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ab270813
ChIP Kit (Transcription factors, ChIP-seq)

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ChIP Kit (Transcription factors, ChIP-seq) datasheet:

www.abcam.com/ab270813

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This product is for research use only and is not intended for diagnostic use.

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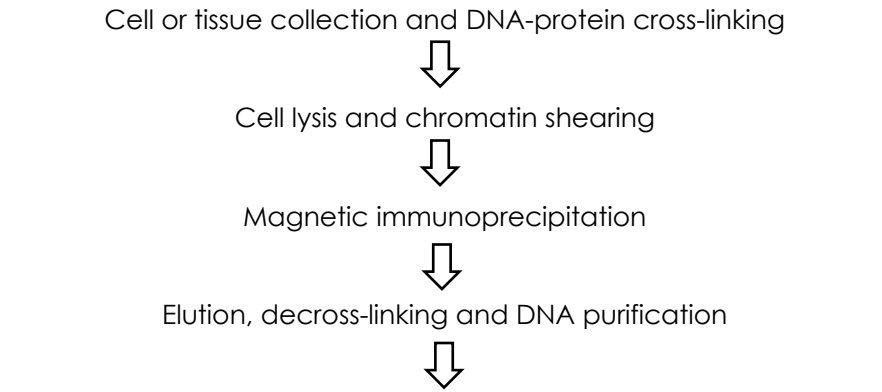
1. Overview

ChIP Kit (Transcription factors, ChIP-seq) (ab270813) is a highly validated solution for robust transcription factor and other non-histone proteins ChIP-seq results, and contains everything you need for start-to-finish ChIP prior to Next-Generation Sequencing. This complete solution contains all buffers and reagents for cell lysis, chromatin shearing, immunoprecipitation, and DNA purification. This kit contains positive and negative control antibodies (CTCF and IgG, respectively) as well as positive and negative control PCR primers pairs (H19 and Myoglobin exon 2, respectively).

Sample size:

Cells (4,000,000 cells per IP)

Tissues (30 mg of tissue per IP)



2. Materials Supplied and Storage

Component	Quantity 24 tests	Quantity 10 tests	Storage
5% BSA (DNA free)	800 µl	380 µl	-20°C
5x ChIP Buffer C1b	6.9 ml	3.4 ml	4°C
Buffer C	1.6 ml	700 µl	4°C
Carrier	72 µl	32 µl	-20°C
ChIP-seq grade CTCF antibody	8 µg	4 µg	-20°C
ChIP-seq grade H19 imprinting control region primer pair (human)	96 µl	42 µl	-20°C
ChIP-seq grade Myoglobin exon 2 primer pair (human)	96 µl	42 µl	-20°C
ChIP-seq grade water	26.6 ml	14 ml	4°C
Protein A-coated magnetic beads	720 µl	300 µl	4°C
Elution Buffer E1	3.4 ml	1.5 ml	4°C
Elution Buffer E2	144 µl	64 µl	4°C
Fixation buffer	8 ml	4 ml	4°C
Glycine	8.8 ml	4.4 ml	4°C
Purification Beads	400 µl	180 µl	4°C (do not freeze)
Lysis Buffer L1b	100 ml	50 ml	4°C
Lysis Buffer L2	60 ml	30 ml	4°C
Protease inhibitor cocktail	80 µl	38 µl	-20°C
Rabbit IgG	8 µg	4 µg	-20°C
Shearing Buffer S1b	6.7 ml	3.4 ml	4°C

