EpiQ™ Chromatin Analysis Kit

Instruction Manual

Catalog #172-5400
#172-5401

BIO-RAD

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EpiQ™ Chromatin Analysis Kit

Introduction

Epigenetic processes such as DNA methylation and histone modification control gene expression by altering chromatin structure. Actively transcribed genes are associated with open or accessible chromatin regions; genes that are transcriptionally silent are often in closed or inaccessible chromatin regions. The EpiQ chromatin analysis kit is an innovative research tool for assessing the chromatin structure of genomic regions in cultured cells using real-time PCR analysis.

Table 1. EpiQ chromatin analysis kit components.

	Quantity	Storage
Chromatin Digest and Analysis Components		
EpiQ nuclease	100 µl	–20°C
EpiQ chromatin buffer	6 ml	–20°C
EpiQ stop buffer	1.5 ml	–20°C
Reference gene primers (human/mouse)	1 ml	–20°C
Control gene primers (human)	1 ml	–20°C
Control gene primers (mouse)	1 ml	–20°C
Genomic DNA Purification Materials		
Mini columns	50	Room temperature
2 ml capless tubes	50	Room temperature
2 ml capped tubes	50	Room temperature
DNA lysis solution	50 ml	Room temperature
DNA low-stringency wash solution	20 ml	Room temperature
DNA high-stringency wash solution	40 ml	Room temperature
DNA elution solution	20 ml	Room temperature
EpiQ Chromatin SYBR® Supermix*		
EpiQ chromatin SYBR® supermix	5 ml	–20°C or 4°C for ≤6 months

* EpiQ chromatin SYBR® supermix is compatible with all real-time PCR instruments except the ABI7000, 7300, 7700, and 7900, which require a higher concentration of ROX passive reference dye. ROX passive reference dye (catalog #172-5858) can be ordered separately.

Necessary Supplies

The following supplies are required but are not included in the EpiQ chromatin analysis kit:

Materials

Cultured cells (48-well plate or in suspension)

100% ethanol

Nuclease-free water

Tris/EDTA (TE), pH 8.0

1.5 ml microcentrifuge tubes

Equipment

37°C incubator for cell culture

Temperature block or water bath set at 37°C

Temperature block or water bath set at 70°C

Instrument for DNA quantification

Real-time PCR instrument

Microcentrifuge

Timer

Using the EpiQ™ Chromatin Analysis Kit

qPCR

The EpiQ chromatin analysis kit requires analysis of samples by real-time PCR (qPCR). We strongly recommend using EpiQ chromatin SYBR® supermix for qPCR analysis. PCR primers that amplify the genomic DNA region of interest must be designed and verified; qPCR cycling protocols must be optimized. See the EpiQ Chromatin Assay Design and qPCR Optimization Guide (www.bio-rad.com/epiq) for details. Analysis of qPCR data can be performed using the EpiQ chromatin kit data analysis tool (www.bio-rad.com/epiq).

Cell Culture

The EpiQ chromatin analysis kit is designed for cells grown in 48-well plates. Cells grown in 24-well plates can also be used, but in that case, the amount of reagents used should be scaled proportionally (that is, doubled), and two mini columns should be used per sample. At harvest, the cells should be healthy and at 80–95% confluence. Cells grown in suspension culture may also be used (see Section E).

Experimental Design

Each experiment requires two treatment groups: an undigested group in which cells are treated with chromatin buffer only, and a digested group in which cells are treated with chromatin buffer containing nuclease. It is recommended that each group contain at least three biological replicates; thus six wells of cultured cells are the minimum recommended for each experiment.

If a comparison between RNA expression and chromatin structure is desired, a separate plate of cells should be used to harvest RNA. Similarly, if a comparison between DNA methylation and chromatin structure is desired, a separate plate of cells should be used to harvest genomic DNA.

Time Estimate

The preparatory work, in situ chromatin digestion, and the stopping of chromatin digestion can be done in ~90 min. Isolation and quantification of genomic DNA takes ~45 min. The preparation of samples for qPCR takes ~45 min; qPCR takes about 2 hr. Thus, the experiment can be done in ~5 hr. The procedure can be paused after DNA isolation or after DNA quantitation.

