Magna ChIP-Seq™

Chromatin Immunoprecipitation and Next Generation Sequencing Library Preparation Kit

Catalog No. 17-1010

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

The macromolecular structure of chromatin in eukaryotic cells is dynamic and various epigenetic marks help to define a static chromatin state (1). This chromatin state is a reflection of accessibility and/or presence of certain protein:DNA or protein:protein interactions in a location or region specific manner. This dynamic and coordinated interaction directly influences the expression of a particular gene locus. Thus, the elucidation of these interactions is essential for gaining a deeper understanding of a variety of biological processes and disease states.

One of the main tools for investigating these interactions is ChIP (chromatin immunoprecipitation). ChIP is a powerful technique classically used for mapping the *in vivo* distribution of proteins associated with chromosomal DNA. These proteins can be histone subunits, transcription factors, or other regulatory or structural proteins bound either directly or indirectly to DNA. Using high quality antibodies, protein-interacting regions of chromosomal DNA, as well as their post-translational modifications can be detected. Typically either end-point or quantitative PCR is performed to verify whether a particular DNA sequence (the gene or region of the genome) is associated with the protein of interest. Using this classical approach, laboratories can evaluate the interactions of the proteins of interest for a limited number of known target genes.

However, as the need to map, characterize, and understand these interactions across the epigenome has grown, labs have turned to genome-wide approaches for the analysis of ChIP using either microarrays (ChIP-chip)[†] or next generation sequencing (ChIP-Seq). This combination of chromatin immunoprecipitation with genome-wide analysis represents a powerful approach that can provide a comprehensive look at transcription-factor binding as well as histone modifications and other chromatin associated proteins (2). Although ChIP-chip is a powerful approach and lends itself to the detailed analysis of specific regions of the genome or gene families using high density arrays, the ChIP-Seq approach provides genome-wide data with a high resolution, and wide dynamic range thus allowing for comprehensive coverage of the genome.

Several instrument platforms and distinct sequencing technologies are available for analyzing genomic DNA or RNA sequences (3). The application of chromatin immunoprecipitation combined with massively parallel sequencing (ChIP-Seq) has been performed on several of these platforms (4). To facilitate this procedure, the Millipore Magna ChIP-Seq kit provides a complete workflow solution to facilitate success in the ChIP-Seq application for both new and experienced laboratories.

[†] For details on genome-wide analysis using microarrays please refer to the Magna ChIP²™ Microarray kit user manual (catalog numbers 17-1000, 17-1001, or 17-1002).

Kit Overview

The Magna ChIP-Seq Kit provides a validated set of reagents, protocols, and in process quality control guidelines to simplify the performance of chromatin IP and sequencing library construction. The approach described in this manual has been successfully used to construct ChIP-Seq libraries from as little as 1 ng of ChIP DNA.

This kit contains three modules: the Magna ChIP™ Protein A/G reagents for chromatin precipitation and purification of ChIP DNA (Magna ChIP A/G (2° to 8°) and Magna ChIP A/G (-20°C) and a set of Library Construction reagents suitable for the preparation of next generation sequencing libraries from genomic DNA.

These modules are designed to provide laboratories flexibility in the performance of the ChIP-Seq application. Each module has unique properties that make it suitable for this purpose. In the ChIP Module I, the use of a magnetic protein A+G blend eliminates concerns about bead selection when antibodies of different species and class are utilized. In addition, the magnetic properties of these beads permit more rapid processing of ChIP reactions through the use of a magnetic separation stand such as the Magna GrIP™ Rack (catalog number 20-400).

Successful ChIP is a key first step of library construction. ChIP Module II contains positive and negative control antibodies and a set of PCR primers that can be used as in process controls or to verify technique. For your specific target, a highly validated antibody demonstrated to enrich for your target of interest is required. Millipore offers a selection of antibodies demonstrated to work in ChIP (ChIP qualified antibodies) as well as a collection of rigorously validated antibodies and control primer sets known as ChIPAb+™ kits (visit www.millipore.com/epigenetics to search a complete list of targets).

The library construction module contains a collection of enzymes and buffers in convenient master mix formulations that are commonly used for the creation of sequencing libraries. Each of these components must pass rigorous quality control standards to ensure success in library construction. All provided reagents are lot controlled, both individually and as a set. Each set of reagents provided is functionally validated through construction and sequencing of a genomic DNA library on an Illumina Genome Analyzer II.

Kit Components

This Kit provides sufficient reagents for 22 individual ChIP reactions and 10 next generation library sample preparations. Synthetic oligonucleotide primers and adaptors are not provided allowing flexibility in your approach to library construction. Please consult http://seqanswers.com or your instrument system manufacturer's guidance for specific sequences for single end, paired end or barcoded library construction.

Magna ChIP-Seq Chromatin Immunoprecipitation and Next Generation Sequencing Library Preparation Kit Contents (Cat. No. 17-1010)
Magna ChIP A/G (2° to 8°C) MAGNA00013
Magna ChIP A/G (-20°C) MAGNA00014
Magna ChIP-Seq Library Construction (-20°C) MAGNA00015

Magna ChIP A/G (2° to 8°) MAGNA0013		
Component	**Catalog #**	**Quantity**
Magnetic Protein A/G Beads	CS204457	450 µL
ChIP Dilution Buffer	CS200624	12.5 mL
Low Salt Wash Buffer	CS200625	12.5 mL
High Salt Wash Buffer	CS200626	12.5 mL
LiCl Wash Buffer	CS200627	12.5 mL
TE Buffer	CS200628	12.5 mL
Cell Lysis Buffer	CS200634	5 mL
Nuclear Lysis Buffer	CS200623	5 mL
ChIP Elution Buffer (w/o Proteinase K)	CS200629	5 mL
10X Glycine	20-282	11 mL
10X PBS	20-281	24 mL
Store the Following at Room Temperature Upon Receipt		
Spin Filters	20-290	22 Filters
Collection Tubes	20-291	22 Tubes
Bind Reagent A	20-292	25 mL
Wash Reagent B	20-293	12.5 mL
Elution Reagent C	20-294	1.5 mL

Magna ChIP A/G (-20°C) MAGNA0014		
Component	**Catalog #**	**Quantity**
Protease Inhibitor Cocktail II, 200X ****Contains DMSO**	20-283	110 µL
Proteinase K (10 mg/mL)	20-298	60µL
RNAse A (10 mg/mL)	20-297	60 µL
Anti-RNA Polymerase II, clone CTD4H8	05-623B	25 µg
Normal Mouse IgG	12-371B	25 µg
Control Primers	22-004	75 µL

