Chromatin Immunoprecipitation (ChIP) Assay Protocol

Can be prepared in advance and stored at -80º

Wash Staph A Cells:

1. Resuspend 1 gram of lyophilized Staph A cells (Pansorbin®, Calbiochem Cat#507862) in 10 mL of 1X Dialysis Buffer without sarkosyl (DB – srk) (difficult to resuspend, try using a P1000 pipetman w/several mL, then after resuspension add remaining volume OR allow 30 minutes to rehydrate to ease resuspension)
2. Transfer to a 15 mL tube and centrifuge at 6,000 rpm for 5' at 4°C, pour off supernatant
3. Resuspend pellet in 10 mL 1X DB – srk
4. Centrifuge at 6,000 rpm for 5' at 4°C, pour off supernatant
5. Resuspend in 3 mL of PBS, 3% SDS, 10% BME (2.25 mL 1X PBS, 450 µL 20% SDS, 300 µL BME) in fume hood
6. Boil for 30' in fume hood
7. Centrifuge at 6,000 rpm for 5' at RT, pour off supernatant into chemical waste
8. Wash with 10 mL of 1X DB – srk
9. Centrifuge at 6,000 rpm for 5' at RT, pour off supernatant
10. Repeat 7, 8 and 9
11. Resuspend in 4 mL of 1X DB – srk
12. Divide into 100 µL aliquots (40x 0.5 mL tubes), snap freeze and store at -80 (or liquid N2) for indefinite time

Must be prepared within two weeks of beginning experiment and stored at +4º
(Prepare 100 µL Staph A for every $5x10^7$ cells, will use half Day 1 and half Day 2)

Blocking Staph A Cells:

1. Thaw 1 tube (100 µL each) of Staph A Cells for approximately every $5x10^7$ cells or one tube for four IPs to be used for ChIP analysis.
2. Add 10 µL of 10mg/mL salmon sperm DNA and 10 µL of 10mg/mL BSA for each 100 µL Staph A aliquot and mix by pipette
3. **For best results, incubate on rotating platform overnight at 4°C, or at 4°C for several (≥3 hrs) hours, (or 2 hrs at RT if time constraint)**
4. Transfer to 1.5 mL tube and microfuge at 14,000 rpm for 3' at 4°C and remove supernatant
5. Wash pellet by resuspending in 1 mL 1X DB – srk
6. Microcentrifuge at 14,000 rpm for 3' at 4°C and remove supernatant
7. Repeat 5 and 6
8. Resuspend the pellet in 100 µL of 1X DB – srk w/ 1mM PMSF (use 1 µL of 100mM PMSF)
9. Washed and blocked Staph A Cells can be stored at 4°C for up to 2 weeks

DAY 0:

A: Preparation of Cross-Linked Cells:

1. Wipe down bench and pipettes with Alconox
2. Cell cultures should be healthy and not density arrested prior to cross-linking, generally use 1 x 10^7 cells per antibody per ChIP (fewer cells, as low as 2 x 10^6 cells, can be used but may result in lower signal to noise ratio)
3. In fume hood, add formaldehyde (37% stock) directly to tissue culture media to a final concentration of 1%
4. In fume hood, incubate adherent cells on a shaking platform and suspension cells on a stir plate for 10' at RT (cross-linking for longer periods of ≥ 30' may cause cells to form aggregates that do not sonicate efficiently)
5. In fume hood, stop the cross-linking reaction by adding glycine to a final concentration of 0.125M continuing to rock/spin for 5' at RT (for adherent cells use 10X (1.25M) soln and for suspension cells add powdered glycine directly to flask)
6. Wash cells with 1X PBS (for adherent cells, pour off media and rinse plates twice with 1X PBS, after second rinse, stand plate up and let PBS run down and then dump again; for suspension cells, wash twice by centrifuging and resuspend in 1X PBS)
7. Optional: For adherent cultures add an appropriate volume of tissue culture trypsin (e.g. 10 mL per 500 cm^2 dish) to coat monolayer and incubate for 10' at 37°C (this step is useful for cells that are difficult to swell)
8. Scrape adherent cells from culture dish to 50 mL tube
9. Rinse the adherent cells culture dish with 30 mL PBS, add the remaining cells to the 50 mL tube and mix
10. Centrifuge adherent cells at 1000 rpm for 10' at 4°C
11. Carefully aspirate supernatant so as to not lose cells
12. Wash cells by resuspending pellet in 50 mL of 1X PBS (thoroughly resuspend and take a small aliquot for a cell count)
13. Pellet again and aspirate the supernatant
14. Cells can be used immediately for ChIP assay or snap frozen in liquid nitrogen and stored in liquid nitrogen or -80°C freezer indefinitely

