012). Unlike most other DNA viruses such as the adenoviruses and herpesviruses, SV40 DNA is found as chromatin in both the virus particle and intracellular minichromosomes and as a consequence undergoes epigenetic regulation during an infection that can be transmitted to a subsequent infection (Milavetz, Kallestad et al. 2012).

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Because of the limited coding capacity of the SV40 genome, it relies on cellular proteins for most steps in transcription and replication. This fact along with the observation that SV40 also causes tumors in susceptible animals has resulted in SV40 being used extensively as a model to study normal eukaryotic molecular biology processes and the origins of cancer. Many fundamental aspects of eukaryotic transcription, replication, and transformation were first identified or characterized using SV40. It is safe to say that SV40 has played a major role in the development of modern molecular biology.

This protocol consists of two major parts. The first describes procedures for preparing high titer infectious virus and isolation of SV40 chromatin directly from virus and minichromosomes from infected cells. The second part describes how the chromatin can be used to study epigenetic regulation including the location of nucleosome, histone modifications, and the location of other bound proteins. A diagram showing the major steps in the preparation of SV40 chromatin and its subsequent use in epigenetic studies is shown in Figure 1.

CAUTION: SV40 is classified as risk group agent 1 or 2 in different countries. See your national classification of microorganisms for the assignment of SV40 to the appropriate biosafety level of laboratories (BSL-1 or BSL-2). Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms (Burnett 2009).

NOTE: All equipment and solutions coming in contact with cells must be sterile, and appropriate sterile technique should be used. All steps must be performed in a Class II Biosafety cabinet. Autoclave all glassware and plastic ware before disposal.

NOTE: All incubations are performed in a humidified 37°C incubator unless otherwise specified.

BASIC PROTOCOL 1

PREPARATION OF DISRUPTED VIRUS

This protocol describes the preparation of SV40 virus, its purification, and disruption to generate SV40 chromatin from the virus. The virus is prepared from African Green Monkey Kidney cells infected with a small amount of virus to inhibit the formation of defective virus using standard cell culture procedures. The virus is concentrated, digested with nuclease to remove surface contaminants and purified by sedimentation. The purified nuclease-digested virus is disrupted by treatment with a combination of dithiothreitol (DTT) and EGTA and purified by sedimentation on a glycerol step gradient.

Materials

High titer stock SV40 virus: Our stock virus (776) was originally obtained from the laboratory of Dr. Daniel Nathans. SV40 can be obtained from the American Type Culture Collection (ATCC) or alternatively by contacting the Milavetz laboratory (barry.milavetz@ad.ndus.edu)

SV40 DNA (Obtained by a modified Hirt procedure (Hirt 1967), see Support Protocol 1)

Four 75 cm^2 T-flasks containing 70% confluent African Green Monkey Kidney (AGMK) cells (ATCC # CCL-26)

75 cm^2 T-flasks (Corning, #430720U)

Cell culture medium MEM (Gibco, #11095-098, see recipe for additional components)

Fetal bovine serum (Gibco, #16140-063)

Gentamycin (Gibco, # 15710-072)

Trypsin (Gibco, # 25300-120)

Nuclease-free water (Ambion, #AM9937)

DNAse I (New England Biolabs, #M0303S)

Agarose (Sigma, # A6877)

SsoAdvanced Universal SYBR Green Supermix (Biorad, # 172–5274)

$T_{10}E$ (see recipe)

EGTA 100 mM (see recipe)

DTT, 1 M (see recipe)

10% glycerol low-ionic strength buffer (see recipe)

Running buffer (see recipe)

Sample buffer (see recipe)

Ethidium bromide or SYBR green (see recipe)

10 µl Graduated Filter Tips

10 µl Pipetman

1000 µl Pipetman

1000ul Filter Tips

15 ml centrifuge tubes (Corning, # 430052)

200 µl Graduated Filter Tips

200 µl Pipetman

37°C heat block

Beckman ultracentrifuge TLA-100 or equivalent small volume ultracentrifuge

BSL-2 biosafety laminar air cabinet (Nuaire, Model NU-425-400 or equivalent)

Eppendorf snap-cap microcentrifuge flex tubes (Fisher Scientific # 022364111)

Power supply (125V)

Real-time PCR machine

Sterile cell culture incubator

Submerged Agarose Gel Apparatus

ChIP DNA Clean and Concentrator kit (Zymo Research, # D5205)

Preparation of High Titer Infectious SV40 Virus

1. Perform all manipulation of materials that contain SV40 virus in a BSL-2 biosafety cabinet. Seed from two to as many as eight, 75 cm^2 T-flasks (T-75) with AGMK cells during routine cell culture in 10 ml MEM (with 10% fetal bovine calf serum, sodium carbonate, and gentamycin) and incubate in a water jacketed incubator at 37°C. Incubate cells either in a 5% CO_2 atmosphere or with the caps on the T-flasks tightly closed in the absence of external CO_2.

   We prefer the latter procedure to decrease the likelihood of accidental contamination of the laboratory with SV40.

2. When the cells are approximately 75% confluent, add 1 μl of a stock SV40 virus preparation originally obtained by plaque purification of virus and incubate the cells until the majority of the cells have died and are no longer attached to the surface of the T-flask.

3. Pool the media from the two or more flasks and store at −20°C until used for the preparation of disrupted virus.

   Typically we pool at least six to eight T-75 plates of virus in order to have sufficient stocks of the same virus for our studies. This crude virus preparation is used to prepare disrupted virus as described immediately following or to prepare SV40 minichromosomes as described in the next section. We refer to this crude virus as our working stock SV40.

4. If desired, titer the virus by real-time PCR to obtain an estimate of the amount of SV40 DNA present.

   We routinely do this to estimate the amount of virus present in our working stocks.

5. We determine the quantity of SV40 DNA by real-time PCR using primers that recognize a portion of the SV40 genome. Routinely we use the primers 5’AAAATGAAGATGGTGGGGAGAAGAA3’ and 5’GACTCGAGGTGAAATTTGTGATGCT3’ which recognize a fragment approximately 250 bp in size found near the termination of both early and late transcription, however, other sets of primers can be used. Amplify one μl of the virus in 25 μl total volume of PCR amplification buffer containing 12.5 μl of 2X BioRad amplification buffer (SsoAdvanced Universal SYBR Green Supermix), 0.25 μl of each primer (100 μM) and nuclease-free water to 25 μl total volume. Preheat the mixture in the PCR machine for 10 minutes to activate the DNA polymerase and then amplify the DNA for 40 cycles at 95°C for 1 minute to denature the DNA, 54°C for 1 minute to anneal the DNA, and 72°C for 1 minute DNA extension. A good preparation of virus typically appears between 13 and

15 cycles using this PCR protocol. If desired an absolute amount of SV40 DNA can be determined using diluted SV40 DNA as standards although we do not typically do this.

Concentrate Crude SV40 Virus

2 Add 1 ml of crude SV40 virus to each of four to six 1.5 ml Eppendorf or other high quality microcentrifuge tubes. The tubes must be able to withstand at least two rounds of ultracentrifugation. Concentrate the crude virus by ultracentifugation at 50,000 rpm (27,050 × g) for 35 minutes at 4°C in a TLA100.3 rotor in a TLA100 ultracentrifuge.

3 Following centrifugation a small white pellet should be observed in the bottom of the microcentrifuge tube and the rest of the liquid should be clear. Remove the clear supernatant liquid taking care not to dislodge the pellet. Typically, we add a second aliquot of 1 ml of crude SV40 virus to each tube and subject the crude virus in the microcentrifuge tube to ultracentrifugation at 50,000 rpm (27,050 × g) for 35 minutes at 4°C.

4 Following this second centrifugation step, remove the supernatant and resuspend the pelleted crude virus in 175 μl of $T_{10}E$ by repeatedly drawing the sample up and down in a pipette tip to break up the white pellet. The resuspension should result in a cloudy liquid in the microcentrifuge tube. Store the resuspended crude SV40 at −20°C.

Remove External Chromatin from the Virus

1. Remove the viral and cellular chromatin associated with the pelleted SV40 virus which is not found within the virions and as such protected from nuclease digestion by DNase I digestion. For each tube of crude pelleted virus, thaw the resuspended pellet, adjust the resuspension to nuclease digestion conditions by the addition of 20 μl of the 10X buffer supplied with the DNase I, and add 5 μl of DNase I (5 units). Following repeated mixing by drawing the suspended virus into and back out of a 200 μl pipette tip, the mixture is incubated at 37°C for 30 minutes in a heating block. After incubation, store the mixture at −20°C.

Freezing and thawing is used in part to help breakup any aggregates.

2. Subject the resuspended SV40 virus to two more rounds of DNase I digestion and subsequent freezing. For each round thaw the mixture, add 5 μl of fresh DNase I to each tube, mix the fresh nuclease by drawing the liquid into and back out of a 200 μl pipette tip and again incubate at 37°C for 30 minutes in a heating block.

The DNase I is not specifically inactivated in any way.

3. In order to remove all fragments of chromatin generated by the nuclease treatment along with residual nuclease, pellet the DNAse I treated virus through a 10% glycerol low-ionic strength buffer. Typically, this step can also be used to concentrate the partially purified SV40. To each of four Eppendorf

microcentrifuge tubes or equivalents, add 1 ml of 10% glycerol low ionic strength buffer. Add the thawed nuclease digested SV40 from one or two tubes of digested virus (200–400 µl) to the top of the glycerol buffer being very careful to prevent mixing between the digested SV40 and the glycerol buffer. Then pellet the nuclease-digested SV40 by ultracentifugation at 50,000 rpm (27,050 × g) for 35 minutes at 4°C in a TLA100.3 rotor in a TLA100 ultracentrifuge. Following centrifugation there should be a small white pellet at the bottom of each of the centrifuge tubes most likely much smaller than what was observed in the pelleting of the crude virus above. Remove all of the liquid in the centrifuge tubes taking care not to dislodge the pellets.

4. Resuspend the pelleted nuclease-digested SV40 in 189 µl of $T_{10}E$ by repeatedly drawing the sample into and back out of a 200 µl pipette tip to break up the white pellet. The resuspension should result in a slightly cloudy liquid. Store the resuspended nuclease-digested SV40 at −20°C until the disruption step below.