Assessment of Assay Conditions

The standard experimental procedure calls for in situ digestion of chromatin for 1 hr with 2 µl EpiQ nuclease. Due to inherent variability in cell lines, it is recommended that an initial assessment of chromatin digestion conditions be performed for each cell line using the reference (epigenetically silenced) gene and control (constitutively expressed) gene primers provided in the kit.

For adherent cells, grow six wells of each cell line to 80–95% confluence in a 48-well tissue culture plate; three wells will remain undigested, and the other three wells will be digested with nuclease. Follow the directions in the experimental protocol sections A, B, and C.

For cells in suspension, follow the experimental protocol described in Section E.

Analyze each sample by qPCR using the reference gene and control gene primers supplied with the kit as described in Section D. Analyze the data using the EpiQ chromatin kit data analysis tool found at www.bio-rad.com/epiq.

The experimental conditions are appropriate if all of the following are true:

1. The difference in quantification cycle (ΔC_q) between the digested and undigested reference gene is ≤1.5.
2. The ΔC_q between the undigested reference gene and the undigested control gene is ≤1.5.
3. The accessibility of the control gene is >95%.

If one of these conditions is not met, refer to the Troubleshooting section (Section H) to optimize assay conditions.

Experimental Procedure (Adherent Cells)

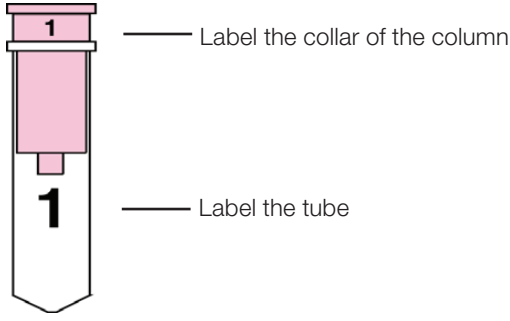
A. Preparatory Work

A-1. Add 80 ml of 100% ethanol to the DNA low-stringency wash solution concentrate to obtain a final volume of 100 ml. Make a check mark in the box on the bottle to indicate that ethanol has been added.

A-2. Label one 1.5 ml tube and one 2 ml capped tube for each sample.

A-3. Transfer 375 µl of DNA lysis solution (brown bottle) to each labeled 1.5 ml tube. Store at room temperature.

A-4. Place a mini column into a 2 ml capless tube. Label both the collar of the column and the tube. Prepare and label one column/tube for each sample.



A-5. Place the DNA elution solution bottle on a heat block set at 70°C until ready to use (step C-9 or F-10).

A-6. Thaw the tube of EpiQ stop buffer at room temperature, and place it on ice.

B. In Situ Chromatin Digestion

B-1. Thaw the EpiQ chromatin buffer at room temperature and vortex gently prior to use. (EpiQ chromatin buffer may appear cloudy.) Label one 1.5 ml tube U (undigested) and another 1.5 ml tube D (digested). Transfer an equal volume of chromatin buffer into both tubes. Each tube should contain 100 µl of chromatin buffer per sample plus an additional 10%, rounded up to the nearest 50 µl (Table 2). Nuclease is added to tube D at a later step. Return remaining chromatin buffer to –20°C for future use.

Table 2. Amount of chromatin buffer based on sample number.

# Samples (per Treatment)	Chromatin Buffer (Add to tubes U and D)
3	350 µl
6	700 µl
9	1,000 µl
12	1,350 µl

B-2. Incubate tubes U and D at 37°C for 10 min.

Note: Complete steps B-3 through B-7 as quickly as possible.

B-3. Add 2 µl of EpiQ nuclease for every 100 µl of chromatin buffer in tube D. See Table 3 for amount of nuclease to use based on sample number. Mix gently and store at room temperature. Do not add nuclease to tube U.

Table 3. Amount of nuclease based on sample number.

# Samples (per Treatment)	Nuclease (Add to tube D only)
3	7 µl
6	14 µl
9	20 µl
12	27 µl

B-4. Remove the cell culture plate from the 37°C incubator. Aspirate the culture media using a 200 µl pipet tip attached to a Pasteur pipet. Tilt the plate at a 60° angle so that all of the culture media can be removed.