The following steps will take you through a control ChIP experiment. We recommend that the first ChIP experiment that you do be a comparison of an antibody to PolII vs. an IgG control (ordering info at the end of protocol). We also recommend using the promoter of the gene for the large subunit of RNA polII for the positive control primer set (since this promoter is active in all cells) and the 3' UTR of the DHFR gene as a negative control primer set (primer sequences at end of protocol). All buffer & solution recipes also found at the end of protocol.

☆ Steps that may vary with cell type

DAY 1:

DATE:
TIME:

*****Washed/Blocked Staph A cells must be ready at this point*****

B. Preparation of Cross-Linked Chromatin and Antibody Incubation

Prepare everything on ice

☆1. Prepare Swelling Solution.
Use 1 mL per $5x10^7$ cells.

Cells: _____
Swelling Buffer volume to Prepare:
_____mL_

Prepare from 10X Stock Autoclaved solutions on ice:

a. Add appropriate amount of Mol. Bio. Grade H_2O
b. Use 10 x (1M) Tris pH 7.6 to make 1X (0.1M) Tris
c. Use 10X (100mM) KOAc & (150mM) MgOAc to make 1X (10mM) KOAc & (15mM) MgOAc
d. protease inhibitors final concentration:
   i. 1mM PMSF
   ii. 0.01mg/mL aprotinin (use aliquot for one day – do not refreeze)
   iii. 0.01mg/mL leupeptin

H_2O: _____µL_
10X (1M) Tris pH 7.6: _____µL_
10X (100mM) KOAc & (150mM) MgOAc: _____µL_
100mM PMSF:____µL_
10mg/mL Aprotinin: _____µL_
10mg/mL Leupeptin: _____µL_

2. Prepare Nuclei Lysis Buffer.
Use 1 mL per $1x10^8$ cells

Cells: _____
Nuclei Lysis Buffer volume: _____mL_

Prepare Nuclei Lysis Buffer from Stock solutions on ice:

a. Add appropriate amount of Mol. Bio. Grade H_2O
b. Use 1M Tris-Cl pH 8.0 to make 50mM Tris-Cl
c. Use 500mM EDTA pH 8.0 to make 10mM EDTA
d. Use 20% SDS to make 1% SDS
e. protease inhibitors final concentration:
   i. 1mM PMSF
   ii. 0.01mg/mL aprotinin (use aliquot for one day – do not refreeze)
   iii. 0.01mg/mL leupeptin

DEPC H_2O: _____µL_
1M Tris-Cl pH 8.0: _____µL_
500mM EDTA pH 8.0: _____µL_
20% SDS: _____µL_
100mM PMSF:____µL_
10mg/mL Aprotinin: _____µL_
10mg/mL Leupeptin: _____µL_

3. Resuspend cells with 1 mL per $5x10^7$ cells of prepared Swelling Buffer

Volume Swelling Buffer: _____mL_

☆4. Incubate on ice for 20' and flick occasionally to resuspend
☆5. Dounce cells on ice using a 2 mL B Dounce with 15 strokes to release nuclei & disperse cell clumps (If using larger volume, dounce only 1 mL at a time or use larger dounce)
6. Transfer sample to 1.5 mL tubes

☆ Steps that may vary with cell type

7. Microfuge at 2,500 x g for 5' at 4°C to pellet nuclei, pour off supernatant
8. Resuspend nuclei in 1 mL per $1x10^8$ cells of prepared Nuclei Lysis Solution (If sample was split into two tubes for douncing, use 1 mL to resuspend the first pellet, then transfer to second pellet and resuspend)