Wash Buffer 1 w/o iso-propanol	2 ml	900 µl	4°C
Wash Buffer 2 w/o iso-propanol	2 ml	900 µl	4°C
Wash Buffer W1	8.4 ml	3.5 ml	4°C
Wash Buffer W2	8.4 ml	3.5 ml	4°C
Wash Buffer W3	8.4 ml	3.5 ml	4°C
Wash Buffer W4	8.4 ml	3.5 ml	4°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- RNase/DNase-free 1.5 ml and 15 ml tubes
- Formaldehyde, 37%, molecular grade
- Phosphate buffered saline (PBS) buffer
- 100% isopropanol
- qPCR SYBR® Green Mastermix
- ChIP/ ChIP-seq grade antibodies
- Fluorescence-based assay for DNA concentration measurement,
- Cell culture scraper (for adherent cells)
- Protease inhibitor cocktail (100 µl per chromatin preparation)
- Dounce homogenizer with loose and tight pestles (2 ml)
- Scalpel blades
- Magnetic rack
- Sonicator
- Microtubes
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- rotator (Rotating wheel)
- Vortex
- Thermomixer

- Fluorometer
- qPCR cycler
- Petri dishes
- Gloves

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Protocol

- Before proceeding please read the Pre-Chip Assay Preparation Guidlines and Protocol for Chromatin Shearing Analysis documents on the product datasheet

- Protocol for the first step differs for cells (step 5.1) and tissues (step 5.2).

- The protocol below describes the chromatin preparation (formaldehyde cross-linking, cell collection, lysis and chromatin shearing) from a batch of approximately 25 million cells or 200 mg of tissue. This will be sufficient for 6 ChIP reactions (using approximately 4 million cells or 30 mg of tissue per IP), 1 input and 1 sample for chromatin shearing assessment.
- Please note that the described protocol uses a prompt direct fixation in a cell culture plate. Fixed and scraped cells cannot be accurately counted. This means that for adherent cells you need to use an approximate estimation of cells number per plate. Alternatively, an additional parallel plate for counting can be prepared. Suspension cells can be counted before the fixation.

5.1 Cell collection and DNA-protein cross-linking.

From cultured cells:

5.1.1 Equilibrate the Fixation Buffer to room temperature before use.

5.1.2 Prepare the cross-linking solution in a fume hood by adding formaldehyde to the Fixation Buffer to a final concentration of 11% (e.g. add 0.596 ml of 37% formaldehyde to 1.407 ml of Fixation Buffer). Add the diluted formaldehyde directly to the cell culture medium in a proportion of 1:10. For a 20 ml cell culture you will need 2 ml of cross-linking solution.

5.1.3 Incubate the cells for 15 mins at room temperature with gentle shaking.
Δ Note: The fixation time might require an additional optimization.

5.1.4 Add Glycine to the cell culture medium in a proportion of 1:10 to stop the fixation. Incubate for 5 mins at room

temperature with gentle shaking. Proceed to the next step immediately.

Δ Note: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the fixed cells can be stored at -80°C for up to 4 months. To freeze the cells, remove the medium, wash the cells once with 20 ml of PBS. Add another 5 ml of PBS, collect the cells by scrapping and/or centrifugation at 500 *x g* for 5 mins and 4°C and discard the supernatant. Store the cell pellet at -80°C.

From adherent cells:

5.1.5 Remove the medium and wash the cells once with 20 ml of PBS. Discard the PBS. Keep everything at 4°C or on ice from now on.

5.1.6 Add 5 ml of cold Lysis buffer L1b to the plate. Collect the cells by scraping and transfer them into a 50 ml tube.

5.1.7 Rinse the flask with 20 ml of Lysis buffer L1b and add this to the 50 ml tube. The total volume of Lysis buffer L1b should be 25 ml per 25 million cells (for up or down scaling use 1 ml of buffer L1b per million of cells). Proceed immediately with step 5.1.11.

From suspension cells:

5.1.8 Transfer cells into a 50 ml tube. Pellet the cells by centrifugation at 500 *x g* and 4°C for 5 mins. Discard the cell culture medium.

5.1.9 Resuspend the cells in 20 ml of ice-cold PBS, centrifuge at 500 *x g* and 4°C for 5 mins and gently discard the supernatant. Keep everything at 4°C or on ice from now on.

