Instruction Manual Version 3 - 01.14

Shearing ChIP kit

Cat. No.: **C01020020** (kch-redmod-100)
C01020021 (kch-redmod-400)

diagenode

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Kit Materials

Kit content

The content of the kit is sufficient for the lysis and the shearing of 100 million cells (kch-redmod-100) or 400 million cells (kch-redmod-400).

Kit components

SHEARING MODULE (steps 1 to 3)				
Component	Description	Quantity (x100)	Quantity (x400)	Storage
Buffer A (Cell collection)	Salt and ion chelator mix included. Add formaldehyde before use.	10 ml	40 ml	4°C
1.25 M glycine	-	10 ml	40 ml	4°C
Buffer B (Lysis 1)	Detergent and ion chelator mix included.	30 ml	120 ml	4°C
Buffer C (Lysis 2)	Salt and ion chelator mix included.	30 ml	120 ml	4°C
Buffer D (Chromatin shearing)	Detergent and ion chelator mix included.	3 ml	12 ml	4°C

Protocol

STEP 1. Cell fixation and collection

Cells can be fixed before scraping (step 1-a.) or cells can be fixed after collection (step 1-b.). Both methods are described below: use the method that is the most suitable for your ChIP study.

Step 1-a. Scraping method:

1. Prepare cross-linking buffer by mixing Buffer A with 37% formaldehyde (w/v) and PBS as shown below (Table 1). The final concentration of formaldehyde is 1%.
Note: Handle formaldehyde in a chemical fume hood.

Table 1

Cross-linking buffer	3x 10e6 cells	10e7 cells	5x 10e7 cells
37% formaldehyde (w:v)	90 µl	270 µl	1350 µl
Buffer A	210 µl	630 µl	3150 µl
PBS	3 ml	9 ml	45 ml
TOTAL VOLUME	**3.3 ml**	**9.9 ml**	**49.5 ml**

Note: Alternatively, the mixture of [formaldehyde 11% final in buffer A] can be prepared and added - at 1:10 (v/v)- directly to the cell culture medium in the cell containing flask. (If cells are grown in suspension, it is the method of choice). Mix immediately and incubate on a shaking platform for 10 minutes at room temperature. Then proceed to step1-a. point 4.

2. Remove completely the culture media from the culture dishes or flasks.

3. Add the freshly prepared cross-linking buffer to the cells (Table 1). Add about 3 ml of buffer per 3x 10e6 cells. Mix immediately and incubate for 10 minutes at room temperature.
Note: The duration of cross-linking varies between cell type and protein of interest. It is possible to optimize the fixation step by testing different incubation time, such as 10, 20 and 30 minutes. Do not cross-link for longer than 30 minutes as cross-links of more than 30 minutes can not be efficiently sheared.

4. Add glycine to the cells to stop the cross-linking. Add about 300 µl of glycine per 3x 10e6 cells (Table 2). The provided stock solution of glycine is 1.25 M and is therefore diluted 10 times to reach a final concentration of 0.125 M. Mix and incubate for 5 minutes at room temperature (that is to quench the formaldehyde).

Table 2

To stop fixation	3x 10e6 cells	10e7 cells	5x 10e7 cells
1.25 M glycine	330 µl	990 µl	4950 µl

Note: Alternatively, if the mixture of [formaldehyde 11% final in buffer A] was added - at 1:10 - directly to the cell culture medium in the cell containing flask. (Method of choice for cells grown in suspension.). Then, add 1:10 (v/v) of 1.25 M glycine to the total volume of [(formaldehyde-buffer A-medium] mixture. Mix immediately and incubate for 5 minutes at room temperature. Then proceed to steps of centrifugation and washes.

5. Aspirate the media completely.
   Note: If cells are grown in suspension, collect the cells by centrifugation at this point.