Volume Nuclei Lysis Buffer Solution:
_____mL_

9. Incubate on ice for 10'
10. Transfer samples to appropriate tubes for sonication – 15mL polystyrene tube are best (may split samples to avoid going over max volume for tubes to be sonicated)
    (See below for tube ranges)

Type of tube:
____mL polystyrene tube_
Volume of samples:
_____

11. Sonicate:
    a. **We use a Diagenode BioRuptor™ Sonicator** kept in a cold room
    b. Prepare samples with following volume range:
        i. 1.5 mL Tube (polystyrene recommended): 100 - 300 µL
        ii. 15 mL Tube (polystyrene!): 500 µL - 2 mL
    c. Wear ear protection and post signs on doorways stating "sonication is in progress and ear protection is required"
    d. Remove tube holder and check that water level is <u>at</u> blue "water level" mark
    e. Use appropriate tube holder and accessories (check Quick Reference Sheet) for your samples and rinse with ethanol before use
    f. Balance tubes in sonicator (similar to centrifuge)
    ☆ g. Input sonication settings: the pulse duration, intensity and number will vary depending on the extent of cross-linking and cell type, you must optimize for your expt. Ideally the least amount of input energy that gives satisfactory fragmentation should be used. We use as starting conditions: 4 pulses for 15 sec with 1 min rest interval = timer 5 min
        i. LMH dial should be set to High
        ii. Set Interval by adjusting red needle (sonication time, usually 15 sec) and the green needle (rest interval, 1 min standard to allow cooling)
        iii. Then set the Timer (only allows "On" = cycle, 5', 10' or 15', so keeping track with a stopwatch might be necessary for times in between)

LMH Dial: _H_
Sonicaton Time: ___x 15 sec_
Rest Interval: __min_
Timer: ___min__sec_

    h. Rinse off used tube holder and accessories with water and then ethanol when finished
    i. Run a gel to check sonication
        i. Use 10 µL sample and add 40 µL H_2O
        ii. Reverse cross-link by adding 2 µL of 5 M NaCl (0.2M NaCl)
        iii. Boil for 15'
        iv. After returning to RT, add 1 µL of 10 mg/mL RNase A at 37° for 10'
        v. Clean with QiaQuick PCR Purification Kit
            a. Add 250 µL PBI buffer to each sample
            b. Add 5 µL of 3M NaOAc pH ~5.0, light vortex
            c. Transfer to Qiagen column (purple)

☆ Steps that may vary with cell type

d. Centrifuge at max speed for 1' and discard flow through
e. Place column back in catch tube and add 750 µL PE buffer to each tube
f. Centrifuge at max speed for 1' and discard flow through
g. Reinsert column into catch tube and spin at max for 1' more
h. Label tube
i. Place column into a clean/labeled 1.5 mL tube, discard catch tube
j. Add 30 µL of EB to column membrane, let sit 2'
k. Centrifuge at max speed for 1' and discard column

vi. Load gel with 1 & 4 µL of sonicated sample

12. If using 15 mL tubes, transfer sonicated chromatin to 1.5 mL tubes
13. Microfuge samples (hinge facing outside) at 14,000 rpm for 10' at 4°C
14. Transfer supernatant to new 1.5 mL tube. (This sonicated chromatin can be frozen and used at a later time.)

Sample storage info:
Location =
Tube Label =

C. Immunoprecipitation of Cross-Linked Chromatin

1. Freshly add ~1 µL of 100mM PMSF per ~100 uL of blocked/washed Staph A cells prepared earlier

Volume B/W Staph A cells in aliquot: _____µL_
100mM PMSF: _____µL_

2. Preclear chromatin by adding blocked/washed Staph A cells w/PMSF (10 µL per 1x 10^7 cells)

Volume B/W Staph A w/PMSF added to each tube: _____µL_

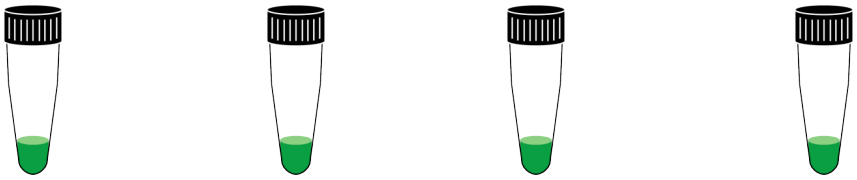
Do NOT throw out remaining Staph A Cells, you will need them for Day 2, Step D2