5.1.10 Resuspend the cells in 1 ml of ice-cold lysis buffer L1b by pipetting up and down several times. Add 24 ml of buffer L1b to obtain a total volume of 25 ml per 25 million cells (for up or down scaling use 1 ml of L1b per 1 million cells). Proceed immediately with step 5.1.11.

5.1.11 Incubate at 4°C for 20 mins with gentle mixing on a rotator. Pellet the cells by centrifugation at 500 *x g* and 4°C for 5 mins and discard the supernatant. Resuspend the cell pellet in 1 ml of ice-cold Lysis buffer L2 by pipetting up and down several times. Add 14 ml of buffer L2 and incubate for 10 mins at 4°C with gentle mixing on a rotator (for up or down scaling, use 600 µl of buffer L2 per 1 million of cells).

5.1.12 Pellet the cells again by centrifugation for 5 mins at 500 *x g* and 4°C and discard supernatant.

5.1.13 Add 8.4 µl of 200x protease inhibitor cocktail to 1.67 ml of Shearing Buffer S1b. This is a complete Shearing buffer needed for 25 million cells. Keep on ice.

5.1.14 Add the complete Shearing buffer S1b to the cell pellet. The cell concentration in the shearing buffer should be 1.5 million of cells per 100 µl of S1b. Resuspend the cells by pipetting up and down several times and incubate on ice for 10 mins. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate sonication microtubes.
Δ Note: The use of correct type of microtubes is essential for the efficiency of sonication.

5.1.15 Shear the chromatin by sonication. Choose the protocol which is adapted to your device:
Δ Note: We recommend performing pilot experiments for each new sample type.

5.1.16 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 *x g* at 4°C for 10 mins. Pool the supernatants which contain the sheared chromatin.

5.1.17 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol is described in a separate section "Protocol for chromatin shearing analysis".
Δ Note: We recommend analyzing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously. The analysis of chromatin shearing can be done in parallel with the following steps of the protocol or can be included in the main workflow. Store the chromatin aliquot at -20°C until analysis.

5.1.18 Use the chromatin for immunoprecipitation or store it at -80°C for up to 2 months.

5.2 Tissue disaggregation and DNA-protein cross-linking.

From tissues:

5.2.1 Equilibrate the Fixation Buffer to room temperature before use.

5.2.2 Prepare the cross-linking solution in a fume hood by adding 54 µl of 37% formaldehyde to 2 ml of Fixation buffer to a final concentration of 1%. Use 2 ml of Fixation buffer for one chromatin preparation.

5.2.3 Put 200 mg of fresh or frozen tissue in a petri dish on ice. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

5.2.4 Chop the tissue into small pieces (between 1-3 mm3) using a scalpel blade and transfer it into a Dounce homogenizer. Add 1 ml of formaldehyde diluted in Fixation buffer.

5.2.5 Immediately disaggregate the tissue using a Dounce homogenizer (loose fitting pestle) to get a homogeneous suspension. Set the timer for a total fixation time of 15 mins and start deducting the fixation time from this point.

5.2.6 Transfer the tissue suspension into a 15 ml tube. Rinse the Dounce homogenizer with an additional 1 ml of diluted formaldehyde and pool with the sample in the same 15 ml tube.

5.2.7 Incubate for a total time of 15 mins (starting at step 5.2.5) at room temperature with gentle rotation on a rotator.
Δ Note: The fixation time might require an additional optimization.

5.2.8 Add 200 µl of Glycine to the tissue suspension to stop the fixation. Incubate for 5 mins at room temperature with gentle mixing on a rotator. Proceed to the next step immediately.
Δ Note: We strongly recommend using freshly fixed tissue for preparation of sheared chromatin prior to ChIP. If not possible, the fixed tissue can be stored at -80°C for up to 4 months. To freeze the tissue, remove the medium and wash the tissue once with 2 ml of PBS.
Store the tissue pellet at -80°C.