B-5. Add 100 µl from tube U (undigested) to each undigested sample well.

B-6. Add 100 µl from tube D (digested) to each digestion sample well.

B-7. Swirl the plate so that the buffer covers the cells. Incubate the plate at 37°C for 1 hr. Incubation time is critical.

Note: The chromatin buffer can change the appearance of the cells and possibly cause them to detach from the plate. This does not affect the results.

B-8. After 1 hr, remove the plate from the 37°C incubator. Add 25 µl of EpiQ stop buffer to each well.

B-9. Swirl the plate, and place it back in the 37°C incubator for 10 min.

B-10. After 10 min, remove the plate from the 37°C incubator. Tilt the plate at a 60° angle to pool the cell lysate in the bottom portion of the well.

B-11. Using a 200 µl pipet, transfer all the cell solution from each well (125 µl) into the corresponding labeled 1.5 ml tube containing DNA lysis solution (prepared in step A-3). Scraping the well with a rubber policeman is not necessary.

B-12. Mix tube contents by inverting several times. Pulse-spin the tubes in a microcentrifuge for 5 sec.

B-13. Add 250 µl of 100% ethanol to each tube. Mix tube contents by inverting several times. Pulse-spin the tubes in a microcentrifuge for 5 sec.

Note: After step B-13, tubes can be stored at room temperature for up to 2 hr.

C. Isolation of Genomic DNA Using a Microcentrifuge

Note: See Section F for a procedure using a vacuum manifold.

C-1. Use a 1 ml pipet tip to transfer the entire contents of one cell lysis tube into the corresponding mini column prepared in step A-4.

C-2. Spin columns in a microcentrifuge at 12,000 rpm (13.4 rcf) for 1 min; discard flowthrough. Return the column to the same capless 2 ml tube.

C-3. Add 650 µl of DNA low-stringency wash solution to each column. Spin columns in a microcentrifuge at 12,000 rpm (13.4 rcf) for 1 min; discard flowthrough. Return the column to the same capless 2 ml tube.

C-4. Add 650 µl of DNA high-stringency wash solution to each column. Spin columns in a microcentrifuge at 12,000 rpm (13.4 rcf) for 1 min; discard flowthrough. Return the column to the same capless 2 ml tube.

C-5. Add 650 µl of DNA low-stringency wash solution to each column. Spin columns in a microcentrifuge at 12,000 rpm (13.4 rcf) for 1 min; discard flowthrough. Return the column to the same capless 2 ml tube.

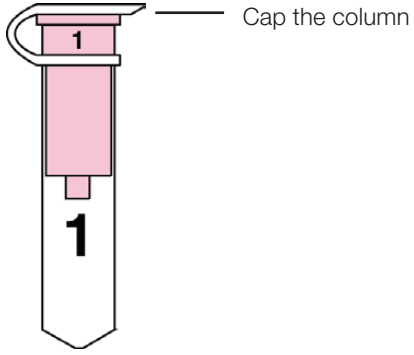
C-6. Repeat step C-5 one more time.

C-7. Spin columns in a microcentrifuge, 12k rpm (13.4 rcf) for 3 min to dry the columns.

C-8. Transfer each column into the corresponding labeled 2 ml capped tube (prepared in step A-2).

C-9. Add 52 µl of DNA elution solution (preheated to 70°C, step A-5) to the center of each column. Wait 2 min. Cap each column (see illustration). Spin columns in a microcentrifuge at 12,000 rpm (13.4 rcf) for 2 min. Retain flowthrough.

Note: In some microcentrifuge rotors, the 2 ml tubes containing the mini column do not fit in adjacent wells; if this is the case, load only every other microcentrifuge well.



C-10. Repeat step C-9 one more time for a total of two elutions (both elutions are collected in the same tube).

C-11. Quantify the amount of genomic DNA in each sample. Expected yield is 5–50 ng/µl.

C-12. Dilute a portion of each genomic DNA sample to 1 ng/µl in TE. Store samples at 4°C for up to 1 month or at –20°C for long-term storage.