Magna ChIP-Seq Library Construction (-20°C) MAGNA0015		
Component	**Catalog #**	**Quantity**
10X End Repair Reaction Buffer (w/ dNTP's)	CS204412	110 µL
End Repair Reaction Enzyme Mix	CS204413	11 µL
10X dA-Tailing Reaction Buffer (w/ dATP)	CS204414	55 µL
Klenow Fragment (DNA polymerase I, 3'-5' exo-)	CS204415	11 µL
5X Ligation Reaction Buffer	CS204416	110 µL
T4 DNA Ligase	CS204417	44 µL
Phusion® HF 2X PCR Master Mix	F-531MP	275 µL

Materials Required But Not Supplied

Reagents

- Cells, stimulated or treated as desired
- Antibody of interest for chromatin immunoprecipitation
- 37% Formaldehyde
- *Taq* DNA polymerase for standard PCR
- dNTPs, 2.5 mM each
- SYBR® Green Master Mix for qPCR (e.g. DyNAmo™ SYBR Green qPCR Kit, New England Biolabs catalog # F-400L)
- DNase and RNase-free sterile H_2O
- Quant-iT™ dsDNA HS Assay Kit, (Life Technologies catalog #: Q32851)
- MinElute® PCR purification kit (QIAGEN, catalog #: 28004) or similar kit
- MinElute® Gel Extraction Kit (QIAGEN, Cat.#: 28604) or similar kit
- 2% Agarose TAE gel with 0.5 µg/mL ethidium bromide and gel running buffer
- 50 bp DNA Ladder (e.g. New England Biolabs catalog # N3236S)
- 6X gel Loading buffer (New England Biolabs, catalog # B7021)

Equipment and Materials

- Magnetic Separator (e.g. Magna GrIP Rack (8 Well), catalog #20-400)
- Vortex mixer
- Rotating wheel/platform
- Microcentrifuge
- Sonicator
- Thermomixer or Hybridization Oven
- Variable temperature water bath or incubator
- Laboratory timer
- Cell scraper
- Microfuge tubes, 1.5 mL
- Thermal cycler
- PCR tubes, 0.2 mL
- Filter-tip pipette tips
- Qubit® system (Life Technologies™ catalog #: Q32857)
- Agilent 2100 Bioanalyzer™ or Bio-Rad Experion™ system and associated DNA ChIP consumables and materials
- Apparatus for conventional agarose electrophoresis DNA
- Gel cutting apparatus or transilluminator
- Variable volume (5-1000 µL) pipettors + tips
- Real-time PCR Instrument & amplification plates

Hazards:

- Wear gloves when using this product. Avoid skin contact or ingestion of all reagents and chemicals used in this protocol.
- Protease Inhibitor Cocktail contains DMSO, avoid contact with skin.
- Chromatin preparation may require use of liquid nitrogen. Use personal protective equipment (PPE) when handling liquid N_2 to avoid burns.
- Use PPE, fume hoods and venting when working with concentrated formaldehyde solutions. Formaldehyde is toxic by inhalation, skin contact and ingestion.
- Bind Reagent A (20-292) provided in this kit contains guanidine thiocyanate. This compound is a skin irritant and can form highly reactive compounds when combined with bleach. Do not add bleach or acidic solutions directly to this buffer or solutions containing this buffer.
- Wash Reagent B contains ethanol and is flammable. Avoid storing or using this reagent around open flames.

Storage and Stability

MAGNA00013 Store at 2-8ºC, good for 6 months from date of receipt when reagents are stored and handled appropriately.
Please note: Some components in this box should be stored at room temperature (18-25ºC) upon receipt. Please see "Kit Components" section for details.

MAGNA00014 Store at -20ºC, good for 6 months from date of receipt when reagents are stored and handled appropriately.

MAGNA00015 Store at -20ºC, good for 6 months from date of receipt when reagents are stored and handled appropriately.

Magna ChIP-Seq Kit Functional Validation:

ChIP modules are functionally tested in quantitative chromatin immunoprecipitation reactions and each set of library construction reagents is functionally validated together through construction and sequencing of a genomic DNA library on an Illumina Genome Analyzer II.

Overview of the ChIP-Seq Procedure

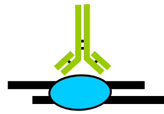
The Magna ChIP-Seq protocol described herein is based upon our existing Magna ChIP protocol. This proven ChIP method is designed to ensure consistent ChIP results and includes all key reagents as well as controls. Similar to the Magna ChIP Chromatin Immunoprecipitation kits (catalog #17-610, 17-611, 17-408, and 17-409), the ChIP portion of this protocol can be completed in a single day, if desired, or performed over the course of two days. Once a successful ChIP reaction is completed the resulting ChIP DNA is enzymatically manipulated using a multi-step process to generate the sequencing library. This library construction process typically takes 1 to 1.5 days to complete.

Because the ChIP-Seq procedure requires multiple days to complete, advance planning is advised. It is strongly recommended that you read the entire protocol before for starting work and that you plan your entire experiment in advance. A generalized overview of the major steps of a typical ChIP-Seq workflow is provided on page 8**.**

The detailed protocols and guidelines presented here contain helpful hints to help you avoid common pitfalls. Three of these are emphasized here. To ensure a successful outcome the following suggestions should considered as you design and plan your experiment. First, choose antibody and source of sheared chromatin demonstrated work well in ChIP. Second, always run the suggested in-process DNA and library QC steps before proceeding. Third, whenever practical, use provided controls to verify the successful completion of each step.

Overview of ChIP-Seq Workflow

Isolate & Fragment Chromatin

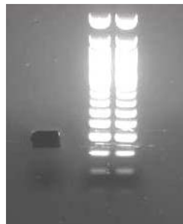


ChIP

Reverse Crosslinks

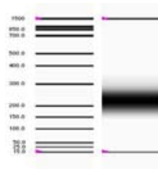
End Repair & Modification

Adapter Ligation

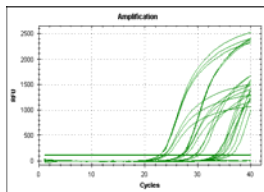


Gel Isolation to size select fragments

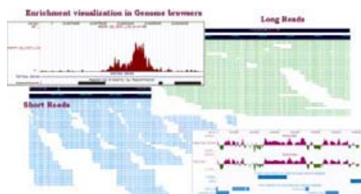
PCR Amplification



QC Library Using Microfluidic or Conventional Electrophoresis



QC Library by Real-time PCR



Next Gen Sequencing

Section I: Chromatin Immunoprecipitation

Tips to Help Ensure a Successful Experiment

IMPORTANT: Please read this section and review entire protocol before starting.