5.3 Cell lysis and chromatin shearing.

From tissues:

Δ Note: An additional 100 µl of protease inhibitors cocktail is required per chromatin preparation.

5.3.1 Centrifuge samples at 850 *x g* for 5 mins at 4°C. Gently discard the supernatant and keep the pellet.

5.3.2 Wash the pellet with 10 ml of ice-cold PBS and centrifuge samples at 850 *x g* for 5 mins at 4°C. Gently discard the supernatant. Keep everything at 4°C or on ice from now on.

5.3.3 Add 50 µl of 200x protease inhibitor cocktail to 10 ml of ice-cold Lysis buffer L1b. This is a complete lysis buffer L1b needed for 200 mg of tissue.

5.3.4 Add 1 ml of ice-cold complete Lysis buffer L1b to the pellet and resuspend by pipetting up and down several times. Add the remaining amount of complete buffer L1b.

5.3.5 Incubate at 4°C for 20 mins with gentle mixing on a rotator.

5.3.6 Pellet the cells by centrifugation at 850 *x g* for 5 mins at 4°C and discard the supernatant.

5.3.7 Add 50 µl of 200x protease inhibitor cocktail to 10 ml of ice-cold Lysis buffer L2. This is a complete Lysis buffer L2 needed for 200 mg of tissue. Add 1 ml of ice-cold complete Lysis buffer L2 to the cell pellet and resuspend the cells by pipetting up and down several times. Add the remaining amount of complete buffer L2.

5.3.8 Incubate at 4°C with gentle mixing on a rotator for 10 mins.

5.3.9 Pellet the cells again by centrifugation at 850 *x g* for 5 mins at 4°C and discard supernatant.

5.3.10 Add 8.4 µl of 200x protease inhibitor cocktail to 1.67 ml of Shearing buffer S1b. This is a complete Shearing buffer needed for 200 mg cells. Keep on ice.

5.3.11 Add the complete Shearing buffer S1b to the pellet. Resuspend the cells by pipetting up and down several times and homogenize using a dounce homogenizer (tight pestle). Incubate for 10 mins on ice.

5.3.12 Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate 1.5 ml microtubes.
Δ Note: The use of correct type of microtubes is essential for the efficiency of sonication.
Shear the chromatin by sonication. Choose the protocol which is adapted to your device. Δ Note: We recommend performing pilot experiments for each new sample type.

5.3.13 Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 *x g* at 4°C for 10 mins. Pool the supernatants which contain the sheared chromatin.

5.3.14 Take an aliquot of 50 µl of sheared chromatin for the shearing assessment.

Δ Note: We recommend analyzing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously. The analysis of chromatin shearing can be done in parallel with the following steps of the protocol or can be included in the main workflow. Store the chromatin aliquot at -20°C until analysis.

5.3.15 Use the chromatin in immunoprecipitation or store it at -80°C for up to 2 months.

5.4 Magnetic immunoprecipitation for cells and tissues.

5.4.1 Determine the amount of IP reactions to be run including the positive and negative control IPs. Take the required amount of Protein A-coated magnetic beads and transfer it to a clean 1.5 ml tube. 30 µl of beads are needed per IP.

5.4.2 Prepare 4 ml of 1x ChIP buffer C1b by mixing the following reagents:

- 3.2 ml ChIP-seq grade water
- 0.8 ml 5x ChIP buffer C1b
- 80 µl of 5% BSA

Keep the diluted ChIP buffer C1b on ice.

5.4.3 Wash the beads 3 times with 1 ml of ice-cold 1x ChIP buffer C1b. To wash the beads, add 1 ml of 1x ChIP buffer C1b directly to the beads suspension, resuspend the beads by pipetting up and down several times and incubate at 4°C with gentle shaking for 5 mins. Spin the tubes and place them in the 1.5 ml magnetic rack. Wait for one minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 2 times.