3. Incubate on rotating platform for no longer than 15' at 4°C
4. Microfuge at 14,000 rpm for 5' at 4°C
5. Transfer supernatant to new tube and measure volume

Volume measured: _____

6. Divide volume equally into 2 mL screw-cap tubes representing ~1x10^7 cells for each immunoprecipitation. Include an IgG negative control sample along with your experimental antibodies

Tube Names, volumes & # cells:



☆ Steps that may vary with cell type

7. Make double the total sample volumes of IP Dilution Buffer:
(_____sample volume) x 2 x (_2_ # Samples) = _____
with protease inhibitors, by adding:
   a. 10 µL/mL PMSF
   b. 1 µL per 1 mL of solution - aprotinin (use aliquot for one day – do not refreeze)
   c. 1 µL per 1 mL of solution - leupeptin

IP Dilution Buffer volume to prepare: _____
100mM PMSF:_____µL_ 10mg/mL Aprotinin: _____µL_ 10mg/mL Leupeptin: _____µL_

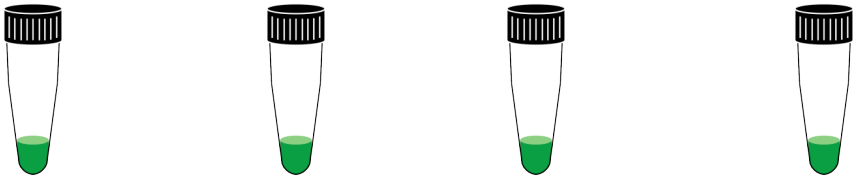
8. Add double the sample volume of prepared IP Dilution Solution to each sample

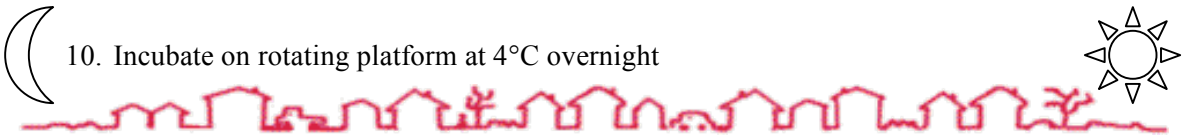
IP Dilution Solution volume added to each sample: _____

9. Add 1-2 µg of primary antibody to each sample (although the optimal amount for each antibody will need to be determined experimentally, 1 µg is a good starting point for most antibodies)

Record lot number of antibodies for future reference!

Tube names & 1º Ab info and 1º Ab amount:





10. Incubate on rotating platform at 4°C overnight

☆ Steps that may vary with cell type

DAY 2:

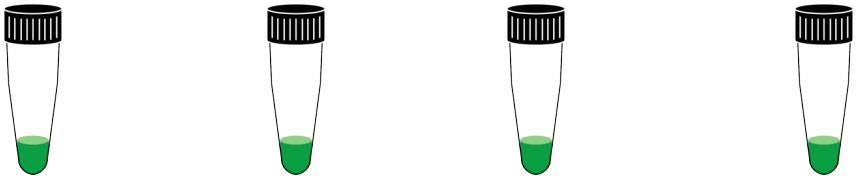
DATE:
TIME:

D. Washing and Cross-Link Reversal

Use 1.5 mL tubes for washing steps

1. If you are using monoclonal antibodies or a polyclonal antibody from a species other than rabbit, add an appropriate amount (typically 1-4 µg) of secondary antibody (e.g. rabbit antimouse for mouse monoclonals or rabbit antigoat for goat polyclonals). Staph A binds rabbit IgG Abs efficiently, therefore a secondary Ab is typically not required if using a rabbit polyclonal.