5. If desired, determine the quality of the nuclease-digested SV40 by analyzing 10 µl of the sample by submerged agarose gel electrophoresis on a 0.9% agarose gel using a TEA buffer system. For best results purify 20 to 30 µl of the digested virus and corresponding amount of the original resuspended virus following concentration using a Zymo ChIP DNA Clean and Concentrate kit or equivalent following the kits directions. We elute the SV40 DNA in a volume equal to the starting volume of the sample. We add a 10 µl sample of the purified SV40 DNA from each sample to 10 µl of sample buffer and analyze the sample along with a sample of pure SV40 DNA prepared by the Hirt procedure. The digested sample should yield primarily only one band corresponding to forms I SV40 DNA, the supercoiled form of SV40 DNA. The crude sample, if analyzed, should also contain the forms of SV40 DNA along with a smear of DNA extending through much of the center of the agarose gel. An example of this type of analysis is shown in Figure 2. Compare the DNA in lane 2 from crude virus to the single band in the DNAse I digested virus in lane 3.

Disrupt the Viral Protein Coat and Purify the SV40 Chromatin

1. Disrupt the resuspended nuclease-digested SV40 (189 µl) by adding 5 µl of EGTA (100 mM) and 6 µl of freshly prepared DTT (1 M). Following addition of the two reagents thoroughly mix the resuspension by repeatedly drawing the liquid into and forcing the liquid out of a 200 µl pipette tip. Following mixing incubate the mixture for 30 minutes at room temperature. Store the mixture at −20°C when the incubation is complete.

2. Continue disruption of the SV40 by two subsequent additions of 6 µl of freshly prepared DTT and incubation for 30 minutes at room temperature followed by freezing after each incubation.

3. Following the 3 disruption steps separate the SV40 chromatin from intact virus by sedimentation on 10% glycerol. Carefully layer approximately 400 µl of disrupted SV40 from two disruption tubes onto 1 ml of 10% glycerol low-ionic

strength buffer in a 1.4 ml Eppendorf tube and centrifuge for 35 minutes at 50,000 rpm (27,050 × g) at 4°C in a TLA100 ultracentrifuge. Depending upon the number of tubes of concentrated virus this will yield either two tubes or three tubes.

4. Following centrifugation take 200 µl fractions starting at the top of the centrifuge tube. Discard the first two fractions that should contain only fragments of SV40. Pool the next three fractions that contain full-length SV40 whose minichromosomes are compacted differently depending upon the proteins associated and keep as the disrupted SV40 chromatin. Discard the rest of the contents of the tube.

5. If desired, determine the quality of the SV40 chromatin by submerged agarose gel electrophoresis as above using a 25 µl sample from the pool of minichromosomes. Mix the sample with 10 µl of sample buffer which contains SDS to remove proteins from the chromatin. High quality chromatin should consist of primarily form I and II DNA corresponding to the supercoiled and relaxed form of the viral DNA. If substantial amounts of linear DNA (form III) or smaller fragments of SV40 DNA are present, it suggests that the SV40 DNA has been degraded and should not be used.

6. If desired, determine the quantity of SV40 DNA as described above by real-time PCR. A good preparation of disrupted viral chromatin appears between 13 and 15 cycles.

BASIC PROTOCOL 2

PREPARATION OF SV40 MINICHROMOSOMES FROM LYTICALLY INFECTED CELLS

This protocol describes the preparation of SV40 minichromosomes from infected African Green Monkey Kidney cells and their purification. Cells are infected with a stock virus containing high levels of SV40 and incubated from 30 minutes to 48 hours post-infection. Times for harvesting the minichromosomes are chosen based upon the biological events that the SV40 minichromosomes are undergoing transcription or replication. Nuclei are prepared from the infected cells at harvest using a low-ionic strength buffer containing magnesium ion to keep the chromatin compacted, followed by extraction of the nuclei in a buffer lacking magnesium ion to free the minichromosomes. The extracted crude SV40 minichromosomes are purified by sedimentation on a glycerol gradient.

Materials

High titer stock SV40 virus, Our stock virus (776) was originally obtained from the laboratory of Dr. Daniel Nathans. SV40 can be obtained from the American Type Culture Collection (ATCC) or alternatively by contacting the Milavetz laboratory (barry.milavetz@ad.ndus.edu)

SV40 DNA (Obtained by a modified Hirt procedure (Hirt 1967), see support protocol 1)

Four 75 cm^2 T-flasks containing 70% confluent African Green Monkey Kidney (AGMK) cells (ATCC # CCL-26)

75 cm^2 T-flasks (Corning, #430720U)

Cell culture medium MEM (Gibco, #11095-098, see recipe for additional components)

Fetal bovine serum (Gibco, #16140-063)

Gentamycin (Gibco, # 15710-072)

Trypsin (Gibco, # 25300-120)

Nuclease-free water (Ambion, #AM9937)

DNAse I (New England Biolabs, #M0303S)

Agarose (Sigma, # A6877)

SsoAdvanced Universal SYBR Green Supermix (Biorad, # 172–5274)

$T_{10}E$ (see recipe)

EGTA 100 mM (see recipe)

DTT, 1 M (see recipe)

10% glycerol low-ionic strength buffer (see recipe)

Running buffer (see recipe)

Sample buffer (see recipe)

Ethidium bromide or SYBR green (see recipe)

Dulbecco's Phosphate Buffered Saline (PBS) (Gibco) 14190-250

Disposable cell scraper (Fisher) #08–773-2

0.05% Triton X-100 (see recipe)

Nuclei isolation buffer (see recipe)

Nuclei extraction buffer (see recipe)

10 µl Graduated Filter Tips

10 µl Pipetman

1000 µl Pipetman

1000ul Filter Tips

15 ml centrifuge tubes (Corning, # 430052)

200 µl Graduated Filter Tips

200 µl Pipetman

37°C heat block

Beckman ultracentrifuge TLA-100 or equivalent small volume ultracentrifuge

BSL-2 biosafety laminar air cabinet (Nuaire, Model NU-425-400 or equivalent)

Eppendorf snap-cap microcentrifuge flex tubes (Fisher Scientific # 022364111)

Power supply (125V)

Real-time PCR machine

Sterile cell culture incubator

Submerged Agarose Gel Apparatus

Infection of Cells

1. The number of 75 cm^2 T-flasks used for the preparation of SV40 chromosomes depends upon the type of experiment. Typically we use one or two flasks for each time-point of infection or treatment. Because the chromosomes are purified by ultracentrifugation we are limited to a total of six flasks in an analysis comparing SV40 chromosomes at different times of infection or different conditions of incubation. Seed T-flasks with AGMK cells using normal cell culture procedures and incubate at 37°C. We use T-flasks so that we can tightly close the cap, which allows us to do all of our manipulations with SV40 virus in a biosafety hood at BSL-2 conditions. When cells are approximately 70% confluent, pour 9 ml of medium from the T-flask into a sterile 15ml centrifuge tube and discard the rest of the medium from the flask. Pour the 9 ml into the T-flask and add 1 ml of a working stock of virus as described above to the flask. Incubate the flask for 30 minutes at 37°C.

2. While the virus is incubated on the cells, warm fresh MEM to room temperature. After the 30-minute incubation to adsorb virus onto the cells and begin the infection process, remove the medium containing virus and then wash each flask twice with 10 ml of the fresh warmed MEM medium. The purpose of the two washes is to remove as much of the unbound virus as possible. This is done to ensure that to the extent possible the infection is synchronized in all of the cells. Following the two medium washes either harvest immediately the infected cells or continue the incubation for the times desired by the experimental conditions.

Isolation of Nuclei from Infected Cells

1. In preparation for isolating SV40 infected cell nuclei, fill one 15 ml conical centrifugation tubes for each T-flask of infected cell with 15 ml of a low-ionic strength isolation buffer (stored at 4°C). Immediately remove 1 ml of the buffer and transfer to a 1.4 ml Eppendorf tube. Store the tubes containing nuclei isolation buffer at 4°C in a refrigerator until ready to be used.

2. Harvest the SV40 chromosomes present in cells infected with SV40 at the desired time in infection by first removing all the medium present in the cell culture flask. Pour off the medium, place the flasks on edge for a minute or two, and remove the residual medium collecting on the bottom with a 1 ml Pipetman.

3. Following removal of the media wash each flask twice with 10 ml of cold PBS removed from the refrigerator immediately before use. Again place the flasks on edge to collect residual PBS for a minute or two. Remove the residual PBS using a 1 ml Pipetman.

4. Harvest the infected nuclei present in cells by adding 1.0 ml of a low-ionic strength isolation buffer from the previously prepared 15 ml centrifuge tubes to each flask. Rock each flask to ensure that the isolation buffer has wet all of the cells in the flask. If viewed in a microscope the cells begin to swell slightly.

5. Using a curved rubber cell scraper, dislodge the cells present in the flask from the bottom of the flask.

6. Following dislodging of the cells, add 1.0 ml of 0.05% Triton X-100 to each flask to remove the lipid membrane surrounding the cells. Draw the liquid containing dislodged infected cells into and force out of the 1 ml pipet used to deliver the triton buffer at least 5 times to further break up the cell membranes. Then centrifuge the buffer containing the disrupted cells in a Beckman Model J6-MI centrifuge for 10 min at 3,000 rpm (3420 × g) to pellet the nuclei which have been released from the cells.

7. Remove the supernatant isolation buffer by pouring off the bulk of the buffer. Then use a 1 ml pipette to remove the rest of the buffer from the pellet. Add 200 μl of isolation buffer from the 1 ml Eppendorf tube using a 200 μl pipette to the pellet and resuspend by drawing the suspension into and out of the pipette at least 5 times. Layer the resuspension onto the remaining 800 μl of isolation buffer in the Eppendorf tube and centrifuge in a Sorvall Biofuge Fresco for 10 minutes at 2,000 rpm (10,820 × g) and 4°C.

8. Following centrifugation remove the supernatant with a 1 ml pipette, and store the pellet at −20°C until used for the next step.

Extraction of Crude SV40 Minichromosomes from Infected Nuclei and Purification of SV40 Minichromomes

1. Thaw the frozen pellets of the nuclei obtained from SV40 infected cells quickly at room temperature by the addition of 100 μl of 4°C nuclei extraction buffer. Vortex the tubes to resuspend the pellet and then centrifuge the tubes in an Eppendorf microfuge for 30 sec at 10,000 rpm (54,140 × g). Remove the supernatant, add 400 μl of fresh nuclei extraction buffer to each tube, and incubate for 1 hour at 4°C in a refrigerator.