D. Analysis of Samples by qPCR

Note: Prior to performing qPCR analysis on the genomic DNA samples isolated in Section C or F, design and verify the PCR primers and optimize the qPCR cycling protocol. Refer to the EpiQ Chromatin Assay Design and qPCR Optimization Guide for details (www.bio-rad.com/epiq).

We strongly recommend using the EpiQ chromatin SYBR® supermix for qPCR. This supermix is compatible with all real-time PCR instruments except the ABI7000, 7300, 7700, and 7900, which require a higher concentration of the ROX passive reference dye. To use EpiQ chromatin SYBR® supermix in these instruments, add 100 µl of ROX passive reference dye (catalog #172-5858) for every 1 ml of EpiQ chromatin SYBR® supermix before proceeding.

Inclusion of qPCR efficiency will improve the accuracy of EpiQ chromatin structure analysis results. Refer to Section G and the EpiQ Chromatin Assay Design and qPCR Optimization Guide (www.bio-rad.com/epiq) for details.

D-1. Place EpiQ chromatin SYBR® supermix at room temperature*.

D-2. Prepare a master mix for each genomic DNA sample and target primer combination with sufficient volume to run each reaction in triplicate (see Table 4). Mix by vortexing gently, and store at room temperature.

Table 4. qPCR master mix preparation for analysis in triplicate.

	Volume	
Reagent	**3.6 x 20 µl reaction (for 96-well plate)**	**3.6 x 10 µl reaction (for 384-well plate)**
Genomic DNA sample	18 µl	9 µl
Primer mix (2 µM each)	18 µl	9 µl
EpiQ chromatin SYBR® supermix	36 µl	18 µl

* qPCR plate setup can be performed at room temperature because the supermix contains a hot-start DNA polymerase.

D-3. Pipet 20 µl of master mix from each tube into three wells of a 96-well PCR plate (use 10 µl for 384-well plate).

D-4. Seal the plate with the appropriate sealing film or cap strips.

D-5. Centrifuge the plate briefly.

D-6. Analyze the plate by qPCR using the optimized cycling protocol. The following is the optimum cycling protocol for the reference gene and the control gene primers supplied with the EpiQ chromatin analysis kit:

qPCR protocol:

Step 1.	96°C for 5 min
Step 2.	96°C for 15 sec
Step 3.	60–67°C for 1 min, plate read
Step 4.	80°C for 30 sec
Step 5.	Go to step 2; repeat 39 times
Step 6.	Melt curve, 70–96°C
END	

D-8. Analyze the qPCR data using the EpiQ chromatin kit data analysis tool (www.bio-rad.com/epiq). This software package processes EpiQ qPCR data to determine the chromatin structure of targeted gene regions.

Appendix

E. Analysis of Cells in Suspension

Note: Cells in suspension should be healthy and actively growing. Between 100,000 and 500,000 cells are needed (250,000 is recommended) in each sample. For each cell type, analyze three undigested samples and three digested samples. Thus, for each cell line you will need between 600,000 and 3 million cells. The following example is based on 250,000 cells per sample.

E-1. Perform steps A-1 through A-6, omitting step A-3.

E-2. Use the Bio-Rad TC10™ automated cell counter (Bio-Rad catalog #145-0001), a hemocytometer, or other cell counting device to estimate the number of cells per ml of culture media.

E-3. Transfer 1.5 million cells into a 15 ml conical centrifuge tube. Pellet the cells by centrifugation at 1,000 rpm for 5 min.

E-4. Aspirate cell culture media; be careful not to disturb the cell pellet.

E-5. Resuspend the cell pellet in 1 ml of phosphate buffered saline. Transfer cells to a 1.5 ml microcentrifuge tube. Pellet cells by centrifugation at 6,000 rpm for 2 min.

E-6. Carefully remove most of supernatant by decanting. Centrifuge cells at 6,000 rpm for 15 sec. Remove remaining supernatant with a 200 µl pipet.

E-7. Resuspend cell pellet in 650 µl of EpiQ chromatin buffer. Pipet up and down to mix.

E-8. Transfer 100 µl of cell solution into each of the 1.5 ml microcentrifuge tubes labeled in step A-2.

E-9. Add 2 µl of EpiQ nuclease to the digested samples. Do not add nuclease to the undigested samples. Vortex gently to mix.