Tube names & 2º Ab info:



2. Incubate on rotating platform at 4°C for 1 hour.
3. Freshly add ~1 µL of 100mM PMSF per 100 uL of blocked/washed Staph A cells prepared earlier

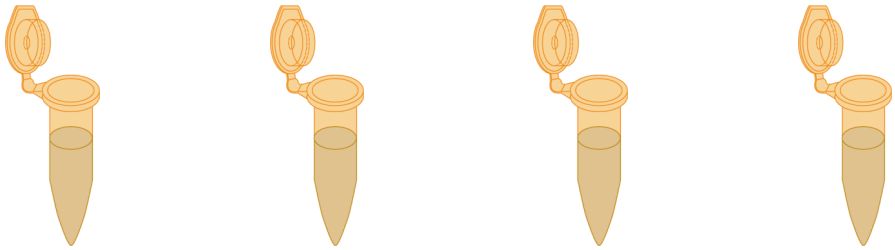
Volume B/W Staph A cells in aliquot: _____µL_
100mM PMSF: _____µL_

4. Add blocked/washed Staph A cells w/PMSF (10 µL per 1x 10^7 cells)

Volume B/W Staph A w/PMSF added to each tube: _____µL_

5. Incubate on rotating platform for no longer than 15' at RT
6. Transfer to new 1.5 mL tube

Tube names:



7. Microfuge samples at 14,000 rpm for 4' at 4°C
8. Save all of supernatant from IgG IP for "Input", and use later in reversing cross-links step D27

Input Info
Location:
Tube Label:

9. Pour off supernatant in other samples

☆ Steps that may vary with cell type

****Be sure to use appropriate monoclonal or polyclonal dialysis and wash buffers****

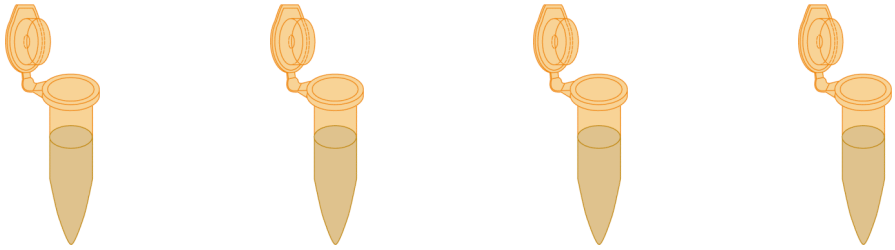
10. Wash pellets with 1 mL of 1X Dialysis Buffer (Add 10 µL of 100mM PMSF per 1 mL of buffer, does NOT contain aprotinin/leupeptin)
    (1 mL x 2 washes) x (____ # samples) = _____mL_

    Total volume 1X Dialysis Buffer prepared: _____mL_
    100 mM PMSF: ____µL_
11. Invert tube 20 times by hand at RT
12. Microfuge at 14,000 rpm for 4' at 4°C, pour off supernatant in labeled waste container
13. Repeat 9, 10 and 11 once more
14. Wash pellets with 1 mL of IP Wash Buffer (Add 10 µL of 100mM PMSF per 1 mL of buffer, does NOT contain aprotinin/leupeptin)
    (1 mL x 4 washes) x (____ # samples) = _____mL_

    Total volume IP Wash Buffer prepared: _____mL_
    100 mM PMSF: ____µL_
15. Invert tube 20 times by hand at RT
16. Microfuge at 14,000 rpm for 4' at 4°C
17. Repeat 13, 14 and 15 three more times, pour off supernatant in labeled waste container
18. Microfuge the pellet again orienting the pellet on outside and aspirate the last traces of buffer
19. Prepare IP Elution Buffer at RT (no inhibitors). Need 100 µL for each IP sample plus 100 µL for the "10% Input" sample:
    (50 µL x 2) x (____ # samples) + 100 µL = _____µL_

    Total volume IP EB (1% SDS and 50 mM $NaHCO_3$) to prepare: _____µL_
    dH2O: _____µL_
    20% SDS: _____µL_
    1M $NaHCO_3$: _____µL_
20. Elute antibody/protein/DNA complexes by adding 50 µL of IP Elution Buffer at RT (no inhibitors)
21. Shake on vortexer for 15' at setting 3 at RT
22. Microfuge at 14,000 rpm for 3' at RT
23. Remove supernatant to a new 1.5 mL tube

