

Supplemental Information

Probiotics Stimulate Bone Formation in Obese Mice via Histone Methylations

Jyotirmaya Behera¹, Jessica Ison¹, Michael J. Voor^{2,3}, Neetu Tyagi^{1*}

¹Bone Biology Laboratory, Department of Physiology, School of Medicine, University of Louisville, Louisville, KY 40202 USA

²Department of Orthopaedic Surgery, School of Medicine, University of Louisville, Louisville, KY 40202 USA

³Department of Bioengineering, Speed School of Engineering, University of Louisville, Louisville, KY 40202 USA

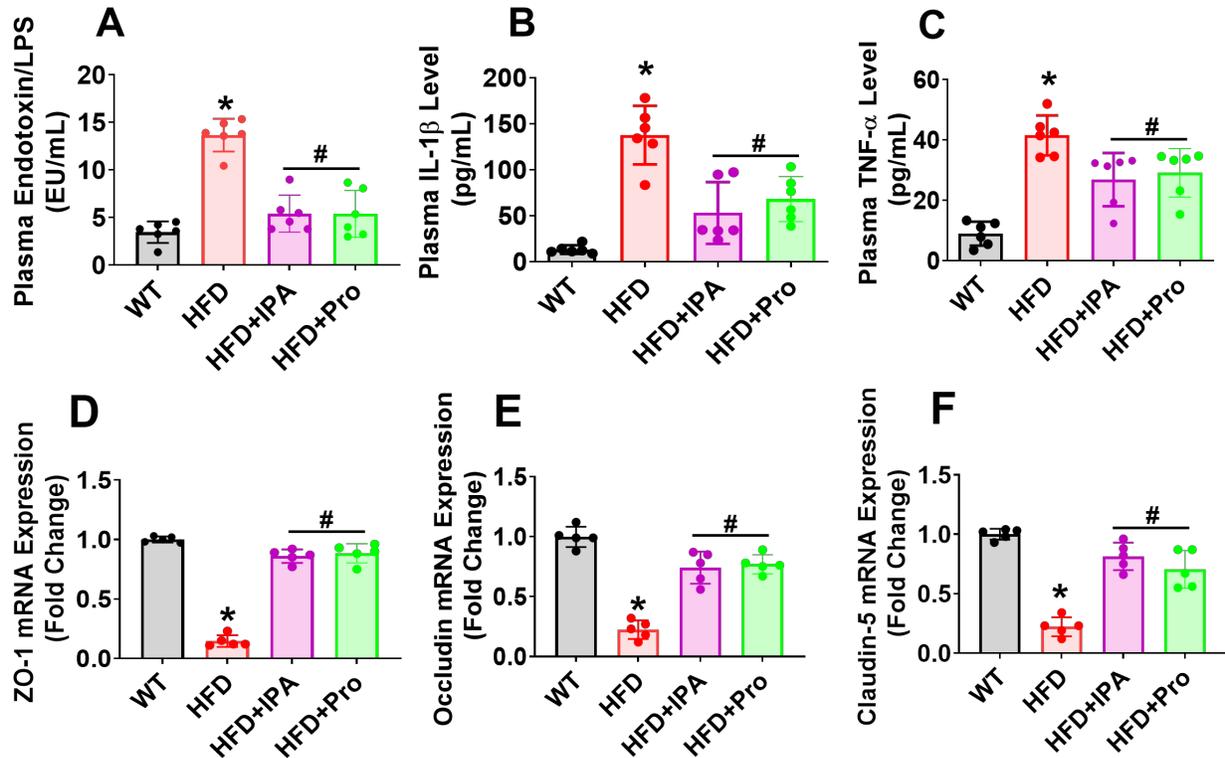
Running title: Gut Microbiota Manipulation Promotes Bone Formation in Obese Mice via Histone Methylations