E-10. Incubate tubes in a 37°C temperature block for 1 hr.

E-11. After 1 hr, remove the tubes from the temperature block. Add 25 µl of EpiQ stop buffer to each tube. Vortex tubes gently to mix and return to 37°C temperature block for 10 min.

E-12. After 10 min, remove the tubes from the temperature block. Transfer 375 µl of DNA lysis solution (brown bottle) to each sample.

E-13. Mix tube contents by inverting several times. Pulse-spin the tubes in a microcentrifuge for 5 sec.

E-14 Add 250 µl of 100% ethanol to each tube. Mix tube contents by inverting several times. Pulse-spin the tubes in a microcentrifuge for 5 sec.

Note: Tubes can be stored at room temperature for up to 2 hr before proceeding to Section C or F.

F. Isolation of DNA Using a Vacuum Manifold

Note: This procedure describes DNA isolation using a vacuum manifold (Bio-Rad catalog #732-6470). Begin the following procedure after step E-14.

F-1. Set up the vacuum manifold apparatus as directed by the manufacturer.

F-2. Insert a mini column into the manifold fitting.

F-3. Pipet the cell lysate into the column. Apply vacuum until all of the lysate has passed through the column.

F-4. Add 650 µl of low-stringency wash solution to the column. Apply vacuum until all of the wash solution has passed through the column.

F-5. Add 650 µl of high-stringency wash solution to the column. Apply vacuum until all of the wash solution has passed through the column.

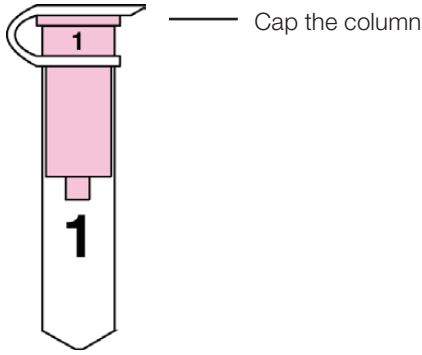
F-6. Add 650 µl of low-stringency wash solution to the column. Apply vacuum until all of the wash solution has passed through the column.

F-7. Repeat step F-6 one more time.

F-8. Transfer the column into a 2 ml capless tube. Centrifuge at 12,000 rpm (13.4 rcf) for 3 min to remove residual wash solution.

F-9. Transfer each column into the corresponding 2 ml capped tube (prepared in step A-2).

F-10. Add 52 µl of DNA elution solution (preheated to 70°C, step A-5) to the center of each column. Wait 2 min. Cap each column (see illustration). Spin columns in a microcentrifuge at 12,000 rpm (13.4 rcf) for 2 min. Retain flowthrough.



Note: In some microcentrifuge rotors, the 2 ml tubes containing the mini column do not fit in adjacent wells; if this is the case, load only every other microcentrifuge well.

F-11. Repeat step F-10 one more time for a total of two elutions (both elutions are collected in the same tube).

F-12. Quantify the amount of genomic DNA in each sample. Expected yield is 5–50 ng/µl.

F-13. Dilute a portion of each genomic DNA sample to 1 ng/µl in TE. Store samples at 4°C for up to 1 month or at –20°C for long-term storage.

G. Determination of Amplification Efficiency

Note: Newly designed primers should be verified for amplification efficiency using a standard curve generated from a serial dilution of genomic DNA. Genomic DNA isolated from an undigested EpiQ sample (Section C or F) or purchased from a commercial source can be used to prepare the genomic DNA dilution series. This section provides an example of how to perform standard curve analysis.

G-1. Label five 1.5 ml tubes and prepare a 5-fold genomic DNA dilution series in TE from 1 ng/µl genomic DNA stock as shown in Table 5. Tube 5 contains TE only to serve as a no-template control (NTC).

Table 5. Genomic DNA dilutions for efficiency analysis.