5.4.4 After the last wash, resuspend the beads in 1x ChIP buffer C1b adding the original volume of beads (this means 30 µl per IP).

5.4.5 Take the required number of 1.5 ml tubes (one tube per IP) and add 30 µl of the washed beads to each tube.

5.4.6 Prepare the ChIP reaction mix as described below for 1 IP. Scale according to the number of IPs including a small excess (0.5 extra reaction).

Δ **Note:** Proceed separately with reaction mixes to be used with different antibodies

- 6 µl of BSA
- 1.8 µl 200x protease inhibitor cocktail
- 20 µl 5x C1b buffer
- (42.2 µl –x µl) ChIP-seq grade water
- Add x µl of ChIP-seq grade antibody

x amount of a ChIP-seq grade antibody.

The total volume of the ChIP reaction mix per IP is 70 µl

Δ **Note:** The required amount of antibody per IP varies. Check the supplier's recommendation or perform a titration curve using different amounts of antibody. Use 1 µg of IgG (negative control antibody) for the negative control IP. If a positive control IP is included in the experiment, use 1 µg of

the CTCF positive control antibody. If required, NaBu (20 mM final concentration) or other inhibitors can be added.

5.4.7 Add 70 µl of ChIP reaction mix to the individual tubes containing 30 µl of washed Protein A-coated magnetic beads. Incubate the tubes for 2-4 hours at 4°C under constant rotation on the rotator.

5.4.8 Briefly spin the tubes containing the ChIP reaction mix and add 250 µl of sheared chromatin. Keep aside 2.5 µl of the sheared chromatin at 4°C to be used as an INPUT. Incubate the tubes overnight at 4°C under constant rotation on the rotator.

5.4.9 Perform the washes as follows: briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Add 350 µl of Wash buffer W1: gently shake the tubes to resuspend the beads and incubate for 5 mins on the rotator at 4°C.

5.4.10 Repeat the washing step as described above once with Wash buffer W2, W3 and W4, respectively.

5.5 Elution, decross-linking and DNA purification for cells and tissues.

5.5.1 Before the first use of the kit, prepare Wash buffer 1 and Wash buffer 2 by adding an equal volume of isopropanol. Wash buffers 1 and 2 should be stored at 4°C. Never leave the bottle open during storage to avoid evaporation.

5.5.2 After removing the last wash buffer, add 100 µl of Elution buffer E1 to the beads, resuspend the beads pellet and incubate for 30 mins on the rotator at room temperature.
Δ Note: If a precipitation is observed in Elution buffer E1, warm it at 37°C until it becomes clear. This will not impair the reaction.

5.5.3 Briefly spin the tubes and place them into the magnetic rack. Wait for one minute, transfer the supernatant to a new 1.5 ml tube and add 4 µl of Elution buffer E2. At the same time add 97.5 µl buffer E1 and 4 µl of buffer E2 to the 2.5 µl INPUT sample. Incubate for 4 hours or overnight at 65°C with shaking.
Δ Note: if desired, include a chromatin sample for shearing assessment at this step. Perform decrosslinking and DNA purification in parallel with the IP and input samples.

5.5.4 Add 2 µl of carrier to each IP and INPUT sample.

5.5.5 Add 100 µl of 100% isopropanol to each IP and INPUT sample.

Δ Note: Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

5.5.6 Resuspend the Purification Beads by vortexing and transfer 10 µl to each IP and INPUT sample.

5.5.7 Incubate the IP and INPUT samples for 10 mins at room temperature on the rotator.

5.5.8 Briefly spin the tubes, place in the magnetic rack, wait 1 minute and discard the buffer.

5.5.9 Add 100 µl of Wash buffer 1 (completed with isopropanol) per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes, place in the magnetic rack, wait 1 minute and discard the buffer.