***Address for Correspondence:**

Neetu Tyagi, Ph.D., FAPS
Associate Professor
Bone Biology Laboratory
Department of Physiology
Health Sciences Centre, A-1201,
University of Louisville
Louisville, KY 40202
Phone: 502-852-4145
Fax: 502-852-6239
E-mail: n0tyag01@louisville.edu

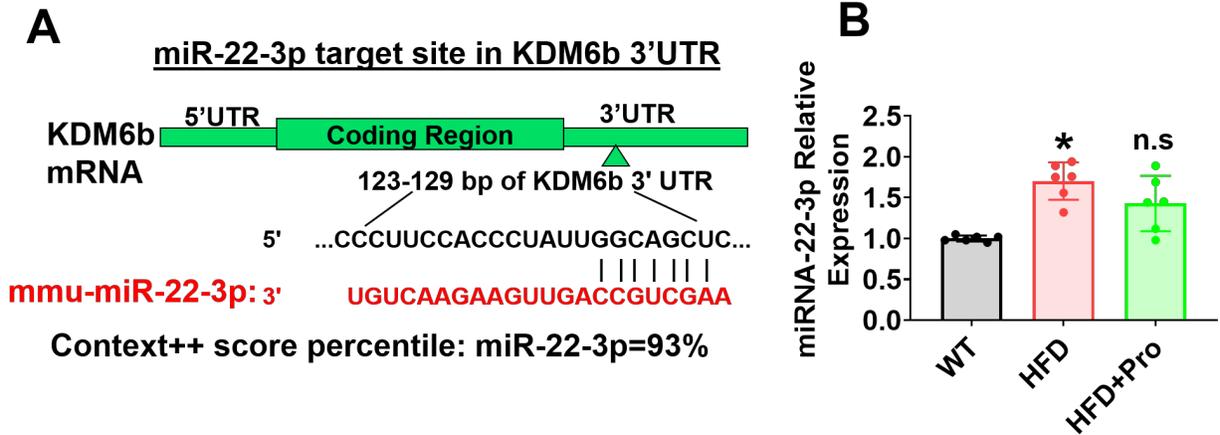
Supplementary Figure.

Supplementary Figure S1



Supplementary Figure S1. Effect of probiotics or IPA treatment on endotoxin and inflammatory cytokine level in HFD mice. **(A)** The plasma endotoxin level was detected using the Limulus Amebocyte Lysate kit. * $p < 0.0001$ compared with the WT control, # $p < 0.0001$ compared with the HFD, by one-way ANOVA followed by a Tukey's multiple comparisons tests. **(B)** Plasma IL-1 β levels were measured using ELISA. * $p < 0.0001$ compared with the WT control, # $p < 0.0001$ (HFD+IPA), # $p < 0.001$ (HFD+Pro) compared with the HFD, by one-way ANOVA followed by a Tukey's multiple comparisons test. **(C)** Plasma TNF- α level was measured using ELISA. * $p < 0.0001$ compared with the WT control, # $p < 0.0095$ (HFD+IPA), # $p < 0.0318$ (HFD+Pro) compared with the HFD. **(D-F)** qPCR analysis of mRNA transcript expression of tight junctions' proteins (ZO-1, Occludin, and Claudin-5) in the experimental group. * $p < 0.0001$ compared with the WT control, # $p < 0.0001$ compared with the HFD for fig. S1D-F, by one-way ANOVA, followed by a Tukey's multiple comparisons test. All data are expressed as mean \pm s.e.m. $n = 5-6$ mice per group. The error bars represent the s.e.m.