2. Following this incubation centrifuge the suspension at 10,000 rpm (54,140 × g) for 30 sec in an Eppendorf microfuge. Transfer the supernatant containing the extracted chromosomes to a new 1.4 ml Eppendorf tube and store at −20°C until the SV40 chromatin is purified. The extracted nuclei can be purified immediately, but we generally prefer to store the extract at least overnight before the purification step.

3. SV40 chromosomes are separated based upon their sedimentation characteristics from virions and partially encapsidated SV40 chromatin by ultracentrifugation in a TLA-100 ultracentrifuge. Layer the 400 µl of extracted SV40 chromatin from infected nuclei carefully onto 1 ml of a low-ionic strength 10% glycerol buffer in a 1.4 ml Eppendorf tube. It is important to use a very high quality tube for this step. Transfer the tubes to a TLA 100.3 rotor and centrifuge for 35 min at 50,000 rpm (27,050 × g) at 4°C.

4. Following centrifugation, sequentially remove 200 µl fractions from the top of the tube to the bottom using a 200 µl pipette. Discard the first two fractions which tend to contain fragments of SV40 chromatin (and possibly cellular chromatin). Collect and pool the next three aliquots (3–5) which contain open form SV40 chromosomes and for subsequent analysis. If desired collect and pool fractions 7 and 8 since they contain SV40 chromatin that are either encapsidating or uncoating depending upon whether the chromatin is obtained late or early in infection, respectively.

SUPPORT PROTOCOL 1

Preparation of SV40 DNA by the Hirt Procedure—Pure SV40 DNA used for comparison during submerged agarose gel electrophoresis and real-time PCR is prepared by a modification of the Hirt procedure (Hirt 1967). The Hirt procedure makes use of the fact that the polyomaviruses like SV40 can be easily separated from the cellular chromatin in an infected cell due to the very small size of the polyomavirus. The infected cells are lysed with a detergent and NaCl added which precipitates the host DNA but not the viral DNA. The viral DNA is then separated by centrifugation and subsequently purified.

Materials

SV40 working stock virus (*prepared as described above*)

Four 75 cm^2 T-flasks containing 70% confluent African Green Monkey Kidney (AGMK) cells (ATCC # CCL-26)

75 cm^2 T-flasks (Corning, #430720U)

Cell culture medium MEM (Gibco, #11095-098, *see recipe for additional components*)

Fetal bovine serum (Gibco, #16140-063)

Gentamycin (Gibco, # 15710-072)

Trypsin (Gibco, # 25300-120)

$T_{10}E$ (*see recipe*)

Lysing Solution (*see recipe*)

5 M NaCl (*see recipe*)

Dulbecco’s Phosphate Buffered Saline (PBS) (Gibco, #14190-250)

1000 µl Pipetman

1000ul Filter Tips

200 µl Graduated Filter Tips

200 µl Pipetman

15 ml centrifuge tubes (Corning, # 430052)

37°C heat block

Eppendorf snap-cap microcentrifuge flex tubes (Fisher Scientific, # 022364111)

1. All manipulation of materials that contain SV40 virus is performed in a BSL-2 biosafety cabinet. Typically seed two 75 cm^2 T-flasks with AGMK cells during routine cell culture in 10 ml MEM (with 10% fetal bovine calf serum, sodium carbonate, and gentamycin) and incubate in a water jacketed incubator at 37°C.
2. When the cells are approximately 75% confluent pour 9 ml of the culture medium into a 15 ml disposable sterile centrifuge tube and discard the rest of the medium in the T-flask. Add the 9 ml of medium back to the T-flask along with 1 ml of our working stock SV40.
3. Incubate the virus on the cells for 48 hours to allow for infection and multiplication of the virus.
4. At 48 hours pour off the medium, wash the cells with cold PBS twice with removal of the PBS after each wash.
5. Next, add 300 µl of lysing solution to each plate and rock the plate with the solution until all of the cells have lysed and are collected as a viscous liquid on the bottom of the flask when held upright.
6. Carefully transfer the viscous liquid to a Eppendorf tube by pouring. A pipette should not be used during this step because it can shear the cellular DNA.
7. Add 100 µl of 5 M NaCl to the Eppendorf tube and store the tube at 4°C overnight.
8. Centrifuge the tube containing SV40 and cellular DNA at 10,000 rpm (54,140 × g) for 10 minutes and remove the pellet with a sterile wood applicator stick.
9. Purify the remaining liquid containing SV40 DNA using the Zymo ChIP DNA Clean and Concentrate according to the protocol supplied with this kit. Elute the DNA with 25 µl of $T_{10}E$.

BASIC PROTOCOL 3

EPIGENETIC ANALYSIS OF SV40 MINICHROMOSOMES

This protocol describes procedures for analyzing SV40 chromatin by next-generation sequencing for the location of nucleosomes and for the location of modified histones and other chromatin binding proteins. The location of nucleosomes is determined by preparing mononucleosomes using micrococcal nuclease to digest the chromatin, purifying the DNA

present in the nucleosomes, and preparing sequencing libraries from the DNA. The location of specific proteins or modified histones is determined by chromatin immunoprecipitation with antibody to the protein/modification of interest followed by sonication, purification of the DNA, and the preparation of sequencing libraries from the DNA. Finally, we describe how the two sets of procedures can be combined to identify the location of nucleosomes in SV40 chromatin containing a specific protein such as RNA Polymerase II. A list of antibodies which we have successfully used for the epigenetic analyses of SV40 is provided in Table 1.

Materials

50/50 mixture of glycerol and Tris-EDTA buffer for diluting micrococcal nuclease (see recipe)

Agarose gel sample buffer (*see recipe*)

AMPure XP PCR purification kit (Beckman Coulter, #A63880)

Antibodies (various sources we typically purchase from Millipore, Abcam)

Certified Molecular Biology Agarose (Bio-Rad, #1613101)

Certified low melting Molecular Biology Agarose (Bio-Rad, #1613112)

ChIP DNA Clean and Concentrator kit (Zymo Research, # D5205)

ChIP kit (Millipore, # 17-295)

EmbiTec PrepOne Sapphire or similar imaging system

Ethanol (Sigma-Aldrich, #459844)

Micrococcal nuclease (NEB, #M0247S)

NEB library kit NEXT Ultra II DNA library prep Kit and E7335S Mulitplex oligos for Illumina

Nuclease-free water (Ambion, #fAM9937)

SsoAdvanced Universal SYBR Green Supermix (Biorad, # 172-5274)

Zymoclean Gel DNA Recovery Kit (Zymo Research, # D400)

10 µl Graduated Filter Tips

10 µl Pipetman

1000 µl Pipetman

1000ul Filter Tips

2 µl Pipetman

200 µl Graduated Filter Tips

200 µl Pipetman

200 µl thin-wall PCR Tubes (VWR, # 20170-012)

Eppendorf snap-cap microcentrifuge flex tubes (Fisher Scientific, #022264111)

Eppendorf MasterCycler Personal PCR

Eppendorf Protein Lobind 1.5 ml tubes (Fisher Scientific, # 0030108116)

Magnetic Stand

Minicentrifuge

Power supply Bio-Rad Power Pac 3000 at 125 constant volts

Sonifier Branson digital sonifier with a water horn model 102C. Set at 50% power for 6 minutes with cold water running through the horn

Submerged Agarose Gel Apparatus

Digestion of SV40 minichromosomes by Micrococcal Nuclease

1. The location of individual nucleosomes in SV40 minichromosomes isolated under varying conditions can be easily determined by fragmenting the SV40 chromatin with micrococcal nuclease, preparing libraries from the fragmented DNA, and sequencing the libraries by paired-end sequencing. A bioinformatics analysis of the resulting sequencing reads indicates the location of nucleosomes.

2. For these studies one can use SV40 chromatin obtained either by disruption of virions or following infection of cells as described above.

3. Digest the SV40 chromatin with micrococcal nuclease using an Eppendorf Mastercycler Personal PCR machine at 4°C in a 200 µl thin-wall PCR tube. Cool the PCR tube at 4°C at least 10 minutes prior to initiation of digestion. Add SV40 chromatin (43 µl) o the tube followed by 1 µl of BSA as supplied with the enzyme by NEB. Next we prepare the stop buffer for each reaction by placing 250 µl of DNA binding buffer from the Zymo ChIP DNA Clean and Concentrator kit into a 1.4 ml Eppendorf microcentrifuge tube. The stop buffer is kept immediately adjacent to the PCR machine. We then arrange in order two 10 µl piptetmen and a 200 µl Pipetman. The 200 µl Pipetman is adjusted to 60 µl and a sterile tip is placed onto it. The first 10 µl Pipetman is loaded with 5 µl of the 10X digestion buffer supplied by NEB. The second 10 µl Pipetman is loaded with 1 µl of NEB micrococcal nuclease as supplied, or with 1 µl of diluted micrococcal nuclease. Dilute the enzyme if required into a buffer containing a 50/50 mixture of glycerol and $T_{10}E$. Whether enzyme is used as supplied or diluted is determined empirically based upon our initial results. In rapid order we add the 10X digestion buffer and the micrococcal nuclease and then mix with the 200 µl and time the reaction. Typically we start with a 5 second digestion and a 30 second digestion with the enzyme. Stop the reaction by adding the contents of the PCR tube to the 250 µl of DNA binding buffer from the Zymo ChIP DNA Clean and Concentrator kit.

4. Purify the DNA using the ChIP DNA clean and concentrator kit according to the protocol supplied by the manufacturer. In the final purification step elute the DNA using 25 µl of nuclease-free water and store until subsequent use.

5. Monitor the extent of digestion by comparing the amount of PCR amplification product obtained from the digested sample DNA to an equivalent sample for which no enzyme was added. Typically we amplify a 250 bp fragment of the SV40 genome found around the transcription termination site although other regions of the genome have also been used. The primers used for this amplification and the conditions for amplification are described above. We perform the PCR in a 25 μl total sample volume using the BioRad 2X buffer diluted with nuclease-free water to the final concentration. Typically for library preparation we prefer approximately a three cycle reduction in PCR amplification product from the digested sample compared to the undigested sample. This is the equivalent of 75% to 90% digested chromatin. We also usually mix samples digested from 2 cycles to 4 cycles to ensure that the correct sized DNA fragments are represented.