5.5.10 Add 100 µl of Wash buffer 2 (completed with isopropanol) per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes and place them into the magnetic rack, wait 1 minute and discard the buffer.

5.5.11 Spin the tubes again and place them on the magnetic rack. Discard the remaining Wash buffer 2 if necessary. Resuspend the beads pellet in 25 µl of Buffer C. Incubate at room temperature for 15 mins on the rotator.
Δ Note: Buffer C is compatible with down-stream applications such as qPCR analysis and library preparation for Next-Generation sequencing.

5.5.12 Spin the tubes and place them into the magnetic rack, wait 1 minute and transfer the supernatant containing the immunoprecipitated DNA into a new labeled 1.5 ml tubes. Discard the beads.

5.5.13 Place the DNA on ice and take 2 µl of IP'd DNA to determine the concentration.

5.5.14 Determine the total number of regions to be analyzed by qPCR for each sample. Take the required volume of INPUT and immunoprecipitated samples for qPCR analysis. Take into account that these samples will be diluted 1/10 and 5 µl will be used per PCR reaction.
Δ Note: The dilution of samples and the volume per PCR may vary depending on a sensitivity of a Master Mix and qPCR cycler used.

5.5.15 Store the remaining DNA at -20°C until further use.

5.6 Quantitative PCR analysis for cells and tissues.

5.6.1 Take an aliquot of immunoprecipitated DNA and a corresponding INPUT and dilute them 1/10 using ChIP-seq grade water.

5.6.2 Prepare the qPCR mix as follows (20 µl reaction volume using the provided control primer pairs):

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer pair
- 4 µl of water
- 5 µl of diluted immunoprecipitated or INPUT DNA

Use the following PCR program:

Step	Time/cycles		Temperature
Denaturation	3-10 mins*		95°C
Amplification	30 secs	40 cycles	95°C
	30 secs		60°C
	30 secs		72°C (acquire fluorescent data)
Melting Curve**	Follow qPCR instrument manufacturer recommendations		

*Carefully check supplier's recommendations about Taq polymerase activation time

**Include and inspect the melting curves based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single specific product

Δ Note: These conditions may require optimization depending on the type of master mix, qPCR system used and user provided primer pair.

5.6.3 Record the threshold cycles (Ct values) from the exponential phase of the qPCR for the immunoprecipitated DNA sample and input for each primer pair.

5.6.4 Calculate the relative amount of immunoprecipitated DNA compared to INPUT DNA for the control regions (% of recovery) using the following formula:

$$\% \text{ recovery} = 2^{((Ct_{input} - 6.64) - Ct_{sample})} \times 100\%$$

- Ct_{sample} and C_{tinput} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and INPUT, respectively.
- 2 is the amplification efficiency
- 6.64 is a compensatory factor to correct the input dilution

Δ Note: This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results, the amplification efficiency with given primer pair has to be close to 100% meaning that for each cycle the amount of product is doubles (E=2). The real amplification efficiency, if known, should be used.

The formula takes into account that 1% of INPUT was used as suggested in the protocol (2.5 µl INPUT vs 250 µl of chromatin per IP). If the amount of INPUT used is different from 1%, an introduction of a compensatory factor in the formula is required to correct the input dilution (x) as follows:

$$\%\text{recovery} = 2^{[(Ct_{input} - \log2(X\%)) - Ct_{sample}]} \times 100\%$$

Where: log2(x) accounts for the INPUT dilution

Example: If you use an INPUT of 5 µl from 250 µl of chromatin used per IP, it corresponds to 50 X dilution. The compensatory factor is equal to log2(50)=5.64 and the formula to calculate the recovery will be as follows:

$$\%\text{recovery} = 2^{[(Ct_{input} - 5.64) - Ct_{sample}]} \times 100\%$$

5.6.5 If an expected enrichment over positive loci with a low background is observed, proceed to the library preparation using an appropriate protocol (not supplied with this kit).

6. Notes

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