6. Wash cross-linked cells twice with of ice-cold PBS. Use 5 ml of ice-cold PBS per wash per 3x 10e6 cells. For each wash, add the buffer to the cells and remove completely.
   Note: If cells are adherent, this washing step is done in the culture dish. If cells are grown in suspension, cells are collected by centrifugation after the washing step with PBS.

7. Add 500 µl of Buffer B per about 3x 10e6 cells (Table 3) and scrape cells from the culture dish or flask.

Table 3			
Cell scraping	3x 10e6 cells	10e7 cells	5x 10e7 cells
Buffer B	500 µl	1.5 ml	7.5 ml

   Note: If cells are grown in suspension, cells are collected by centrifugation at this point rather than by scraping.

8. Transfer to new conical tubes. Proceed to step 2-.

Step 1-b. Trypsinisation method

1. Pre-warm PBS, culture media and trypsin-EDTA.

2. Remove old media and rinse cells with pre-warmed PBS (Table 4). Shake dishes for 2 minutes. Remove the PBS.

Table 4			
Cell rinsing	3x 10e6 cells	10e7 cells	5x 10e7 cells
PBS	3.5 ml	10 ml	50 ml

3. Add sterile trypsin-EDTA to tissue culture flask or dish still containing adherent cells (Table 5). Brief treatment with trypsin-EDTA removes adherent cells from tissue culture flask bottom.
   Note: Each cell line will differ in the degree of adherence it has, and this will be described in the literature that accompanies it. If necessary, place flask back into incubator for about 1-2 minutes.

Table 5			
Cell detachment	3x 10e6 cells	10e7 cells	5x 10e7 cells
Trypsin-EDTA	1 ml	3 ml	15 ml

4. Check after a minute to see if cells have come off the flask bottom.
   Note: Prolonged treatment with trypsin may damage cells. This can be observed macroscopically as sheets of floating cells will be visible.

5. When cells are detached, add immediately culture medium to the cells (Table 6). The addition of medium will inactivate the trypsin.

Table 6			
Trypsin neutralisation	3x 10e6 cells	10e7 cells	5x 10e7cells
Culture medium	2 ml	6 ml	30 ml

6. Wash down sides of culture flask with a portion of the [cell/culture medium] mixture. Pipette the cells and transfer to a 15 ml or 50 ml centrifuge tube.

7. Count the cells.

8. Spin for 10 minutes at 500 g (1,600 rpm) at RT.

9. Resuspend the cells in culture medium. Use 1 ml of medium per 5 million cells (Table 7).

Table 7			
Cell resuspension	3x 10e6 cells	10e7 cells	5x 10e7 cells
Culture medium	600 µl	2 ml	10 ml

10. Then, prepare a mixture of Buffer A plus formaldehyde as shown below (Table 8). Add the freshly prepared cross-linking buffer to the [cells and medium] for fixation. Incubate under gentle rotation for 10 minutes at room temperature. This is the cross-linking step.

Notes: - Handle formaldehyde in a chemical fume hood.
- After the cross-linking, keep the samples ice-cold at all times.
- Prepare fresh buffers just before use.

Table 8			
Cross-linking buffer	3x 10e6 cells	10e7 cells	5x 10e7 cells
Buffer A	45 ul	140 µl	700 µl
formaldehyde	20 µl	60 µl	300 µl

11. Add glycine to the cells (using the stock solution of 1.25 M) corresponding to about 1:10 (v/v) of the total volume of [cells and medium] solution (Table 9). Mix evenly. That is to quench the formaldehyde.

Table 9			
To stop fixation	3x 10e6 cells	10e7 cells	5x 10e7 cells
1.25 M glycine	66 µl	220 µl	1100 µl

12. Centrifuge for 5 minutes at 500 g (1,600 rpm) at 4°C, to pellet cells. Get rid of the supernatant.

13. Wash the pelleted cells with ice-cold PBS. Add 15 ml of PBS per 5x 10e7 cells and resuspend the cells by pipetting up and down. Centrifuge immediately for 5 minutes at 500 g (1,600 rpm) at 4°C. Discard the supernatant.