ChIP-Seq experiments are composed of multiple steps that are carried out over multiple days. Because this application represents a significant investment of time and materials, it is **strongly recommended** you carefully plan the entire experiment and decide how best to manage your time before you begin.

To help ensure a successful experiment, it is critical to review and follow the suggestions and guidelines provided. It is also important to take the time to evaluate the samples being prepared after key steps in the protocol. This can help prevent the waste of time and materials as well as the generation of an unsatisfactory ChIP-Seq result.

Chromatin Immunoprecipitation Experimental Considerations

- Chromatin size is critical to the success of ChIP-Seq. This protocol works best when the chromatin size is between 200-600 bp. Shearing of the chromatin varies greatly, depending on cell type, growth conditions, quantity, volume, crosslinking, and equipment. It may be necessary to optimize sonication conditions by changing the power settings, cycle number and ratios of time ON and time OFF. The quality of the chromatin can be analyzed visually by agarose gel or microfluidic electrophoresis.
- Cell number equivalents of chromatin required per ChIP reaction is dependent on the quantity of available epitopes in the cell of interest as well as the quality of antibody being utilized. In general, $1X10^6$ to $1x10^7$ cells per ChIP is recommended. The Magna ChIP A/G approach used in this manual recommends preparing chromatin in batch format providing approximately 10 ChIP samples per preparation when using cultured cells. Sufficient buffers for chromatin preparation are provided to enable chromatin preparation from up to five 15 cm plates of cultured cells.
- The success of ChIP-Seq is very dependent on how efficiently you can immunoprecipitate your chromatin. It is important to know the quality of your antibody and level of enrichment for a target locus before performing a ChIP-Seq experiment. To assess the enrichment of your ChIP sample prior to library construction, a mock IgG or negative antibody control ChIP reaction may be performed to determine fold enrichment. Alternatively, a negative locus control may also be used to minimize chromatin and ChIP reagent requirements. If you are inexperienced in the methodology of ChIP or unsure of the performance of your antibody in ChIP, you may consider conducting a classical ChIP experiment using a kit such as Millipore's EZ-Magna ChIP™ kits (Cat. # 17-408 & 17-409).
- Construction of a control Input library sample is recommended for comparative analysis in ChIP-Seq experiments.
- In order to isolate sufficient DNA for ChIP-Seq library construction (1-10 ng of purified ChIP DNA as determined by a PicoGreen® assay), it may be necessary to pool individual ChIP reactions. If your epitope is not as abundant as RNA Polymerase II or certain modifications on histone proteins, pooling 3 individual ChIP samples for the ChIP target of interest is recommended as a starting point. If a mock control is being utilized, it is not necessary to pool mock IP ChIP reactions unless a mock IP library is desired.

Detailed Protocol

Section I: Chromatin Immunoprecipitation –Please Read Entire Protocol First

A. *In vivo* Crosslinking and Lysis (Cultured Cell Protocol)

Prior to starting this section:

- Stimulate or treat, if necessary, adherent mammalian cells at ~80-90% confluency in a 150 mm culture dish containing 20 mL of growth media.
  - For HeLa cells, this is approximately 1 x 10^7 cells. This will generate a preparation of chromatin sufficient for up to 10 separate immunoprecipitations.
  - The volume of buffers supplied in the kit are sufficient to generate chromatin from up to five 150 mm plates of cultured cells, each plate providing up to 10 chromatin preparations (varies with cell and assay type). Chromatin from alternate culture vessels can be isolated with slight modifications to the protocol based on cell number and culture vessel surface area.
  - Include one extra plate of cells to be used solely for estimation of cell number.
  - Cell numbers can be scaled according to the performance of the antibody. For example, Magna ChIP control antibodies can perform successful ChIP on as few as 1 X 10^5 HeLa cells. For simplicity, this protocol is written using 1 X 10^6 cells per ChIP to ensure optimal performance of the control antibodies.
- Obtain ice for incubation of PBS (Step 3) and for incubating the culture dish (Step 6).
- Prepare 42 ml of 1X PBS (4.2 mL 10X PBS and 37.8 mL water) for each 150 mm culture dish and put on ice. This will be used for washes and must be ice cold.
- Warm Nuclear Lysis Buffer to room temperature and ensure SDS is in solution before proceeding with cell lysis.
- Remove Protease Inhibitor Cocktail II and thaw at room temperature for use in Steps 3 and 13. This product contains DMSO and will remain frozen below 18.4°C.

Cross Linking and Cell Lysis Protocol

1. Fix cells in 1% formaldehyde by adding 550 µL of 37% formaldehyde (or 1100 µL of fresh 18.5% formaldehyde) to 20 mL of growth media. Gently swirl dish to mix.

   Important: Use high quality formaldehyde. Do not use formaldehyde that is past the expiration date suggested by the manufacturer.

2. Incubate at room temperature for 10 minutes. Agitation of the cells is not necessary.
3. During 10 minutes incubation period, remove 2 mL of ice cold 1X PBS and place in a separate tube for every dish to be processed. Add 5 µL of Protease Inhibitor Cocktail II to each 1 mL of 1X PBS and put on ice.
4. Add 2 mL of 10X Glycine to each dish to quench unreacted formaldehyde.
5. Swirl to mix and incubate at room temperature for 5 minutes.
6. Place dishes on ice.
7. Aspirate medium, removing as much medium as possible, being careful not to disturb the cells.
8. Add 20 mL of cold 1X PBS to wash cells.

9. Remove PBS and repeat PBS wash step 1X including removal of PBS.
10. Add 2 mL cold PBS containing 1X Protease Inhibitor Cocktail II to each dish (prepared in Step 3).
11. Use sterile cell scraper collect cells from each dish and place into a separate microfuge tubes.
12. Spin at 800 x g at 4°C for 5 minutes to pellet cells.
13. During spin, for each microfuge tube from step 11, combine 0.5 mL of cell Lysis Buffer with 2.5 µL of Protease Inhibitor Cocktail II.
14. Remove supernatant.

    Tip: For chromatin preparations from cultured cells, fixed cell pellets (without buffer) can be snap frozen in liquid nitrogen at this point and stored at -80°C for several months if desired.