6. If we find that digestion with undiluted enzyme results in 4 or more cycles of digestion we dilute the enzyme 1/10 and redo the 5 second and 30 second digestions. If that is still too much we do a 1/100 dilution and repeat the preparation. This is repeated until we obtain the desired 2 to 4 cycle reduction in DNA as determined by PCR amplification. Store the digested chromatin at −20°C until used for the preparation of libraries.

Analysis of SV40 Minichromosomes by Chromatin Immunoprecipitation

1. In order to determine the relationship between the various histone modifications and their location in the SV40 genome, we have used variations of chromatin immunoprecipitation (CHIP) (Balakrishnan and Milavetz 2005, Balakrishnan and Milavetz 2006, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2007, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014). SV40 chromatin from virus or infected cells can be used for these studies.

2. Bind an antibody of interest to agarose in an Eppendorf protein lobind 1.5 ml tube. Add 10 μg of an antibody to a tube followed by 1 ml of chromatin binding buffer and 200 μl of protein A agarose from the Millipore ChIP kit. Rotate the mixture end over end for 4 hours to completely bind the antibody to the protein A agarose. Next, sediment the agarose in an Eppendorf microcentrifuge set to 2000 (10,820 × g) for 1 minute. Remove and discard the liquid. Add 1 ml of fresh chromatin binding buffer followed by 50–100 μl of SV40 chromatin either from disrupted virus or minichromosomes. The amount depends upon how much SV40 chromatin is present in the sample. We routinely measure this by PCR amplification and compare the results to a sample of known SV40 chromatin. Then rotate the antibody bound to agarose end over end overnight to bind up the target SV40 chromatin.

   If the amount of SV40 chromatin is too low as determined from the ChIP experiment, the repeat procedure with more chromatin.

3. The next morning centrifuge the tube to sediment the agarose and remove and discard the supernatant. Wash the agarose using the buffers supplied in the Millipore kit according to the protocol of the kit. During each step of the washes rotate the sample end over end for 10 minutes to thoroughly mix the agarose and wash buffer. At the end of the final wash centrifuge the sample and remove the supernatant.

4. Move the sample into a laminar air hood for further preparation. First transfer each sample from the binding tube to a fresh Eppendorf protein lobind tube using 150 µl of $T_{10}E$. Centrifuge both tubes using a Southwest Science minicentrifuge to pellet the agarose. Remove a small portion of the $T_{10}E$ from the new tube and use it to re-suspend any remaining agarose present in the original tube. Transfer the any residual re-suspended agarose to the new tube. Finally, sediment the agarose and remove and discard the buffer.

5. Next fragment the SV40 chromatin bound to the agarose either by sonication or micrococcal nuclease digestion. When fragmented by sonication, add a fresh 150 µl of $T_{10}E$ to the tube and place the tube in a water-cooled sonication horn associated with the Sonifier. Sonicate the tube for 6 minutes at 50% power with the cold water moving rapidly through the bath. Following sonication return the tube to the hood for further preparation.

   We have previously shown that these sonication conditions generate appropriately sized fragments. This is confirmed when we analyze the libraries prepared from the DNA by submerged agarose gel electrophoresis where we observe a significant amount of the library migrating as DNA between 200 and 400 bp in size.

6. When the bound chromatin is fragmented by micrococcal nuclease digestion, prepare two tubes of chromatin bound to agarose and digest them in tandem. Re-suspend the agarose of each tube in 87 µl of $T_{10}E$ and add 2 µl of BSA as described above. Using the same procedure described above for the digestion of SV40 chromatin, we add in rapid succession 10 µl of 10X digestion buffer and 1 µl of enzyme. Mix the contents of the tube using a 200 µl Pipetman set to 110 µl and incubate one tube for 5 seconds and the other tube for 30 seconds at 4°C. Following each digestion, stop the reaction by the addition of 10 µl EGTA (100 mM) to a final concentration of 10 mM. Again return the two tubes are to the hood.

7. Following fragmentation of the bound SV40 chromatin, centrifuge the tubes to sediment the agarose and allow for the separation of the bound fraction present on the agarose from the liberated chromatin present in the supernatant liquid. Depending upon the purpose of the ChIP, either discard or retain the supernatant for subsequent analysis.

8. If the supernatant is retained, transfer it to a fresh tube, sediment to remove any residual agarose and transfer to another fresh tube. Repeat this once more to

remove all of the agarose and freeze the supernatant or subject it to a second ChIP.

9. Wash the agarose from the original ChIP twice with 150 μl of $T_{10}E$. Add the $T_{10}E$ to the tube, vortex the tube, centrifuge the tube, and remove and discard the supernatant. Repeat the wash a second time to remove as much of the original supernatant as possible.

10. Elute the bound fragmented SV40 DNA from the agarose using the lysing solution supplied in the Millipore Kit. Add 100 μl lysing solution to each tube, vortex the tube, and incubate at room temperature for 15 minutes. Following the incubation, centrifuge the tube and transfer the supernatant to a new tube. Repeat this procedure and add the lysing solution from the second incubation to the first supernatant.

11. Purify the DNA obtained from the ChIP (both the bound and liberated fractions) using a ChIP DNA Clean and Concentrator kit. Elute the purified DNA in 15 μl to 50 μl of nuclease-free water depending upon its subsequent use. If used for PCR analysis we generally elute in 15 μl if the DNA is going to be size selected by agarose gel electrophoresis and in 50 μl if it is being used directly for library preparation using the NEB kit.

Preparation of Libraries for Sequencing

1. We presently prepare our libraries for sequencing from micrococcal nuclease digested SV40 chromatin and ChIP analyses using an NEBNEXT kit designed to work with the Illumina sequencing system. Perform all manipulations in a PCR hood.

2. First, end-repair the DNA in a 200 μl thin-walled PCR tube using a thermal cycler with a heated lid (Eppendorf Mastercycler Personal PCR). Transfer 50 μl of purified fragmented SV40 DNA (regardless of source) to a sterile PCR tube and mix with 7 μl of end repair buffer and 3 μl of end repair enzymes as supplied in the kit. Incubate the solution in the thermal cycler at 20°C for 30 minutes and at 65°C for an additional 30 minutes with the lid closed. Then cool the solution to 4°C.

3. Return the tube to the hood for ligation of the adapter onto the fragmented end-repaired DNA. To each tube we add in order, 1 μl of ligation enhancer, 2.5 μl of diluted adapter, and 30 μl of ligation mix. With a 200 μl Pipetman set to 90 μl thoroughly mic the solution and incubate at 20°C for 15 minutes. Return the solution to the hood and add 3 μl of USER to the tube and then incubate the tube at 37°C for 15 minutes. Dilute the adapter supplied by NEB immediately before use in a dilution buffer supplied by NEB. For micrococcal nuclease digested whole SV40 chromatin we found that a 1/10 dilution was optimal. For fragmented DNA prepared by ChIP analysis we used a 1/50 dilution of the adapter.

4. Purify the DNA library using AMPure. Add 95 µl AMPure to the tube and mix the contents thoroughly before transferring to a fresh 1.5 ml Eppendorf tube. Incubate the contents in the tube at room temperature for 10 minutes to bind the DNA to the substrate.

5. Following binding of the DNA, centrifuge the tube for 5 seconds to force the contents to the bottom and place into a magnetic stand to separate the DNA bound to the magnetic substrate from the liquid. We do this with the stand on its side and the mixture of beads and liquid in the middle of the side of the tube. Place the tubes on their side instead of upright so that it is much less likely that any of the magnetic beads are accidentally removed during removal of the original liquid or wash liquids. Following a 10-minute incubation at room temperature, right the stand to allow the liquid to collect on the bottom of the tube and remove the liquid from the bound beads.

6. Wash the beads twice with a freshly prepared 80% ethanol in water solution. Following the washes dry the beads for 10 minutes in the air and remove the tube from the magnetic stand.

7. Add 15 µl of water to the tube followed by vortexing the contents to mix the magnetic beads and the water. Incubate the mixture for 10 minutes and centrifuge the mixture for 15 sec to force the contents to the bottom of the tube.

8. Place the tube back into the magnetic stand for 5 minutes to allow the beads to separate from the water, which now contains the eluted library DNA.

9. Carefully remove the water containing the library with a 10 µl Pipetman being very careful to not touch the magnetic beads in the process.

10. Following preparation of the library determine the quality of the library by PCR amplification followed by submerged agarose gel electrophoresis on a 2.6% agarose gel. Amplify 1 µl of the freshly prepared library in a final volume of 25 µl which consisted of 12.5 µl of BioRad 2X PCR amplification buffer , 1 µl of the NEB universal primer, 1 µl of a NEB specific index primer, and 10.5 µl of water. Initiate the PCR with a 10 minute incubation at 95°C to activate the polymerase followed by 40 cycles of 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. Following amplification cool the sample to 4°C until analyzed by electrophoresis.

11. We determine the quality of each library by submerged agarose gel electrophoresis. Add 10 µl of sample buffer to the PCR tube containing the amplified DNA for a total volume of 35 µl. Load the sample a well in a 2.7 % agarose gel in TAE buffer along with a size marker. The agarose gel and TAE buffer contained ethidium bromide (5 µl). Electrophorese the sample and size marker for approximately 1 hour and 15 minutes with the voltage set to 125 volts. Following electrophoresis, visualize the amplified DNA and marker by UV light and photographed.

A good library should contain a smear of DNA from approximately 250 bp in size toward the top of the agarose gel. Prominent bands found below 250 bp

probably represent dimers from the adapters loaded onto the fragmented SV40 DNA during the ligation step. Alternatively, smaller products can also be generated by dimers formed from the primers used for PCR.

12. If a suitable library has been prepared we then size select for only those DNA species in the library that are approximately 250–300 bp in size. Since the adapter and primers together add about 100 bp to the size of the library DNA this ensures that we will be analyzing during sequencing only fragments around 100 to 200 bp in size which would be expected for nuclease digested mononucleosomes.