Tube names:



24. Repeat 19-22 on same pellet, combining supernatant in same new tube (100 µL total)
25. Microfuge samples again at 14,000 rpm from 5' to remove any traces of Staph A cells

☆ Steps that may vary with cell type

26. Transfer supernatant to new 0.5 mL tube

Tube names:

27. Add 4 µL of 5M NaCl (0.2M NaCl final) to each IP sample tube.
28. For "10% Input" use IgG supernatant saved earlier in step D7 & measure the volume. Take 10% of this volume and put in a properly labeled "10% Input" 0.5 mL tube. Bring the volume up to 100 µL with IP Elution Buffer. bring volume up to 100 µL with IP Elution Buffer, then add 4 µL of 5M NaCl (0.2M NaCl final).

_____µL is 10%
+ _____µL IP EB
= 100 µL of 0.1%

29. Add 4 µL of 5M NaCl (0.2M NaCl final) to this 100 µL sample of 10% Input.
30. Incubate all samples at 67°C for 4 hrs to overnight to reverse formaldehyde cross-links (recommended) or boil for 15' if time constraint and then freeze

☆ Steps that may vary with cell type

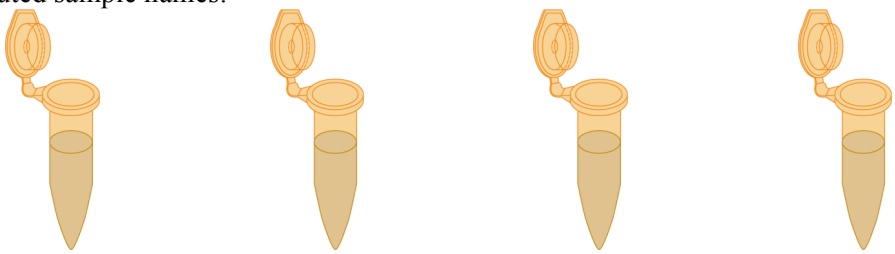
DAY 3:

DATE:
TIME:

E. Column Purification and PCR Analysis

1. Add 1 µL of 10mg/mL RNase A to each sample (including your "10% Input") and incubate for 30' at 37°C
2. Column purify each sample using Qiagen Qiaquick PCR purification Kit
   a. Transfer samples to labeled 1.5 mL tubes
   b. Add 500 µL PBI buffer to each sample
   c. Add 10 µL of 3M NaOAc pH 5-5.2 to make solution less basic, light vortex
   d. Transfer to Qiagen column (purple)
   e. Centrifuge at max speed for 1' and discard flow through
   f. Place column back in catch tube and add 750 µL PE buffer to each tube
   g. Centrifuge at max speed for 1' and discard flow through
   h. Reinsert column into catch tube and spin at max for 1' more
   i. Label 1.5 mL tubes
   j. Place column into a clean/labeled 1.5 mL tube, discard catch tube
   k. Add 50 µL of EB to column membrane, let sit 2'
   l. Centrifuge at max speed for 1' and discard column

Eluted sample names:



3. Use 1 uL of each ChIP sample for PCR reaction. Make 1:50 and 1:10 dilutions of 10% Input in Qiagen EB and use 1 µL for PCR analysis, as well as, 1 µL of undiluted (UD) 10% Input.

PCR Info for 20 (µL) RXNs:
+ Control = Pol2 Promoter
− Control = DHFR3' UTR

#	DNA (Ab specific) 1µL Each	Primer F/R (10 µM)	Volume Primer (µL)	Mol. Bio. Grade H_2O (µL)	10X Buffer (15mM $MgCl_2$)	BSA (µL)	4 mM dNTPs (µL)	Applied Biosystems Amplitaq (µL)
1	Pol2	+ Control	1	13.75	2	1	1	0.25
2	IgG	+ Control	1	13.75	2	1	1	0.25
3	(1:50) Input	+ Control	1	13.75	2	1	1	0.25
4	(1:10) Input	+ Control	1	13.75	2	1	1	0.25
5	UD Input	+ Control	1	13.75	2	1	1	0.25
6	Pol2	- Control	1	13.75	2	1	1	0.25
7	IgG	- Control	1	13.75	2	1	1	0.25
8	(1:50) Input	- Control	1	13.75	2	1	1	0.25
9	(1:10) Input	- Control	1	13.75	2	1	1	0.25
10	UD Input	- Control	1	13.75	2	1	1	0.25