15. Resuspend cell pellet in 0.5 mL of cell Lysis Buffer containing 1X Protease Inhibitor Cocktail II.
16. Incubate on ice for 15 minutes, vortex the cell suspension briefly every 5 minutes.
    - Optional: At the end of the incubation, homogenize the cell suspension 10 times in a Dounce homogenizer to facilitate the release of the nuclei.
17. Spin the cell suspension at 800 x g at 4°C for 5 minutes in a microcentrifuge.
18. During spin, for each microfuge tube to be processed combine 2.5 µL of Protease Inhibitor Cocktail II with 0.5 mL of Nuclear Lysis Buffer.
19. Carefully remove supernatant being careful not to disturb the cell pellet then resuspend cell pellets in 0.5 mL of Nuclear Lysis Buffer.

    Important: For every 1 x 10^7 HeLa cells, 0.5 mL of Nuclear Lysis Buffer is recommended for this protocol. Adjust accordingly if different cell concentrations are used as the ratio of lysis buffer to cell density is important for reliable cell lysis.

20. If optimal conditions for sonication have been determined, proceed to Section B.

B. Sonication of Isolated Chromatin to Shear DNA

Prior to starting this section:

- Optimal conditions need to be determined to shear cross linked DNA to ~200-600 base pairs in length (Figure 1). Once shearing conditions have been optimized, proceed with the steps below.

Sonication Protocol

1. Sonicate cell lysate on wet ice.
    - Sonication conditions must be empirically determined. The efficiency of sonication depends upon cell type, cell equivalents and instrumentation. Where possible, consult your instrument manufacturer's guidelines.
    - Always keep cell lysates ice-cold. Sonication produces heat, which can denature the chromatin. It is important to allow sufficient time between sonication cycles to prevent sample overheating.

2. Spin at a minimum of 10,000 x g at 4°C for 10 minutes to remove insoluble material. However, do not exceed 15,000 x g to prevent loss of chromatin.

3. Carefully transfer supernatant to fresh microfuge tube. Store in 50 µL aliquots.
    - To evaluate chromatin quality, remove one 5 µL aliquot for agarose gel analysis of the sheared DNA and purify for analysis as directed in section D. Although this step is optional it is strongly recommended that chromatin quality be evaluated before proceeding.
    - Each 50 µL aliquot contains ~1 x 10^6 cell equivalents of lysate sufficient for one immunoprecipitation.
    - Sheared cross linked chromatin can be stored at -80°C for up to 3 months.

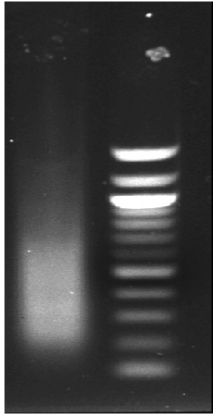


Figure 1: DNA Sonication
Representative sonicated HeLa chromatin prepared from 1 x 10^6 HeLa cells following Section A (all steps) Section B (steps 1-3) and of the Magna ChIP protocol. A 5 µl input sample was purified and eluted in 50 µl. 20 µL of this sample was then separated through a 2% agarose gel by electrophoresis and stained with ethidium bromide. Lane 1 shows a majority of the DNA is sheared to a length between 200 bp and 600 bp.

C. Immunoprecipitation (IP) of Cross linked Protein/DNA

Prior to starting this section:

- Thaw Protease Inhibitor Cocktail II at room temperature for use in Step 3. Note that this solution contains DMSO and will remain frozen below 18.4°C.
- In advance of step 8, ensure the following buffers are placed on ice
    - Low Salt Immune Complex Wash Buffer
    - High Salt Immune Complex Wash Buffer
    - LiCl Immune Complex Wash Buffer
    - TE Buffer

Immunoprecipitation Protocol

1. Prepare enough Dilution Buffer containing protease inhibitors for the number of desired immunoprecipitations and store on ice.
    - Each IP requires the addition of 450 µL of Dilution Buffer and 2.25 µL of Protease Inhibitor Cocktail II.

2. For each immunoprecipitation reaction prepare a microcentrifuge tube containing 50 µL of sheared cross linked chromatin (Section B, step 3) and put on ice. If chromatin has been previously frozen, thaw on ice.

3. Add 450 µL of Dilution Buffer containing Protease Inhibitor Cocktail II into each tube containing 50 µL of chromatin. Prepare sufficient ChIP reactions for pooling (if necessary) to achieve the minimum quantity of immunoprecipitated DNA for library construction. If the number of samples required is unknown, start with 3 reactions for each ChIP target plus an additional sample if a mock IP is planned.

4. Remove 25 µL (5%) of the diluted chromatin as "Input" and save at 4°C until Section D, step 1. Only one input sample is required for each type of chromatin being processed.
5. Add the immunoprecipitating antibody and 20 µL of **fully resuspended** protein A/G magnetic beads.

   Important: Make sure the magnetic bead **slurry** is **well mixed** before removing volume for IP, as magnetic beads will settle on the bottom of the tube over a short period of time.

   The amount of antibody providing the highest level of enrichment should be titrated by performing ChIP in advance of the ChIP-Seq experiment.

6. Incubate immunoprecipitation reaction for **2 hour** to **overnight** at 4°C with rotation or agitation sufficient to keep beads from settling to the bottom of the tube.

   Note: Magna ChIP experiments can be performed following either **one day** or **two day** protocols. It may be possible to reduce the incubation time of the IP from overnight to 1-4 hrs. This depends on many factors (antibody, gene target, cell type, etc.). Ideal conditions should be determined in advance.

7. Separate Protein A/G magnetic beads with a magnetic separation device compatible with 1.5 mL tubes (e.g. Millipore Magna GrIP Rack (8 Well), Cat. # 20-400). Allow the beads to completely separate then carefully remove the supernatant without disturbing the beads.
8. Wash the Protein A/G bead-antibody/chromatin complex by resuspending the beads in 0.5 mL each of the cold buffers in the order listed below and incubating for 3-5 minutes on a rotating platform between each wash followed by magnetic separation and careful removal of the supernatant fraction:
   a. Low Salt Immune Complex Wash Buffer, **one wash**
   b. High Salt Immune Complex Wash Buffer, **one wash**
   c. LiCl Immune Complex Wash Buffer, **one wash**
   d. TE Buffer, **one wash**

D. Elution of Protein/DNA Complexes and Protein/DNA Complex Crosslink Reversal

Prior to starting this section:

- Warm the ChIP Elution Buffer to room temperature and ensure the SDS is in solution before proceeding.