13. The purified unamplified library DNA is size selected by submerged agarose gel electrophoresis using a mixture of high purity low melting temperature agarose and regular agarose such as Bio-Rad Certified Molecular Biology Agaroses. For purification we use a mixture containing 0.7 g of low melting temperature agarose and 0.1 g regular molecular biology grade agarose in 50 ml of the diluted running buffer for optimal resolution of the library. When the agarose has dissolved add 5 μl of SYBR green to the dissolved agarose immediately prior to pouring into the electrophoresis apparatus. Since the amount of library DNA is too small to easily visualize we load the library between marker DNA on the left and the right. Typically there are two or three empty lanes between the marker DNAs and the library DNA.

14. Following electrophoresis, dry the surface of the agarose gel with a paper towel or Kimwipe and visualize the marker DNA on an EmbiTec PrepOne Sapphire. Based upon the position of the marker DNAs cut a line into the gel just below each marker at 250 bp. Again dry the surface of the gel with a paper towel or Kimwipe and cut a line connecting the two marker DNAs through the width of the gel using a metal spatula. Next, use the spatula at a 90° angle to the previous cut to slice the agarose on the right of the lane. This cut is made adjacent to the marker dye present in the library lane near the bottom of the gel, which would include the majority of the library DNA present. Remove the piece of gel on the right of the library sample using the spatula and make a second similar cut immediately to the left of the library sample again using the marker dye as a guide.

This results in more or less the complete lane containing the library DNA being separated from the rest of the gel.

15. For each library being purified, we remove two gel slices for isolation of the library DNA. The first contains fragments from approximately 200 to 250 bp in length and is obtained by taking a slice of the gel in the library lane approximately 4 mm below the line connecting the250 bp size marker. The second contains fragments from 250 to 300 bp in length and is obtained by taking a 4 mm slice immediately above the line connecting the 250 bp size marker.

16. Purify the library DNA in each slice using a Zymoclean Gel DNA Recovery Kit using the reagents and protocol supplied in the kit. Following purification with the kit, elute the library DNA with 25 μl of nuclease-free water.

17. Determine the quality of the size-selected purified library by PCR amplification and submerged agarose gel electrophoresis using the same procedures as described for the initial libraries. A good library should appear as a single band on the agarose gel approximately 250 bp in size. In preparation for making the final library, note the number of PCR cycles at which the amplification products reach a plateau from this amplification.

18. In the final step of the library preparation, amplify the size-selected library by PCR and purify the products for sequencing. Amplify the library in two 50 μl reactions using the same PCR conditions described above for the number of cycles at which the amplification first reaches the plateau stage as determined from the previous step. Purify the amplified DNA using 90 μl of AMPure according to the protocol supplied by the supplier.

19. Sequence the purified amplified library. We sequence our libraries on a MiSeq using paired-end sequencing with 150 cycle chemistry and between 10 and 20 libraries per sequencing chip.

Generally we have around 100,000 reads at each base for each library.

20. Analyze the sequencing results using the bioinformatic programs preferred by the investigator. We do a preliminary quality control analysis using FASTQC v. 0.11.2 (Andrews 2010). If trimming is required we use scythe v0.981 (Buffalo 2011), while for quality trimming we use sickle v1.33 (Joshi and Fass 2011). We set our quality threshold at a phred score of 33 and also discard all reads below 45 bps. We align reads to the SV40 genome (RefSeq Acc: NC_001669.1) using Bowtie2 (Langmead, Trapnell et al. 2009) Contaminating cellular sequences are aligned to Chorocebus_sabeus1.1 and hg19 and discarded. In order to take into account the fact that the SV40 genome is circular, we arbitrarily cut the SV40 genome at 0 and 2666 and generate two sets of data; one aligned to the genome cut at 0 and the other at 2666.

21. We use DANPOS-2.2.2 to determine nucleosome occupancy (Chen, Xi et al. 2013). The results from this program are displayed as heatmaps where the intensity of color correlates with the frequency in which a base is found to be present in a read. A typical example is shown in Figure 3 in which we compare the location of nucleosomes in minichromosomes obtained at 48 hours post-infection (top) to the corresponding location in chromatin from disrupted virus (bottom). As shown in this figure there are a number of bands which overlap in the two forms of chromatin indicating that nucleosomes are located at the same sites in both forms of chromatin. There are also regions in which the bands appear to be shifted to the right or left indicating that nucleosomes are located at very different sites in the two forms of chromatin. From the nucleotide number of the center of the band, we correlate the position of the nucleosome to potential

regulatory sequences in the SV40 genome. For example, the prominent set of bands located around nt 360 on the left of the figure from the disrupted virus, corresponds to a nucleosome located over the late transcription start site in this chromatin. This set of bands appears to be significantly reduced in the minichromosomes isolated 48 hours post-infection and instead a new prominent band appears at nt 212.

REAGENTS AND SOLUTIONS

Ethidium Bromide Stain (0.5 μg/μl)

50 mg ethidium bromide, add water to 100 ml

Chromosome Preparation Nuclei Buffer [pH 7.4]

50 ml glycerol (5%), 0.84 g HEPES (2mM), 5ml 0.1 M $MgCl_2$ (0.5mM), 5ml 0.1 M PMSF (0.5mM), 5ml 0.1 M DTT (0.5mM), add Q water to 1L; sterilize by autoclaving. Note: add PMSF and DTT (filter sterilized) after autoclaving

Chromosome Extraction Buffer [pH 7.5]

0.24 g HEPES (10mM), 1ml 0.1 M EDTA (1mM), 0.5 ml 0.1 M DTT (0.5mM), 0.1 ml 100mM PMSF (0.1mM); add Q water to 100 ml; filter sterilize

Chromosome Isolation Buffer [pH 7.5]

1.19 g HEPES (10mM), 0.186 g KCl (5mM), 5ml 0.1 M EDTA (1mM), 1ml 0.1 M $MgCl_2$ (0.2mM), 2.5 m l 0.1 M DTT (0.5mM). Add appropriate amount of glycerol (see below), add water to 500ml; sterilize by autoclaving.

For 10% glycerol: add 50 ml glycerol

For 50% glycerol: add 250 ml glycerol

10% SDS

100g SDS, add Q water to 1.0 L

Lysing Solution

6.0ml 10% SDS, 2.0 ml of 0.1 M EDTA, add water to 100 ml

5M NaCl

292.2 g NaCl, add Q water to 1L

$T_{10}E$

1 ml 1 M Tris, 1 ml 0.1 M EDTA and 98 ml Q water, filter sterilize

EDTA stock (0.5M)

93.05g of EDTA dissolved in 400ml of water. Adjust pH to 8 with NAOH then top of the solution to a final volume of 500ml.

TAE stock (50X)

242g of tris base dissolved in 750ml water. Add 57.1ml glacial acetic acid and 100ml EDTA. Adjust final volume to 1L. Bring the pH to 8.5.

TAE Electrophoresis Running Buffer

To 20 ml TAE (50X) stock buffer, add 980ml water.

EGTA

38 mg in 1 ml water

Dithiothreitol (DTT, 1M)

77mg DTT in 500 µl water

COMMENTARY

History of SV40

Although not the first member of the polyomavirus family of tumor causing agents to be identified, a distinction reserved for mouse polyomavirus (Gross 1953), the fact that SV40 was identified as a contaminant of the polio vaccines being used at the time (Sweet and Hilleman 1960) resulted in significant interest in the properties of SV40. With the parallel observations that SV40 could cause lytic infection of African Green Monkey kidney cells, cause tumors when injected into new born hamsters, and transform other cells in culture, a number of laboratories began a systematic analysis of the molecular biology of SV40. In a sense one could say that SV40 was the right virus for this kind of study at the time, since the development of interest in SV40 and its relative ease of use paralleled the development of modern molecular biology and its reliance on the then new recombinant DNA technologies. Thus, much of what we presently know of eukaryotic molecular biology has roots in work with SV40. This includes our understanding of transcription, replication, and transformation.

The typical laboratory strain of SV40 contains 5243 bp of closed-circular double-strand DNA organized as two transcription units separated at their starts by a complex regulatory region and at their terminations by a second less complex regulatory region (Tooze and Acheson 1981). The start sites and termination sites are located approximately 1800 apart on the circular genome. The early transcription unit codes for the T-antigen and t-antigen, the former involved in regulation of the virus and dysregulation of the host cell, while the latter is involved primarily in dysregulation of the host. The late transcriptional unit codes for the three major structural proteins of the capsid, VP1, VP2, and VP3 and a regulatory protein referred to as the agnogene protein. As their names imply the early genes are expressed primarily early in infection and are important in the establishment of the infection, while the

late proteins are expressed along with replication of the genome and are responsible for the generation of new virus (Tooze and Acheson 1981).

Regulation of transcription and replication are actually very complex with the involvement of multiple cellular factors along with the viral T-antigen. Interestingly T-antigen regulates its own expression through a feedback inhibition mechanism occurring when the newly-synthesized T-antigen interacts with what is known as binding Site I. T-antigen also binds to a second critical site known as Site II where it places a pivotal role in initiating DNA replication (Tooze and Acheson 1981). The fact that SV40 relies heavily on cellular proteins for most of its molecular biology combined with the fact that SV40 is organized into typical chromatin structure with its reliance on epigenetic regulation has made it a very useful model.

Disruption of SV40 virions

The strategy for disruption of the SV40 virion is based upon two observations concerning the structure of the polyomavirus virion. The first is that treatment of the mouse polyoma virions with sodium dodecyl sulfate in the absence of a reducing agent like DTT results in a product which sediments like an intact virion but lacks VP2 and VP3 while the addition of DTT causes the virion to disaggregate, suggesting that sulfhydryl bridges play a role in keeping the virions together (Walter and Deppert 1975). The second observation was that Ca ++ also plays a role in stabilizing the virion (Brady 1977). While the earlier work was done using mouse polyoma virus, the role of Ca++ and sulfhydryl bridges has been confirmed by X-ray crystallography of SV40 (Stehle, Gamblin et al. 1996). Our procedure has been modified only slightly from the procedure previously described for efficient disruption of mouse polyoma virus (Brady 1977). In our procedure we add a step to digest the virions with DNase I in order to remove any viral or cellular chromatin, which may co-purify with the SV40. This is done to ensure that the SV40 chromatin obtained from virions is representative of the chromatin actually present in the virions and not intracellular SV40 chromatin.