151.25 µL + 22 µL + 11 µL + 11 µL + 2.75 µL
= 189 µL
➜18 µL for each sample

PCR Cycling Conditions:

95° 3'
95° 30"
60° 30"
72° 1'
} x33
10° ∞

☆ Steps that may vary with cell type

SOLUTIONS:

Dialysis Buffer
2 mM EDTA
50 mM Tris-Cl pH 8.0
0.2% Sarkosyl (omit for monoclonal antibodies)

Swelling Buffer (used in this protocol) Made 10X Tris and 10X KOAc/MgOAc and Autoclaved
0.1 M Tris pH 7.6
10 mM KOAc
15 mM MgOAc
Before use, add protease inhibitors

Swelling Buffer (alternative) 5 mM PIPES pH 8.0
85 mM KCl
Before use, add 1% NP40 and protease inhibitors

Nuclei Lysis Buffer
50 mM Tris-Cl pH 8.0
10 mM EDTA
1% SDS
Before use add protease inhibitors

IP Dilution Buffer
0.01% SDS
1.1% Triton X 100
1.2 mM EDTA
16.7 mM Tris-Cl pH 8.0
167 mM NaCl
Before use add PMSF

IP Wash Buffer
(use stated Deoxycholic Acid, Sodium Salt; this product from other companies can have a hard time going into solution)
100 mM Tris-Cl pH 9.0 (8.0 for monoclonal antibodies)
500 mM LiCl
1% Igepal (aka NP40)
1% Deoxycholic Acid, Sodium Salt (Fisher Scientific MW 414.5 Cat. # BP349-100)
Before use add PMSF

IP Elution Buffer – Made fresh from 1M $NaHCO_3$ and 20% SDS
50 mM $NaHCO_3$
1% SDS

☆ Steps that may vary with cell type

Protease Inhibitors – PREPARE WITH CAUTION
100 mM PMSF in ethanol, use at 1:100, use mask when weighing out or use fume hood (prepared 100 µL aliquots)
10 mg/mL aprotinin in 0.01 M HEPES pH 8.0, use at 1:1000 (prepared 5 µL aliquots)
10 mg/mL leupeptin in water, use at 1:1000 (prepared 5 µL aliquots)

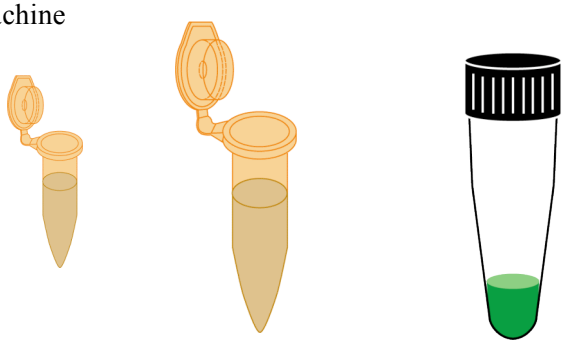
Primers Info

+ Control Pol2 F	5' AGATGAAACCGTTGTCCAAACT 3'
+ Control Pol2 R	5' AGGTTACGGCAGTTTGTCTCTC 3'
- Control DHFR3' UTR F	5' CTGATGTCCAGGAGGAGAAAGG 3'
- Control DHFR3' UTR R	5' AGCCCGACAATGTCAAGGACTG 3'

Antibodies Info
IgG from Rabbit Serum, Sigma Cat#15006-10MG
Rabbit Anti-Mouse IgG, MP Biomedical Cat#55436
RNA Polymerase II 8WG16 Monoclonal Antibody, Covance Cat#MMS-126R

EQUIPMENT/MATERIALS

Knotes Dounce Tissue Grinder 2 mL Capacity, VWR# KT885300-0002
Refrigerated Microcentrifuge/Centrifuge with 1.5/2.0 mL Tube Rotor
Rotator
PCR Machine



☆ Steps that may vary with cell type