Protocol

1. Prepare final elution buffer for all IP tubes as well as all Input tubes (prepared in Section C, step 4). For each tube, prepare elution buffer as follows:

ChIP Elution Buffer	100 µL
RNase A	1 µL

2. Add 100 µL ChIP Elution Buffer/RNase A mixture to each tube and incubate at 37°C for 30 minutes with shaking or agitation (approximately 950 rpm on orbital mixer). During this incubation period thaw the Proteinase K for use in step 3. Once thawed store Proteinase K on ice).
   - Shaking and 37°C incubation can be accomplished with equipment such as an Eppendorf Thermomixer® system, a Labnet Shaking incubator, or a standard roller bottle hybridization oven.

3. Add 1 µL proteinase K to each sample and Incubate at 62°C for 2 hours with shaking or agitation (approximately 950 rpm on orbital mixer).
4. Incubate at 95°C for 10 minutes.
5. Allow sample to cool to room temperature over an approximately 5 minute period.
6. Separate beads using a magnet separation device. Carefully remove and transfer the supernatant to a new 1.5 mL microcentrifuge tube.

E. DNA Purification Using Spin Columns

1. Remove one Spin Filter with Collection Tube and a second Collection Tube for each sample tube from Section D.
2. Add 0.5 mL of Bind Reagent "A" to each 100 µL DNA sample tube (Immunoprecipitations and Inputs) and mix well.
    - 5 volumes of Bind Reagent "A" should be used for every 1 volume of sample.
    - A white precipitate may be observed. This will not interfere with this procedure.
3. Transfer the sample/Bind Reagent "A" mixture to the Spin Filter in Collection Tube.
4. Centrifuge for 30 seconds at a minimum of 10,000 x g. Do not exceed 15,000 x g.
5. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
    - If a precipitate formed in Step 2, it may be observed in the bottom of the Collection Tube and this will not interfere with this procedure.
6. Put the Spin Filter back into the same Collection Tube.
7. Add 500 µL of the Wash Reagent "B" to the Spin Filter in Collection Tube.
8. Centrifuge for 30 seconds at a minimum of 10,000 x g. Do not exceed 15,000 x g.
9. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
10. Place the Spin Filter back into the same Collection Tube.
11. Centrifuge for 30 seconds at a minimum of 10,000 x g. Do not exceed 15,000 x g.
12. Discard the Collection Tube and liquid.
13. Put the Spin Filter into a clean Collection Tube.
14. Add 50 µL of Elution Buffer "C" directly onto the center of the white Spin Filter membrane.
15. Centrifuge for 30 seconds at a minimum of 10,000 x g. Do not exceed 15,000 x g.
16. Remove and discard Spin Filter. Eluate is now purified DNA. It can be analyzed immediately or stored frozen at -20°C.

F. Real-time Quantitative PCR to Verify ChIP DNA Enrichment

It is extremely important to verify enrichment of your ChIP DNA prior to proceeding with the library construction portion of the protocol. Verification of enrichment can be performed using relative standard curve method of qPCR analysis to compare DNA from a mock IP using IgG vs. DNA immunoprecipitated using your ChIP antibody, or can

alternatively be compared using a relative standard curve with two PCR amplicons, a positive control binding region and a negative control region (i.e. region of DNA not enriched by ChIP of your target). Primers should be designed to produce an amplicon of 60-80 base pairs. Note that these primers can also be used to verify enrichment in your final library (see Section II: Library Construction, Part H; Enrichment Verification for additional details)

Input DNA is required whether using relative standard curve method or the comparative Ct (delta-delta Ct) method. Target gene enrichment relative to IgG of greater than 8-10 fold is recommended for library construction. An example of appropriate fold enrichment relative to an IgG mock IP is shown in figure 2.

Protocol

1. Add 2 µL of the ChIP or input sample to a PCR plate suitable for your real time instrument (Performing triplicate of qPCR reactions per ChIP sample is recommended).
2. Prepare a qPCR master mix as shown in Table II. Prepare a volume of master mix at least 10% greater than required for all samples to account for pipette carryover.
3. Add 23 µL of qPCR master mix to 2 µL of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions. Please refer to figure 2 for real-time PCR result.

qPCR reagent assembly for 1 reaction

ddH_2O	9.5 µL
SYBR Green Master Mix	12.5 µL
Primer mix	1.0 µL
Total	23.0 µL

qPCR Parameters:

1. Initial Denaturation 94°C 10 min
2. Denature 94°C 20 sec
3. Anneal and Extend: 60°C 1 min. (modify annealing temperature as required for Tm of primer set)
4. Repeat steps two and three for a total of 50 cycles

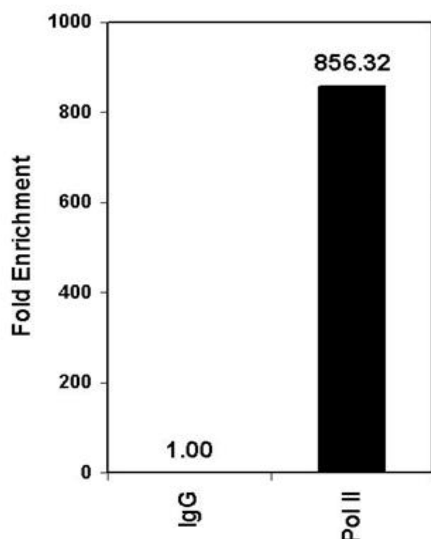


Figure 2: Real-Time PCR Results

Sonicated chromatin prepared from untreated HeLa cells was subjected to chromatin immunoprecipitation using 1 µg of mouse purified IgG (12-371B) or Mouse Anti-RNA Pol II (05-623B) and the Magna ChIP Kit with overnight IP. Successful immunoprecipitation of RNA Pol II associated DNA fragments was verified by qPCR using control primers flanking a binding site in the human GAPDH promoter (22-004).

Section II: Sequencing Library Construction

Tips to Help Ensure a Successful Experiment

IMPORTANT: Please read this section and review entire protocol before starting

Library construction is a multistep process utilizing enzymatic manipulation of your samples, sample clean-up and in process quality control steps. Because this application represents a significant investment of time and materials, it is **strongly recommended** that you carefully review this section and plan the entire experiment and decide how best to manage your time before you begin.