14. Add 15 ml of ice-cold Buffer B per pellet of 5x 10e7 cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing. Proceed to step 2-.

STEP 2. Cell lysis

Notes: - Use a minimum of 3x 10e6 cells and a maximum of 5x 10e7 cells per sample.
- Keep the samples ice-cold at all times.

6. Centrifuge for 5 minutes at 500 g (1,600 rpm) at 4°C. Discard the supernatant, keep the pellet.

7. Add 15 ml of ice-cold Buffer C per pellet 5x 10e7 cells. Scale according if using less cells! Resuspend the pellet by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.

8. Pellet again by centrifugation for 5 minutes at 500 g (1,600 rpm) at 4°C, and discard supernatant.
Note: During the centrifugation, dissolve the protease inhibitor mix tablet in 400 µl of water. It is then a 25x stock solution. After use, store at -20°C.

9. Add Protease Inhibitor mix (P.I.) to ice-cold Buffer D. For each sample of three million cells, add 4 µl of P.I. per 100 µl of Buffer D.

Note: Prepare the volume of [P.I.-Buffer D] that is needed on the day (see Table 10).
P.I. (kch-207-001) is not provided in the kit.

10. Add 30 µl of freshly prepared [P.I.-buffer D] per each sample of one million cells (Table 10). Resuspend the pellet in the [P.I.-buffer D]. This is the chromatin containing sample ready to shear (step 3-.).

Table 10

Cell resuspension	3x 10e6 cells	10e7 cells	5x 10e7 cells
P.I.- BufferD	90 µl	300 µl	1500 µl

Note: The number of cells to be used per IP has to be determined in function of the abundancy of the target and the quality of your antibody. Please scale accordingly.
Use freshly prepared buffer and discard what is not used on the day.

STEP 3. Chromatin shearing

Note: This protocol is very successful for a large variety of mammalian cells. However you might want to optimize shearing conditions for your specific cell type and fixation protocol. Therefore, to start with a small sample (3x 10e6 cells) and check the shearing efficiency is advised. Once conditions have been optimized for your cells following - and the analysis step, prepare sheared chromatin for ChIP using one of Diagenode's high quality ChIP kits.

7. Transfer the chromatin containing sample in appropriate tubes:
- do not add more than 300 µl per 1.5 ml tubes
- do not add more than 2 ml per 15 ml tubes.

Note: Keep some unsheared chromatin for future controls (analysis step).

8. Shear the samples of chromatin using the Bioruptor® from Diagenode (catalog # UCD-200, UCD-300, B01060001). Maintain temperature of the samples close to 4°C. Samples are sonicated for 1 to 3 runs of 5 to 10 cycles [30 seconds "ON", 30 seconds "OFF"] at high power setting. Briefly vortex and spin between each run.

Notes:
- The above shearing conditions were tested with many mammalian cell lines and were excellent for subsequent ChIP experiments.
- Optimal shearing conditions are important for ChIP efficiency. The conditions of shearing are to be optimized for each cell type, fixation protocol and sonicator apparatus. A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.

9. After shearing, if 15 ml tubes were used, transfer the content to new 2 ml tubes.

10. Centrifuge for 5 minutes at 14,000 g (13,000 rpm) 4°C to remove debris. Keep the supernatant. That is the sheared chromatin sample.

Note: At this point, the sheared chromatin can be used directly or stored for subsequent ChIP experiments and for analysis of shearing efficiency. The chromatin can be stored in liquid nitrogen for several months or weeks depending on your ChIP target. Do not freeze/thaw.