Tube	Concentration DNA	Volume DNA	Volume TE	DNA Dilution	Amount DNA in qPCR
1	1,000 pg/µl	50 µl	0	1,000 pg/µl	5,000 pg
2	1,000 pg/µl (= tube 1)	10 µl	40 µl	200 pg/µl	1,000 pg
3	200 pg/µl (= tube 2)	10 µl	40 µl	40 pg/µl	200 pg
4	40 pg/µl (=tube 3)	10 µl	40 µl	8 pg/µl	40 pg
5	0	0	50 µl	0	NTC

G-2. For a 96-well plate, use 5 µl of the genomic DNA dilution prepared in Step G-1 in a 20 µl qPCR reaction (see Table 5) to yield a 4-point standard curve with starting DNA quantities of 5,000 pg, 1,000 pg, 200 pg, and 40 pg. Refer to Section D for details on how to prepare the qPCR reactions for triplicate analysis.

G-3. Analyze the genomic DNA dilutions and NTC by qPCR analysis using the optimized cycling protocol established using the EpiQ Chromatin Assay Design and qPCR Optimization Guide (www.bio-rad.com/epiq).

G-4. Determine qPCR amplification efficiency from the slope of the plot of log (starting DNA quantity) (y-axis) versus the corresponding C_q value (x-axis) based on the equation:

Efficiency (%) = $(10^{(-1/slope)}-1) \times 100$

Most Bio-Rad real-time PCR software packages (except Opticon Monitor™ software) report both slope and qPCR efficiency values from standard curve analyses. Other real-time PCR analysis software packages only report the slope values. In such cases, calculate the qPCR efficiency from the slope using the EpiQ chromatin kit data analysis tool (www/bio-rad.com/epiq). Select primer sets that exhibit >80% qPCR efficiency.

H. Troubleshooting

H-1. **Excessive digestion of the reference (epigenetically silenced) gene.** If the difference in quantification cycle (ΔC_q) between the undigested and digested reference gene is >1.5, one or both of the following may have occurred:

- Excessive nuclease for your cell line may have been used
- The reference gene (RHO) is in a partially accessible chromatin configuration in your cell line

Do the following:

- Use less nuclease: Decrease the amount of nuclease to one half or one quarter of the original amount in a dose-response experiment
- Change reference genes: The hemoglobin gene (HBB) can also be used as a reference gene in most cell lines.

Primer sequences that amplify the HBB promoter in human cells:

hHBB-F: AAGCCAGTGCCAGAAGAGCCAAGGA
hHBB-R: CCCACAGGGCAGTAACGGCAGACTT

Primer sequences that amplify the HBB promoter in mouse cells:

mHBB-F: GAGTGGCACAGCATCCAGGGAGAAA
mHBB-R: CCACAGGCCAGAGACAGCAGCCTTC

H-2. **Excessive digestion of the control gene in the undigested samples.** Some cell lines exhibit a high level of endogenous nuclease activity, which results in digestion of the control gene promoter in the undigested samples. This digestion is likely a result of endogenous nucleases attacking the control gene because it is in accessible chromatin; the reference gene is not affected because it is inaccessible.

If the ΔC_q value between the control gene and the reference gene for the undigested samples is >1.5, decrease the duration of the in situ chromatin digestion in a time course experiment. We found that nuclease treatments as short as 10 min work well. Also, the amount of nuclease may need to be increased to compensate for the shorter digestion time.

H-3. **Low accessibility value for the control gene.** The accessibility value of the control gene should be >95%. If the control gene accessibility value is low, increase the amount of nuclease in a dose-response experiment.

H-4. **Difference in genomic DNA yield between undigested and digested samples.** In some instances, a difference in genomic DNA yield may be observed when comparing the undigested and digested samples. This does not affect chromatin accessibility results.

H-5. **Negative accessibility.** Rarely, analysis of a silenced gene promoter may result in a negative accessibility value. Such results imply that the DNA region studied is in a tightly closed chromatin conformation that is less accessible than the reference gene.

Ordering Information

Catalog #	Description
172-5400	EpiQ Chromatin Analysis Kit, 50 preparations
172-5401	EpiQ Chromatin Analysis Kit, 100 preparations
172-5402	EpiQ Chromatin Preparation Kit, 50 preparations
172-5403	EpiQ Chromatin Preparation Kit, 100 preparations
172-5404	EpiQ Chromatin SYBR® Supermix, 5 ml
172-5405	EpiQ Chromatin SYBR® Supermix, 10 ml

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