To help ensure a successful experiment, it is critical to review and follow the suggestions and guidelines provided. It is also important to take the time to perform the in process quality control checks to help prevent the waste of time and materials as well as the generation of an unsatisfactory ChIP-Seq result.

General Guidelines and Protocols for Sequencing Library-Preparation:

- The size of the chromatin used is key success factor for ChIP-Seq library construction. Ensuring that the chromatin is of an appropriate size before beginning library construction is strongly recommended. If the input chromatin used for the ChIP protocol outlined in section I was sheared to an appropriate size of 200-600 typically, the enriched immunoprecipitated chromatin will be of an appropriate size for library construction.

- Although good sequencing libraries can be generated from as little as 1 ng of starting material, whenever possible use 10 ng of purified ChIP DNA. In order to obtain sufficient DNA for your target of interest, you may need to pool individual ChIP samples. Pooling 3 individual ChIP samples for the ChIP target of interest is recommended as a starting point.

- Pooling may be not required for abundant epitopes. However, it is important to assess the success of your ChIP reactions by quantifying the amount of DNA recovered after ChIP. To accurately determine the amount of material recovered, a sensitive DNA assay that will detect DNA in the pg/µL range such as a Qubit fluorimeter with a PicoGreen assay, Agilent Bioanalyzer system or alternative method is suggested.

- To reflect the genome wide distribution of clonable fragments in the starting chromatin sample, an input library is always recommended as a comparative control for the ChIP-Seq library preparation. Use caution and standard laboratory practice to avoid template cross-contamination when preparing ChIP and Input libraries simultaneously. A single input library can be used for multiple ChIP samples if the ChIP samples are derived from the same batch of chromatin as the input sample.

- Enzymes and other temperature sensitive reagents are used for library construction. Maintain all vials of temperature sensitive reagents on ice when setting up reactions and return the vials to -20°C storage as quickly as possible.

ChIP-Seq Library Construction Protocols

IMPORTANT: Please Read Entire Protocol First

A. Measure Concentration of ChIP DNA:

It is important to accurately quantify the amount of starting material before starting library construction. The use of a Qubit system or similar approach that allows sensitive and accurate quantification is recommended.

Additional material and equipment required:

- Qubit system, (Life technology, Cat. #: Q32857)
- Quant-iT dsDNA HS Assay Kit, (Life technology, Cat. #: Q32851)

Follow the manufacturer's protocol using 10-20 µL ChIP DNA for measurement. A ChIP sample prepared form 1,000,000 cells using the Millipore Magna ChIP protocol typically yields ~ 1-10 ng of DNA depending on the target protein and antibody used.

B. End-Repair Protocol

Additional materials required:

- ChIP DNA : 1-10 ng
- MinElute PCR purification kit (QIAGEN, Cat.#: 28004)

1. Combine the following components into a 0.2 mL microfuge tube and mix well.

ChIP enriched DNA	1-44 µL
End repair reaction buffer (10X)	5 µL
End repair enzyme mix:	1 µL
Sterile nuclease free H2O	Variable
Total	50 µL

If the ChIP DNA concentration is less than 1-10 ng/44 µL, increase the reaction volume to 100 µL and double the amount of 10X reaction buffer and enzyme mix. If using a recommended input library use an equivalent amount of material.

2. Incubate in thermal cycler for 30 minutes at 20°C.
3. Purify DNA sample using MinElute column following suppliers protocol and elute in 40 µL of EB.

C. dA-Tailing Protocol (if required for adapter mix)

Additional Material required:

- MinElute PCR purification kit (QIAGEN, Cat.#: 28004)

1. Add the following components into a 0.2 mL PCR tube and mix well.

End –repaired DNA	40 µL
dA-Tailing buffer (10X):	5 µL
Klenow Fragment (3'-5' Exo^{-})	1 µL
Sterile nuclease free H2O:	4 µL
Total volume:	50 µL

2. Incubate in thermal cycler for 30 minutes at 37°C
3. Purify DNA sample using a MinElute column following suppliers protocol and elute in 35 µL of EB.

D. Adapter Ligation Protocol

Additional Material required:

- MinElute PCR purification kit (QIAGEN, Cat.#: 28004)
- Adaptors appropriate for library construction approach and instrument platform

1. Add the following components into a 0.2 mL microfuge tube and mix:

dA-Tailed DNA	35 µL
Fast ligation buffer(5X):	10 µL
Adaptor mix (1.5 µM):	1 µL
Fast T4 DNA ligase:	4 µL
Total volume:	50 µL

2. Incubate in thermal cycler for 15 minutes at 20°C
3. Purify DNA sample using a MinElute column according to the suppliers protocol and elute in 15 µL of EB.

E. DNA Size Selection Protocol

Additional materials and equipment required:

- 2% TAE agarose gel with ethidium bromide
- Agarose gel box and power supply
- 50 bp DNA Ladder
- 6X gel Loading buffer
- MinElute Gel Extraction Kit (QIAGEN, Cat.#: 28004)

IMPORTANT: Thoroughly clean and rinse the gel box with distilled water and use fresh buffer each time you are generating a library to minimize potential for cross contamination.

1. Prepare 2% TAE agarose gel with 0.5 µg/mL ethidium bromide. Note that ethidium bromide is a mutagen, use appropriate precautions when handing.
2. Combine 6X gel loading dye with 1 µg of 50 bp ladder mix well and load into one lane of the gel.
3. Combine 6X gel loading dye with the purified ligated sample. To avoid the potential for cross contamination of adjacent wells, load the purified ligated sample **at least one lane away** from the ladder. Take care not to allow any of the 50 bp ladder to spill over into the sample lane. If you prepare the input library with the ChIP-Seq library at the same time, make sure you separate both samples with at least one lane or isolate samples from separate gels if desired.
4. Run gel at 120V for ~1 hour or until the tracking dye ~2/3 down length of the gel.
5. Visualize 50 bp ladder using an appropriate transilluminator (see "Gel cutting tips below). The ligated DNA will not be visible on the gel. Excise a gel slice from the sample lane in the range of 175-225 bp to ensure ligated adaptors with sequencer compatible inserts are isolated.

Note: Fragments greater than 225 bp may be acceptable depending upon sequencing instrument. Please follow your instrument manufacturer's guidelines for appropriate fragment size ranges. Fragments smaller than

approximately 175 bp often represent adaptor only ligation products. Failure to remove these short product clones can result in PCR amplification bias and loss of mappable data in the downstream sequencing application.