Bringing it all together: Diagenode's ChIP-seq workflow

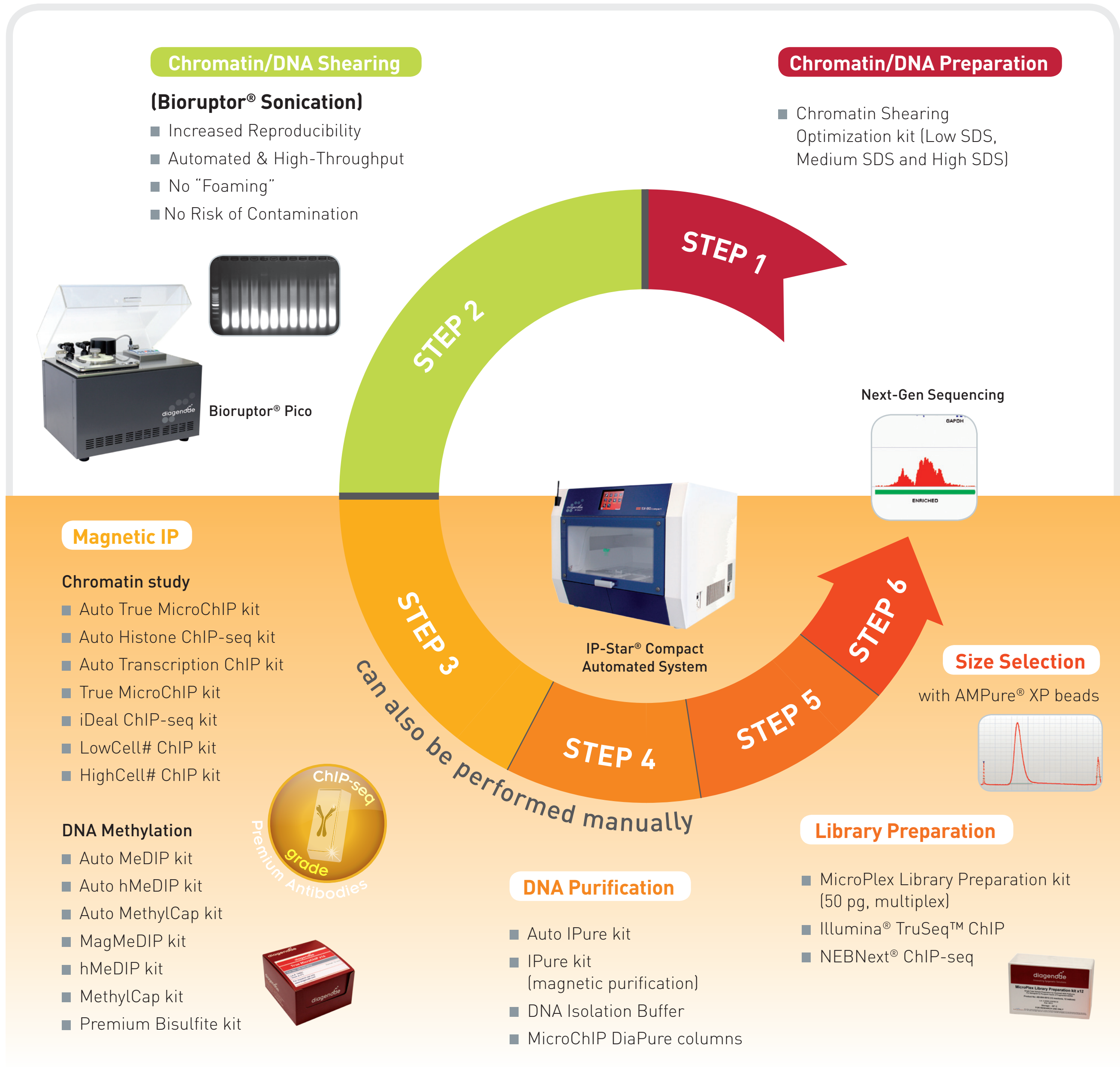

Figure 1. Diagenode provides a full suite of manual and automated solutions for ChIP experiments.

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

Technical Assistance & Ordering Information

At DIAGENODE we pride ourselves on the quality and availability of our technical support. Our Technical Services Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of DIAGENODE products. If you have any questions, or experience any difficulties regarding the SX-8G IP-Star or DIAGENODE products in general, do not hesitate to contact us.
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