Isolation of intracellular SV40 minichromosomes

From the first demonstrations that polyoma virus and SV40 exist as nucleoprotein complexes in infected cells resembling cellular chromatin (Green, Miller et al. 1971, White and Eason 1971), a number of laboratories have described procedures for isolating minichromosomes. These isolation procedures were developed to some extent depending upon the reason for isolating the minichromosomes. The protocol described here was developed specifically to obtain minichromosomes which were capable of limited in vitro DNA synthesis (Tsubota, Waqar et al. 1979). The concentrations of the constituents of each buffer and its pH was determined empirically by measuring the amount of DNA synthesis obtained from minichromosomes isolated using a particular set of buffers. Because the procedure was optimized for DNA synthesis many of the proteins involved in synthesis appear to remain associated with the minichromosomes. We have confirmed this more recently using ChIP analyses to show that RPA70 a protein involved in DNA replication is present on SV40 minichromosomes using this isolation technique (Balakrishnan and Milavetz 2009). Although at the time the procedure was not tested for retaining proteins

involved in transcription, our subsequent work has also shown that RNA polymerase II and p300 are present on SV40 minichromsomes along with appropriately modified histones isolated using this procedure (Balakrishnan and Milavetz 2006, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2007, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014).

Although not recognized when this isolation procedure was developed, SV40 virions can undergo disruption during the isolation of minichromosomes (Garber, Seidman et al. 1980). This can be a serious problem depending upon the isolation conditions and the status of the cells used to prepare the minichromosomes. While our results suggest that it is not a major issue using the protocols as described here since we observe significant differences between the chromatin structure of minichromosomes and chromatin from virions, it is something that should be kept in mind.

Digestion of SV40 chromatin with Micrococcal nuclease

Since the initial observation that micrococcal nuclease can be used to digest chromatin into mononucleosomes (Noll and Kornberg 1977), the enzyme has been used extensively in the study of chromatin structure. More recently micrococcal nuclease digestion of chromatin has been combined with next-generation sequencing to identify the location of nucleosomes over an extended region of chromatin (Mensaert, Denil et al. 2014, Meyer and Liu 2014). Thus, the combination nuclease digestion and next-generation sequencing have become a powerful new way to investigate changes in nucleosome positioning during molecular biology events, as long as certain limitations with the use of micrococcal nuclease are taken into account, such as a preference for micrococcal nuclease to cleave within AT rich regions and to potentially cleave DNA associated within the nucleosome core (Chung, Dunkel et al. 2010, Allan, Fraser et al. 2012). By analyzing multiple samples, using paired-end sequencing, and focusing only on nucleosome sized DNA, we have successfully characterized the organization of nucleosomes in the chromatin from SV40 virions and minichromosomes isolated early and late in infection (Kumar, Christensen et al. 2017).

ChIP analyses

The principles of chromatin immunoprecipitation as used in this protocol was first described in 1988 (Hebbes, Thorne et al. 1988), although it has undergone significant evolution (Kuo and Allis 1999). The procedure described in this protocol has been used extensively by us to investigate the role of histone acetylation and histone methylation in the SV40 life cycle. During the course of our investigations of SV40 chromatin we have modified the basic ChIP procedure in two ways to exploit the fact that SV40 chromatin can be easily obtained as a closed circular intact minichromosome in relatively high amounts. We developed procedures to immunoprecipitate the intact minichromosome in the first step and then to fragment the chromatin subsequently to either investigate the location of the bound and unbound fragments on the genome or to use the bound and unbound chromatin fragments as input in a second ChIP analysis (Milavetz 2004, Balakrishnan and Milavetz 2005, Balakrishnan and Milavetz 2006, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2008, Balakrishnan and Milavetz 2009, Balakrishnan, Gefroh et

al. 2010, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014).

Next-generation sequencing

DNA sequencing has undergone a significant evolution over time. Initially a time-consuming and difficult chemical procedure (Maxam and Gilbert 1977), sequencing was significantly shortened and simplified using the dideoxy nucleotide chain termination procedure (Sanger, Nicklen et al. 1977). While the latter sequencing procedure worked well for smaller genomes, it was not sufficiently robust to cheaply and efficiently be used for the total sequencing of large genomes. Driving this evolution was the desire to cheaply sequence the human genome as a routine event. Although a number of new sequencing strategies have been developed which are referred to as next-generation sequencing, the one that is the basis of the protocol described here is the Illumina system. We do all of our sequencing on an Illumina MiSeq. With the Illumina system libraries of DNA from some source such as micrcooccal nuclease digested chromatin or a ChIP, are prepared which contain a binding sequence which allows the DNA to bind to a substrate which is part of the sequencing system and an index sequence. The DNA is bound to the system and then paired-end sequenced. Paired-end sequencing is allowed in the Illumina system as a consequence of how the bound library DNA is amplified on the substrate. Paired-end sequencing is important because it allows the fragment sequence to be confirmed by comparison to the known SV40 genome sequence, and also allows the sequence to be size-selected. In our analyses we arbitrarily analyze only the sequencing reads that contain from 120 to 150 bp which would be expected to be derived from mononucleosomes. Indexing is also very important in our protocol because it allows us to analyze multiple libraries at the same time. We have successfully sequenced as many as 12 samples simultaneously with large numbers of reads at each nucleotide in the SV40 genome. While 100 to 200 reads at each base is usually considered sufficient, we generally obtain 100,000 or more reads per base with SV40.

Although we initially used the Illumina kit for the preparation of sequencing libraries from SV40 samples, we have found the New England Biolabs kit to be much more sensitive. We now routinely prepare all of our sequencing libraries using the NEB library kit NEXT Ultra II DNA library prep Kit.

Critical Parameters and Troubleshooting

Preparation of SV40 virus—Both the inoculating SV40 virus and the cells are critical for the preparation of working stocks of the virus. It is good practice to sequence the original virus used for the preparation of the working stock. Because of the small size of SV40 this is a fairly simple and inexpensive step. Sequencing of the virus is particularly necessary for working with SV40 mutants or other genetically manipulated forms of SV40 in order to ensure that the identity of the SV40 virus is correct. It is also important to use only a small amount of inoculum in preparing the working stock of virus. SV40 like other polyomaviruses produces defective viruses characterized by the substitution of cellular sequences for viral sequences. We have recently characterized the defective virus found in stocks of SV40 using paired-end next-generation sequencing. Specifically we have looked at

paired end sequences in which one end contained SV40 DNA and the other end contained cellular sequences. We have found that defective viruses using our approach are relatively rare, occurring with a frequency of approximately one sequencing read containing SV40 at one end and cellular DNA at the other end for each 5000 reads which were SV40 at both ends.

The status of the cells used for infections is also extremely important. We routinely start new cells every four to six months using cells obtained from ATCC. We have found that as cells continue in culture they show increased evidence of natural transformation and a decreased ability to harbor SV40 infections and produce good stocks of virus. For this reason we always prepare our stock virus with essentially new stocks of cells. We start a culture from a frozen stock obtained from ATCC and expand it to a 75 cm^2 T-flask. The next few sets of plates from this first T-flask are used for the preparation of the stock virus.

Disruption of SV40 virus—There are three critical steps during the preparation of chromatin from disrupted SV40 virus, the concentration of the virus, the disaggregation of the virus from other nuclear constituents along with nuclease digestion, and the actual disruption of the virus with DTT and EGTA.

The concentration of virus by centrifugation tends to be problem free as long as a high quality disposable plastic tube is used during centrifugation. We use high quality Eppendorf tubes and have not had problems with tubes cracking. However, in the past when we used other tubes we would occasionally have problems with cracking.

The second step of the preparation involves repeated treatment with DNase I to remove all chromatin which is not present within the virions. Associated with the nuclease treatment are repeated cycles of freezing and thawing along with resuspension of the material by drawing the material present in the Eppendorf tube into a 200 μl pipette and forcing the liquid and associate aggregated cellular material back into the Eppendorf tube. The nuclease digestion and disaggregation of any constituents of the nucleus are important to free up as much clean virus as possible. As the final step in this part of the procedure the disaggregated nuclease digested SV40 is sedimented on a 10% glycerol step gradient. It is important that this step is done so that any residual DNase is removed from virus. If the nuclease is not removed it could potentially digest the chromatin released in the next step of the disruption. The efficiency of the nuclease treatment is monitored by submerged agarose gel electrophoresis of an aliquot of the initial concentrated virus and the virus after nuclease treatment. The initial concentrate should contain distinct bands for SV40 but also smears corresponding to cellular chromatin. The smears corresponding to cellular chromatin should be absent after nuclease treatment.

The final step is the disruption of the virus by breaking the covalent linkages holding the VP1 proteins present in the capsid together. These linkages include sulfhydryl bridges between adjacent cysteines and ionic stabilization by Ca^{2+}. The sulfhydryl bridges are broken using DTT while the Ca^{2+} is removed with EGTA. This is a critical step since too much or too little of either reagent can result in reduced yields. At the end of the disruption the chromatin sedimenting as minichromosomes is separated for partially disrupted and

intact virions by centrifugation on a 10% glycerol step gradient. The amount of intact virus can be estimated by resuspending any pellet and analyzing an aliquot by PCR amplification.

We routinely monitor the amount of SV40 at each step in this procedure using real-time PCR amplification. By following the steps this way it is possible to determine if one of the steps was not working properly.

Preparation of SV40 minichromosomes—Contamination by cellular chromatin and disruption of intact SV40 virions are the two most important potential problems that need to be considered during the preparation of minichromosomes. Both the status of the cells and the quality of the input virus are critical for minimizing these two potential problems.

The procedure for isolating minichromosomes was determined empirically and is based on the small size of the minichromosome and the fact that high magnesium concentrations causes chromatin to compact. Thus in the first step of the isolation in which infected nuclei are prepared, magnesium is used to keep chromatin condensed in the nuclei. In the second step of the preparation, the magnesium concentration is reduced allowing the SV40 minichromosomes to escape the nuclei. However, it is likely that the change in magnesium also affect other processes, which are likely to impact that quality of the minichromosomes. For example, the presence of magnesium may also activate cellular nucleases, which contribute to contamination. Also the integrity of the SV40 virion depends in part on calcium crosslinking of the viral capsid protein VP1. Removal of the calcium in the isolation step also results in more or less disruption of the intact virions. Disruption of intact virions appears to be a problem whenever minichromosomes are prepared regardless of the procedure used (Garber, Seidman et al. 1980)

As the cells used for preparing minichromosomes age in culture we see a consistent reduction in the yield of minichromosomes and an increase in both cellular contaminants and disruption of virions. The problem appears to be made worse when virus containing less infectious SV40 is used for the preparation of the minichromosomes. For these reasons we use cells that have been in culture for no more than 4 to 6 months and working stocks of virus which contain high amounts of SV40 based upon PCR quantitation.