Gel cutting tips:

- A dark reader transilluminator or appropriate gel isolation system is recommended to visualize the gel and avoid excess exposure to short wave UV (302 nm or lower).
- If using the UV light, try to put the lane containing the ligated DNA library at the edge of the UV box to avoid direct exposure to UV. The lane containing DNA ladder should be put under UV to visualize the correct size.
- When excising a band under UV light, try to work as fast as possible.

6. Purify the DNA sample from the gel slice using QIAGEN MinElute Gel Extraction Kit according to the supplier protocol and elute in 20 μL EB.

F. High Fidelity PCR Amplification Protocol

Additional material required:

- MinElute PCR purification Kit (QIAGEN, Cat.#: 28604)
- PCR primer 1 and Primer 2 appropriate for sequencing approach and platform

1. Add the following component into a 0.2 mL PCR tube and mix:

Size selected DNA	20 μL
Phusion master mix (2X)	25 μL
PCR primer 1* (25 μM stock):	1 μL
PCR primer 2* (25 μM stock):	1 μL
Sterile nuclease free H2O	3 μL
Total volume:	50 μL

2. Perform amplification reaction using following PCR conditions.

   A. 30 sec at 98°C

   B. Denature 10 seconds at 98 °C

   Anneal 30 seconds at 65 °C

   Extend 30 seconds at 72 °C

   Repeat for 17 more cycles (18 cycles is recommended). You can also optimize the PCR cycle number in following section.)

   C. 5 minutes at 72 °C

   D. Optional hold at 4°C

   Note: PCR primer sequences must be compatible with chosen sequencing platform. Consult manufacturer's suggestions for your instrument as necessary. Annealing temperature used should be based on Tm of actual primers used for this step.

3. Purify the amplified material with MinElute PCR purification kit according to supplier's protocol and elute in 15 μL of EB.

G. Library Analysis and Enrichment Verification

These steps are designed to verify that appropriate size inserts are present in your library (library analysis step) and that there has been suitable enrichment relative to the input sample prior to sequencing (enrichment verification step).

Additional materials and equipment required:

- Agilent Bioanalyzer, Bio-Rad Experion and DNA analysis chips and reagents or equivalent system and reagents
- Real-time PCR instrument and real-time PCR reagents

Library Analysis Protocol

1. Using either a Bioanalyzer or Experion system, load 1 µL per sample of the prepared ChIP-Seq library per well on the DNA chip according to the manufacture's protocol. Whenever possible, examine the input library as well as the ChIP library.
2. Use the data generated from either instrument to determine the size, purity and concentration of the samples.
   a. The library size will be slightly bigger than what you originally excised from the gel due to the overhang of the PCR primer.
   b. Ensure the sample is free of amplification products that reflect the size of adaptors with minimal inserts (generally discrete product bands smaller than 175 bp). An example of a library with an appropriate size distribution is presented in figure 3.
   c. A library concentration of at least 10 nM is suggested for sequencing. However, this can vary with different systems. Consult your instrument provider's specifications for the amount of material required for sequencing. In general, the amount of sample produced after amplification and clean-up is sufficient for several sequencing runs.

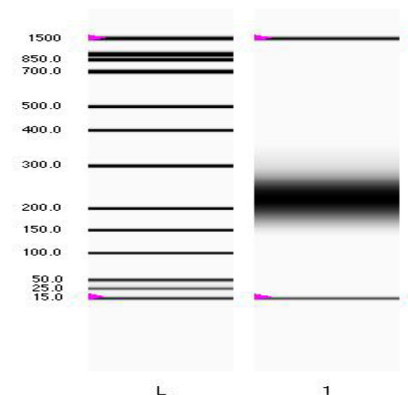


Figure 3: ChIP-Seq Library Analysis
Sp1 ChIP-Seq library quality was assessed using a Bio-Rad Experion system. This ChIP-Seq library was generated from 1 ng of Sp1 ChIP DNA with 18 cycles of PCR. The size is distribution of the library is approximately 175-250 bp.

Library Enrichment Verification

Prior to performing a sequencing run, it is important to confirm relative enrichment of the ChIP-Seq library relative to the input sample to verify sufficient enrichment of your final library to merit a sequencing run. Real-time PCR using a SYBR Green master mix and control primers are used for this purpose. The same primer sets used to assess fold enrichment after the initial ChIP reactions can be used for this purpose (see Section I: Chromatin Immunoprecipitation, part F). After PCR, the relative fold enrichment of enriched library vs. unenriched input sample is easily determined using either the delta Ct method or the delta-delta Ct method. Both approaches are described below. Follow good PCR practices to avoid cross contamination of your reactions.

Library Enrichment Verification Protocol

1. Dilute a sample of both the input library and the ChIP DNA library to 1 nM based on the Bioanalyzer or Experion reading obtained in the "Library Analysis" section above.
2. Set up real-time PCR reaction for either delta-delta Ct analysis or delta Ct analysis as outlined below.

   Delta-delta Ct Method Reaction Set-Up

   Prepare separate real-time reactions for both the chIP enriched library and the input library using both positive and negative control primers from section I, part F as outlined below. Triplicate reactions are recommended for each sample type listed below.

   i) ChIP DNA library using positive control primer Ct= X
   ii) ChIP DNA library using negative control primer Ct= Y
   iii) Input DNA library using positive control primer Ct= Z
   iv) Input DNA library using negative control primer Ct= W

   Delta Ct Method Reaction Set-Up (alternative to above approach)

   Prepare separate real-time reactions using the chIP DNA library and the input DNA library using the positive control primer from section I part F only.

   i) ChIP DNA library using positive control primer Ct= X
   ii) Input DNA library using positive control primer Ct= Z

3. The creation of a master mix for each sample type is advised. To ensure sufficient material for your replicate sample prepare at least 25% excess material for each primer set to account for pipette carry over. Add 2 µL of diluted ChIP library DNA or input library DNA to the appropriate reactions.

   qPCR reagent assembly for 1 reaction:

Reagent	Volume
ddH_2O	9.5 µL
SYBR Green Master Mix	12.5 µL
Primer mix	1.0 µL
Total	23.0 µL

   Add 2 µL of a 1 nM dilution of ChIP-Seq library or input library to each PCR reaction as appropriate. Place PCR reactions into real-time system and run cycling program.

qPCR Cycling Parameters

1. Initial denaturation 94°C 10 min
2. Denature 94°C 20 sec
3. Anneal and Extend: 60°C 1 min (modify annealing temperature to be consistent with Tm of primer set)
4. Repeat steps two and three for total of 40 cycles.

