Sample preparation for Chromatin Immunoprecipitation sequencing (ChIP-seq)

ChIP-seq is a multi-step process that is crucial for the success and quality of the final sequencing data. The goal is to isolate and purify DNA fragments that were specifically bound to your target protein *in vivo*.
The main steps involve cross-linking, chromatin preparation (lysis and shearing), immunoprecipitation, reversal of cross-links, and DNA purification.
This documentation provides a general workflow for the ChIP-seq sample preparation. Empirical testing and optimization at some steps may be required.

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1. Starting Material and Cross-linking

This initial step preserves the protein-DNA interactions that exist in the cell.

Cell/Tissue Preparation

- **Cells:** Start with a sufficient number of cells (e.g., 10^7 cells for common targets; more for low-abundance targets like some transcription factors). Adherent cells must be gently washed and trypsinized/scraped; suspension cells are pelleted.
- **Tissue:** Fresh or flash-frozen tissue (5-30 mg) needs to be finely minced or homogenized in ice-cold PBS with **protease inhibitors**.

Cross-linking

- **Reagent:** Typically, **formaldehyde** is used, often at a final concentration of **1%**.
- **Incubation:** Incubate the cells/tissue with formaldehyde for a short duration, usually **5 to 15 minutes** at room temperature, with gentle mixing.
    - **Optimization is critical:** Insufficient cross-linking leads to loss of complexes, while over-cross-linking can mask the protein's epitope, reducing antibody binding and making sonication difficult.
- **Quenching:** Stop the cross-linking reaction by adding a quenching agent, usually **glycine** (e.g., to a final concentration of 125 mM), and incubating for about 5 minutes.
- **Washing:** Wash the quenched cells/tissue pellet multiple times with cold PBS containing protease inhibitors to remove excess cross-linker.
- ***Note:** the extent of Crosslinking may need to optimized empirically due to sample variation.*

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2. Chromatin Preparation and Shearing

The goal is to lyse the cells/nuclei and then fragment the cross-linked chromatin into an optimal size.

Cell/Nuclear Lysis

- Resuspend the washed pellet in a **cell lysis buffer** (containing detergents and protease inhibitors) to break the cell membrane and release nuclei.
- Then, lyse the nuclei with a **nuclear lysis buffer** (often more stringent, containing stronger detergents like SDS) to release the chromatin. Incubate on ice.

Chromatin Shearing (Fragmentation)

- The chromatin must be fragmented to a size range that is appropriate for sequencing and maximizes the resolution of binding sites.
- **Target Size:** The ideal DNA fragment size for ChIP-seq is typically **200 to 500 bp** (including the protein and cross-linked DNA).
- **Method: Sonication** (using a probe sonicator or, preferably, an adaptive focused acoustic device like a Covaris sonicator) is the standard method for random fragmentation.
- **Optimization:** Sonication conditions (time, power, duty cycle) must be **empirically optimized** for each cell or tissue type and instrument to achieve the correct fragment size.
- **Validation:** After sonication, reverse the cross-links on a small aliquot, purify the DNA, and run it on a **1-2% agarose gel** or use a **Fragment Analyzer/TapeStation** to confirm the fragment size distribution.
- *Optimization may be required.*

Clarification

- Centrifuge the sonicated lysate at high speed to pellet debris and collect the **soluble chromatin supernatant**. This is the input material for immunoprecipitation.
- **Input Control:** Set aside a small aliquot (e.g., 1-10%) of the soluble chromatin before immunoprecipitation. This is the **Input DNA** sample, which is critical for sequencing data normalization and background correction.

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3. Immunoprecipitation (ChIP)

This step uses a specific antibody to pull down the target protein-DNA complexes.

Antibody Binding

- Dilute the soluble chromatin and incubate it with a high-quality, **validated, ChIP-grade antibody** specific to your target protein (or histone modification).
- Include a **negative control** sample, typically using a non-specific **IgG antibody** from the same host species as the primary antibody.
- Incubate the mixture (e.g., overnight at 4C) on a rotating mixer to allow the antibody to bind to its target epitope.

Isolation of Complexes

- Add **Protein A/G magnetic or agarose beads** (which bind to the Fc region of the antibody) to the antibody-chromatin mixture.
- Incubate the mixture (e.g., overnight at 4C) to allow the antibody-protein-DNA complexes to bind to the beads.
- **Washing:** Wash the beads extensively with a series of buffers of increasing stringency to remove non-specifically bound chromatin. The final wash is often a low-salt buffer like TE.

Elution

- Add an **elution buffer** (e.g., containing SDS) to the washed beads and incubate (e.g., at room

temperature or 65C) to dissociate the complexes from the beads and collect the immunoprecipitated (IP'd) material.

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4. Reverse Cross-linking and DNA Purification

This final stage prepares the DNA for sequencing library construction.

Reverse Cross-linking

- Incubate the eluted IP and Input samples (and the Input control) at a high temperature (e.g., **65C, 8-16 hrs or overnight**) to reverse the formaldehyde cross-links, freeing the DNA.
- Treat the samples with **RNase** to digest RNA and then with **Proteinase K** to digest proteins.

DNA Purification

- Purify the DNA using a **spin column purification kit** (e.g., Qiagen QIAquick PCR purification kit or MinElute kit) or a standard phenol-chloroform extraction followed by ethanol precipitation.
- **Crucial Note:** Avoid the use of carrier DNA (e.g., salmon sperm DNA) during the IP and purification, as it can be sequenced and reduce the overall data quality.

Quantification and Quality Control

- **Quantification:** Accurately measure the concentration of the purified DNA, especially the Input DNA, using a **fluorescent dye-based assay** (e.g., Qubit dsDNA HS Assay), as NanoDrop is less accurate for low-concentration samples. The amount of DNA recovered from the IP is often very low (<10 ng).
- **Initial QC:** Perform **quantitative PCR/qPCR** on a few known positive and negative control genomic regions to verify the enrichment of the target (e.g., 1-10% of the Input DNA should be recovered in the IP).

The purified ChIP and Input DNA samples are now ready for **Next-Generation Sequencing (NGS) library preparation**.