We also find it useful to monitor the quality of the minichromosomes, which are isolated and used in subsequent experiments. We routinely determine the yield of minichromosomes by PCR amplification following their isolation and when the yield drops off, start fresh monkey kidney cells. Similarly, we monitor the extent of micrococcal nuclease digestion or the efficiency of our ChIP analyses and when the minichromosomes do not appear to be of high quality again start new cells.

Micrococcal nuclease digestion of SV40 chromatin—While digestion with micrococcal nuclease is the procedure of choice for determining the location of nucleosomes in chromatin when combined with next-generation sequencing, it is difficult to use when applied to SV40 chromatin for a number of reasons. As described above the yield of SV40 chromatin as well as the extent to which it is contaminated by cellular chromatin is highly variable depending upon the status of cells and the specific conditions of isolation (drug

treatment, time in infection, etc). We have seen differences of as many as 10 cycles of real-time PCR in different preparations of SV40. While we can control to some extent for the amount of SV40 chromatin using real-time PCR amplification, the result is only an approximation and we have no easy way to control for the presence cellular chromatin and/or the presence of endogenous nucleases or other cellular contaminants. Moreover, the extent of digestion appears to depend upon the total amount of chromatin present in a sample and not on the amount of SV40 alone. For these reasons it is necessary to determine the optimal amount of nuclease to use empirically. As described in the protocol we use various amounts until we have an amount that results in approximately a 75–90% digestion of the SV40 chromatin as judged by the reduction in amount of SV40 DNA when amplified by a set of primers that recognize a 250 bp fragment outside of the promoter. This can be a challenge since some of the samples being digested have been isolated in only small amounts. However, it is the only way we have found to date that allows us to eventually obtain sufficient high quality nucleosome sized chromatin for library preparation and sequencing. One of the problems appears to be the fact that most suppliers of micrococcal nuclease supply a high specific activity enzyme, while for our purposes a lower activity might be more useful.

Chromatin immunoprecipitation of SV40 chromatin (ChIP analyses)—The ChIP analyses done with SV40 chromatin use the reagents supplied in the Millipore kit and follows the protocol supplied with the kit with one major exception. Because of the nature of SV40 chromatin we have not found it necessary to crosslink proteins to the DNA. In our first publications using ChIP analyses we compared formalin cross-linked chromatin to chromatin which had not been cross-linked and found no differences in the results (Balakrishnan and Milavetz 2006, Balakrishnan and Milavetz 2007). Since then we have not been using formalin cross-linking since it tends to give better yields of recovered DNA. However, using formalin with these procedures should also work.

Perhaps the most important consideration for ChIP analysis with SV40 is the quantity and quality of the input chromatin. We have found that the best analyses occur when we use minichromosomes or chromatin from disrupted virus which appear to be present in relatively large amounts based upon the cycle number during real-time PCR of an aliquot. We generally have the best results when the input chromatin (1 μl of crude chromatin) is found to be present at between 15 and 20 cycles by real-time PCR. For the antibodies that we have used, we find that there is typically from a 3 cycle to a 12 cycle reduction in the amount of input chromatin that is immunoprecipitated. If the DNA obtained following a ChIP is less than approximately 30 cycles we find that it can be very difficult to obtain sequencing libraries as described in the next step. A second consideration, which we have not investigated fully, is whether all of the SV40 chromatin obtained by a particular procedure is equally accessible to the antibodies used for a ChIP. We believe that this may be especially important with chromatin from disrupted virus. We do not know if the VP1 which makes up the capsid and which is disrupted during our preparation is lost from the chromatin or whether it remains bound and can block access to antibodies. The latter could account for some of the variability, which we have observed from preparation to preparation. Similarly, we do not know to what extent the presence of contaminating cellular chromatin impacts the

ChIP results. We know that cellular chromatin will be immunoprecipitated but we do not know how the amount of product compares between the SV40 chromatin and cellular chromatin for any given antibody.

Preparation of sequencing libraries from SV40 chromatin—We have prepared sequencing libraries using kits from Illumina and New England Biolabs (NEB) and have found that the NEB kit is better for SV40 chromatin because of its increased sensitivity. As indicated above, the amount of SV40 chromatin obtained from micrococcal nuclease digestion or ChIP with certain antibodies can be relatively small so that a highly sensitive kit like the NEB NEXT Ultra II DNA library prep Kit is required to obtain useful libraries.

We have also found it to be critical to gel purify the library that is initially generated. We find that typically the initial library contains high molecular weight DNA which is likely a result of either incomplete fragmentation or cellular contamination. By selecting for DNA fragments from about 100 to 200 bp in length we ensure that the majority of the sequencing reads that are obtained will correspond to nucleosome-sized fragments. We find that it is necessary to use a marker DNA approximately 250 bp in size for the gel selection and to take a slice about 4 mm in length below this marker to obtain the correct size DNA for sequencing. This is particularly true when using the NEB library kit because the selected libraries are prepared for sequencing by PCR with index primers. These index primers add approximately 50 bp onto the size of the library DNA and result in a final library, which is proportionally larger than the original size selected on the gel.

Of course, we also do a final size selection with AMPure following the amplification of the final library for sequencing. This step is necessary because over amplification can occur depending upon the quality of the purified library. As indicated in the protocol we amplify only to the beginning of the PCR amplification plateau but when the initial library does not contain a lot of DNA this can be a problem. If this occurs there are two options to address the problem. One can amplify less but prepare more than the usual 100 μl of amplification product or one can try to do a two-step AMPure purification. One can consult AMPure literature to determine the optimum conditions for a two-step purification, but we have found that we do this empirically on the few occasions that we have had to resort to this approach.

Anticipated Results

Following the protocols for the preparation of SV40 chromatin will allow an investigator to prepare SV40 minichromosomes and chromatin from virions that can be used in many types of subsequent investigations. Although not discussed in this set of protocols, the minichromosomes isolated will have limited biological function including DNA synthesis and extension of pre-existing transcripts. Moreover, the SV40 chromatin can be obtained from infected cells, which have undergone various treatments including small molecule inhibitors, siRNA treatment, or other forms of cellular manipulation. While the protocols have been described using the wild-type strain 776 SV40, it can be used with all other wild-type strains as well as natural or designed recombinant mutants of SV40.

The protocols described for epigenetic analyses of SV40 chromatin will allow an investigator to determine the location of any individual protein or protein complex on SV40

chromatin. This includes critical regulatory proteins or functional proteins such as RNA polymerase II or complexes such as nucleosomes. The protocols also allows for the investigation of modifications in these proteins. Finally, the protocols make use of the small size and limited organization of SV40 chromatin so that the location of multiple elements in the same chromatin can be identified. For example, the location of nucleosomes in minichromosomes carrying RNA polymerase II can be determined under a set of circumstances. While the protocols have not yet been used with transfected plasmids carrying portions of SV40 such as the early promoter, it is likely that epigenetic regulation in plasmid constructs can also be addressed using these combinations of protocols.

Time Considerations

The preparation of working stocks of SV40 virus generally takes approximately two weeks from infection to optimal cell death and virus yield. The preparation of disrupted virus from these stocks is relatively quick and can generally be done over no more than three days. We use freeze/thaw as a way to breakup aggregates which is the primary reason for the longer times. If lower yields of SV40 chromatin from disrupted virus are acceptable the time can be significantly shorter. The preparation of SV40 minichromosomes can be very quick. From infection to harvest is a maximum of 48 hours. Going longer increases the likelihood that there will be significant contamination of the minichromosomes by chromatin from naturally disrupted virus as described above and also by cellular chromatin. The actual harvest can be accomplished over no more than an hour. Extraction of the minichromosomes takes a little over an hour, and the purification of chromatin on a glycerol gradient takes about an hour in total.

Preparation of fragmented chromatin by micrococcal nuclease digestion takes approximately one hour until the start of the PCR amplification. The ChIP analyses take more time primarily because we do the binding of the chromatin to the antibody bound to agarose overnight. The actual preparation of the immunoprecipitated DNA in a simple ChIP takes about 2 hours from the first wash until the DNA is stripped from the agarose. The more complex ChIPs where we may use two antibodies take proportionally more time because there is generally two incubations with antibody bound to agarose and two preparations of bound chromatin before the DNA stripping stage.

Sequencing libraries can be prepared with the NEB kit in approximately three hours. The first two steps takes a little over an hour, the ligation of adapter and its cleavage takes another 30 minutes, and the purification of the library with AMPure takes approximately an hour. Purification of the library by submerged agarose gel electrophoresis takes about two hours from the time the samples are loaded onto the agarose gel until the DNA is ready for PCR amplification.