4. Calculate the relative fold enrichment.

If using the delta-delta Ct approach, calculate the relative fold enrichment of the ChIP DNA library versus the input DNA library using the formula: $2^{(-(X-Y)-(Z-W))}$

X= Ct of ChIP DNA library using positive control primer

Y=Ct of ChIP DNA library using negative control primer

Z= Ct of Input DNA library using positive control primer

W= Ct of Input DNA library using negative control primer

If using delta Ct approach, calculate the relative fold enrichment of the ChIP DNA library versus the input DNA library using the formula: $2^{(-(X-Z))}$

X= Ct of ChIP DNA library using positive control primer

Z= Ct of Input DNA library using positive control primer

Libraries suitable for sequencing typically show approximately 10 fold enrichment. An example of enrichment data is shown in figure 4. Although lower enrichment values may provide reliable sequence data, sequencing of libraries with low relative enrichment values is not recommended without validation on the instrument of choice. If you are unsure, consult your system provider for specific guidance.

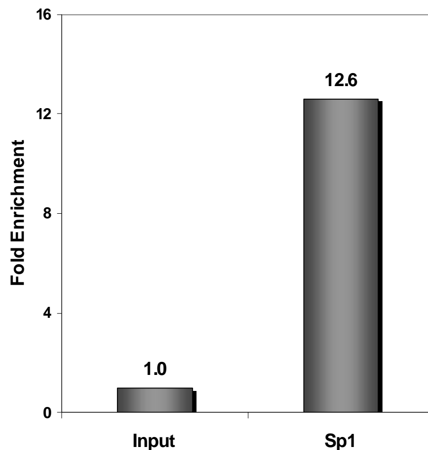


Figure 4. ChIP-Seq Library Enrichment Verification A Sp1 ChIP-Seq library is evaluated by qPCR using mass normalized quantities to determine Fold Enrichment relative to an input sample library.

CHROMATIN IP OPTIMIZATION AND TROUBLESHOOTING

Step	Potential Problems	Experimental Suggestions
Crosslinking	Not enough or too much crosslinking	The amount of formaldehyde and time of crosslinking must be determined empirically. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time. HINT: Histones may not need to be cross linked since they are tightly associated with DNA.
Cell Lysis	Inefficient disruption of cells	It is important to have sufficient lysis buffer for the number of cells processed. Follow the guidelines in this protocol. Also, verify cell lysis by viewing a 10 µL portion of the cell lysate under the microscope to confirm lack of intact cells.
Chromatin Shearing	Not enough/too much sonication	It is important to optimize sonication conditions. Prepare chromatin using different time and power settings to optimize fragmentation.
	Denaturation of proteins from overheating sample	Keep the sample on ice during sonication. Shorten the time of each sonication and increase the number of times the sample is sonicated. Allow sufficient time for sample to cool between pulses.
Addition of Primary Antibody	Antibody doesn't recognize protein in fixed chromatin	Choose an antibody directed to a different epitope of the antigen. Decrease the amount or time of formaldehyde fixation.
	Not enough or too much chromatin	Perform IP from a dilution series of antibody with a fixed amount of chromatin or vice versa.
	Insufficient incubation time	• Incubate the antibody of interest with the chromatin at 4°C overnight. • Select a different antibody with higher affinity. • Perform a Western blot of the immunoprecipitated protein to verify the antibody can precipitate the antigen of interest.
	Primary antibody is not compatible with A/G beads	Use a bridge antibody or secondary antibody to compatible with A/G beads
Addition of Secondary Reagent – Protein A/G beads	Not enough beads	The magnetic beads settle to the bottom of the tube over time. Be sure the Protein A/G magnetic beads are well mixed prior to removing the appropriate volume for IP.

Step	Potential Problems	Experimental Suggestions
Washing	Not enough washing time	Increase number of washes for each wash buffer.
	Aspiration of the beads during buffer removal	• Carefully remove supernatant and make sure there are no beads in the supernatant prior to removing it. • Use rack with magnets capable of firmly holding beads in place (e.g. Magna GrIP Rack cat# 20-400)
Elution and Reversal of crosslinking	Incomplete elution	When performing elution, make sure that the temperature is near 60°C. Proteinase K will be inactivated by prolonged incubation at temperatures above 65°C.
	Excessive Crosslinking	Excessive cross linking may not be reversible. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time.
PCR	Incorrect Annealing Temperature or Amplification Conditions	• Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct T_m. • Perform PCR on genomic DNA to confirm amplification conditions and ability of primers to generate a single DNA product of the expected size.
	Bad primers	Follow suggestions for primer design in section “Chromatin IP Assay Overview, section B”.
	No PCR product	• Increase amount of DNA added to the PCR reaction. • Increase the number of cycles for the amplification reaction.
	PCR product is a smear	• Decrease amount of DNA added to the PCR reaction. • Use HotStart *Taq* polymerase to avoid non-specific annealing of primers.

Step	Potential Problems	Experimental Suggestions
Not enough library DNA generated	Not enough starting material	• Pool and precipitate several ChIP DNA samples • Perform a second sonication on the ChIP DNA to cut the size down to around 100-600 bp. • The Qubit system is recommended for quantifying small amounts of DNA.
	Sample lost in one of the purification or size selection step	• Repeat the library prep process and execute the purification step with extra caution. • Practice the gel cutting process before the actual size-selection. Always try to load your maker and sample towards the middle of the lane to avoid uneven running.
Appearance of second band in library	The higher molecular weight band due to concatenation or secondary structure	• Order new HPLC purified oligos.
	The lower molecular band is due to primer dimmer	• Re-purify the library by gel purification to exclude contaminating low molecular weight products. • Run the gel longer and cut the band carefully avoiding fragments below 175 bp.
Over amplification of library	PCR cycle number is too high	• Use fewer PCR cycles.
QC of the library	Library is not enriched when compared with input library using the mass normalization	• Use QC primer or a negative control primer to normalize the library • Cross contamination with input samples can occur during library construction. Use PCR practices to minimize potential cross contamination. Decontaminate work bench and equipment and use fresh reagents and filter tip pipettes to set-up reactions.
No product after library construction	The library preparation is suddenly not working after a few success	• Order new adaptors and primers directly from your vendor
Multiplexing the library	Try to sequence multiple libraries together	• Add bar code to the end of the adaptor.

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