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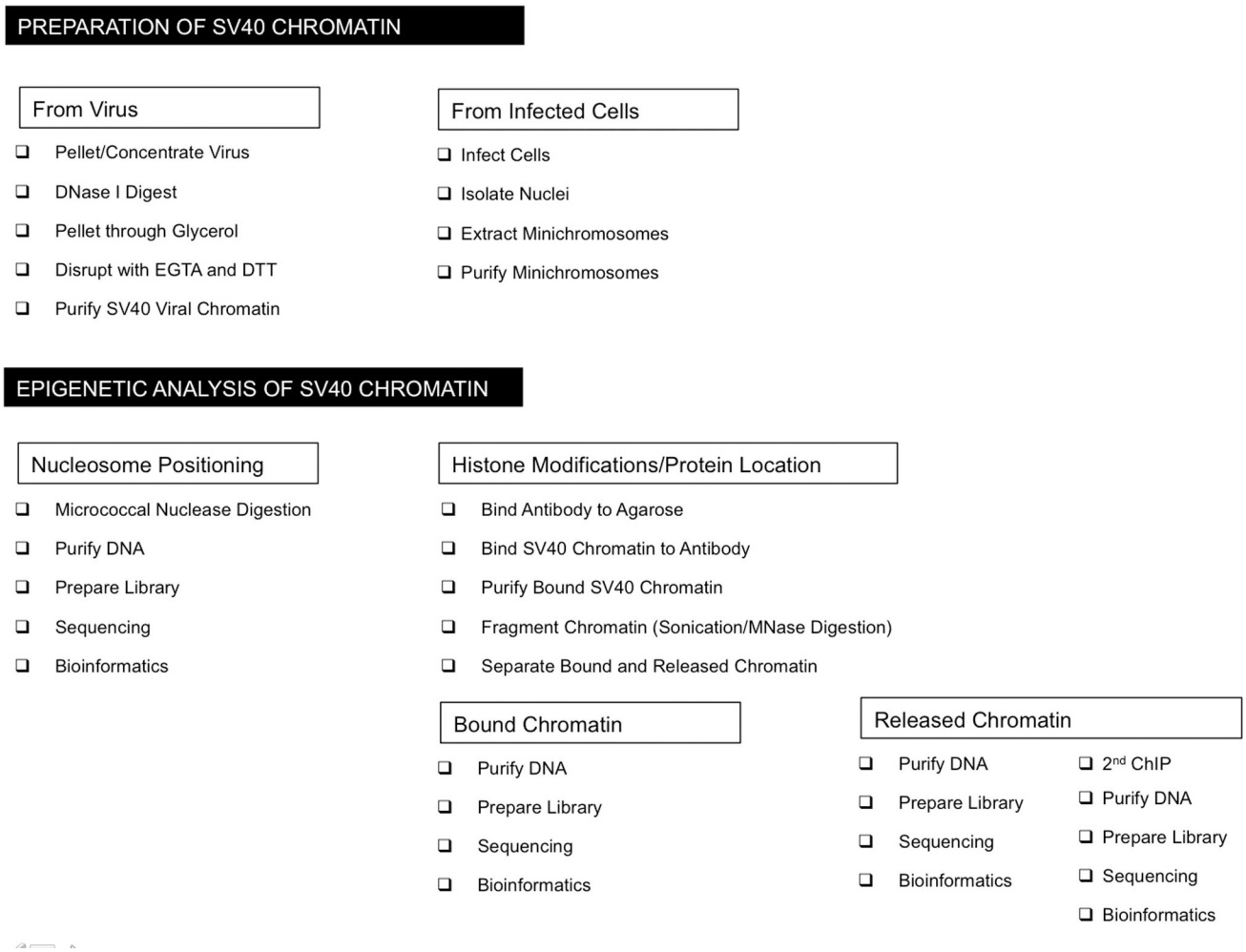

Figure 1.
Flowchart of Preparation of SV40 Chromatin and Subsequent Epigenetic Analysis

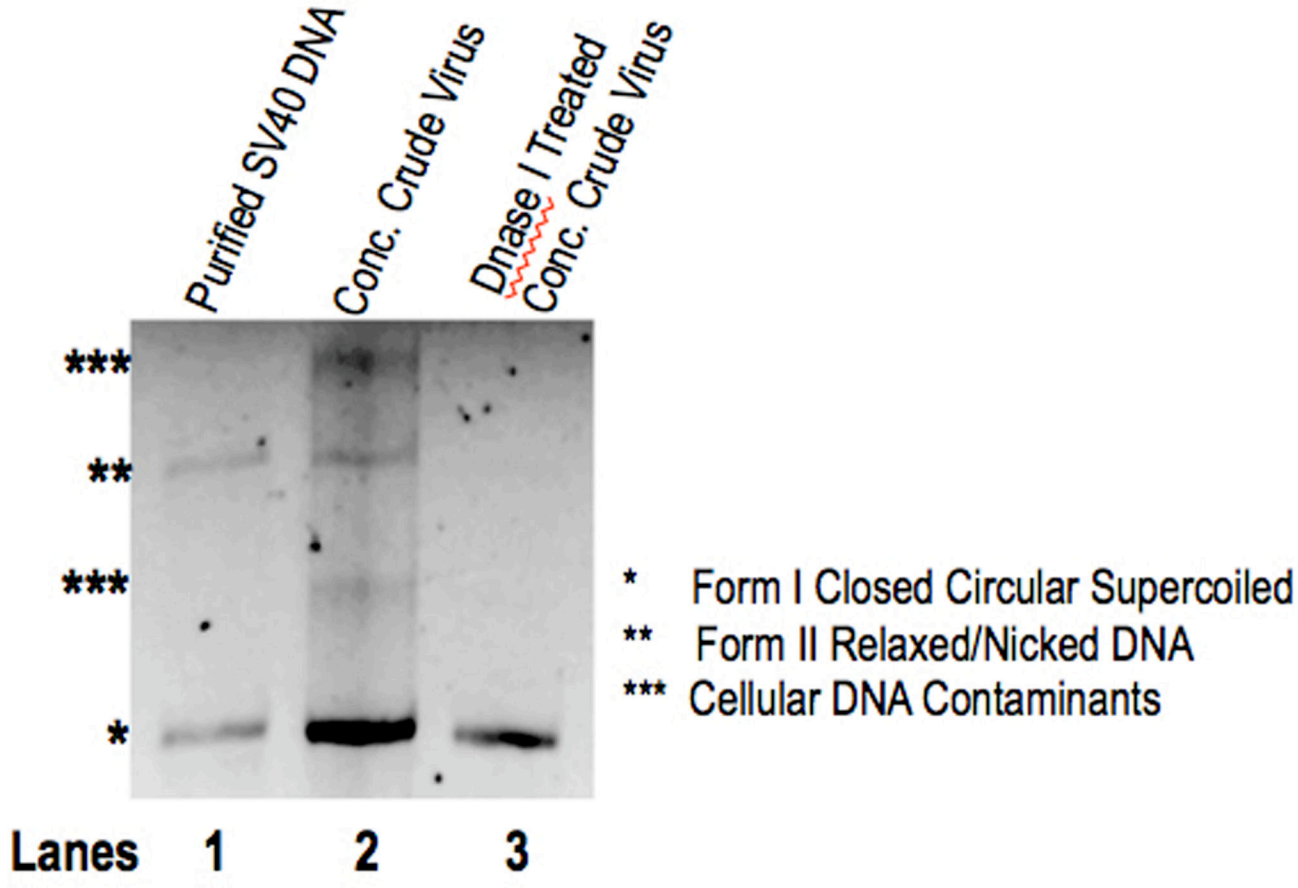

Figure 2. A comparison by agarose gel electrophoresis of crude SV40 virus before and after DNAse I digestion

Crude SV40 virus was concentrated by centrifugation, resuspended in buffer and digested with DNAse I. For comparison purposes purified SV40 DNA prepared by the Hirt procedure was also analyzed. Lane 1, Hirt SV40 DNA, Lane 2, Crude concentrated SV40 before digestion, and Lane 3, Concentrated SV40 after DNAse I digestion. The positions of form I (supercoiled SV40 DNA0 and form II (relaxed SV40 DNA) are indicated on the side of the figure.

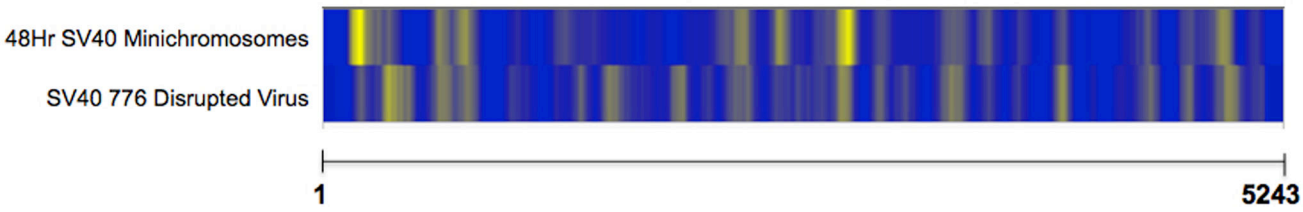

Figure 3. Next Generation Sequencing analyses of micrococcal nuclease digested SV40 chromatin obtained from minichromosomes and virions

Libraries were prepared from micrococcal nuclease digested SV40 minichromosomes obtained 48 hours post-infection (top) and from disrupted SV40 virions (bottom) using the protocols described in the text. The libraries were sequenced by paired-end sequencing using an Illumina MiSeq. Sequencing reads were plotted against the SV40 genome linearized between nucleotide number 5243 and 1. The sequencing reads from at least four biological replicates were then normalized and merged to generate the heatmap comparison shown. The intensity of the yellow color indicates the frequency in which a nucleosome was found located at a particular site in the SV40 genome with the brightest yellow corresponding to the highest frequency.

TABLE 1

ANTIBODY	COMPANY	CATALOG #	REFERENCE
Hyperacetylated H3	Millipore	06-599	(Balakrishnan and Milavetz 2005, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2009)
Hyperacetylated H4	Millipore	06-599	(Balakrishnan and Milavetz 2005, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2007, Balakrishnan and Milavetz 2008, Balakrishnan and Milavetz 2009, Milavetz, Kallestad et al. 2012)
H3K4me1	Millipore	07-436	(Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
H3K4me2	Active Motif	39141	(Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
H3K4me3	Millipore	04-745	(Balakrishnan and Milavetz 2009, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
H3K9me1	Abcam	ab9045	(Balakrishnan and Milavetz 2009, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
H3K9me2	Abcam	ab1220	(Balakrishnan and Milavetz 2009, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
H3K9me3	Abcam	ab8898	(Balakrishnan and Milavetz 2009, Milavetz, Kallestad et al. 2012, Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
H4K20me1	Active Motif	39175	(Balakrishnan, Gefroh et al. 2010, Milavetz, Kallestad et al. 2012)
H4K20me2	Millipore	07-031	(Balakrishnan, Gefroh et al. 2010)
H4K20me3	Millipore	07-463	(Balakrishnan, Gefroh et al. 2010)
RNA Polymerase II	Millipore	05-623	(Kallestad, Woods et al. 2013, Kallestad, Christensen et al. 2014)
RPA70	SantaCruz Biotech	sc-900	(Balakrishnan and Milavetz 2008, Balakrishnan and Milavetz 2009, Balakrishnan, Gefroh et al. 2010)
P300	SantaCruz Biotech	sc-584	(Balakrishnan and Milavetz 2008)