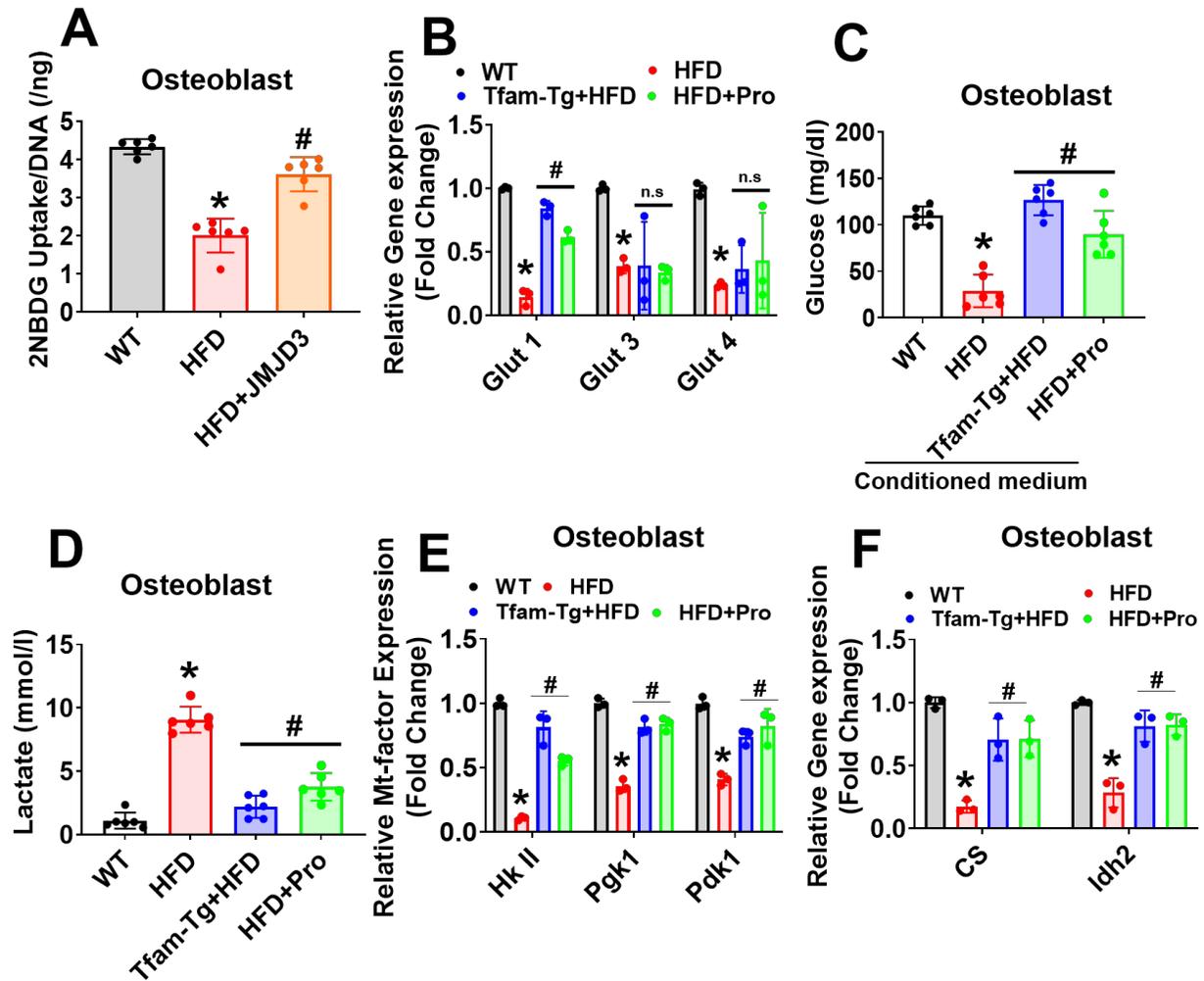
Supplementary Figure S2



Supplementary Figure S2. The prediction of the miR-22 binding site and its expression.

(a) Schematic representation of the miR-22-3p binding sites in histone demethylase, Kdm6b/Jmjd3. (b) miR-22-3p expression in osteoblast. * $p < 0.0003$ compared with the WT control and $n.s.p < 0.1458$ compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. $n = 6$ mice per group. All data are expressed as mean \pm s.e.m. The error bars represent the s.e.m.

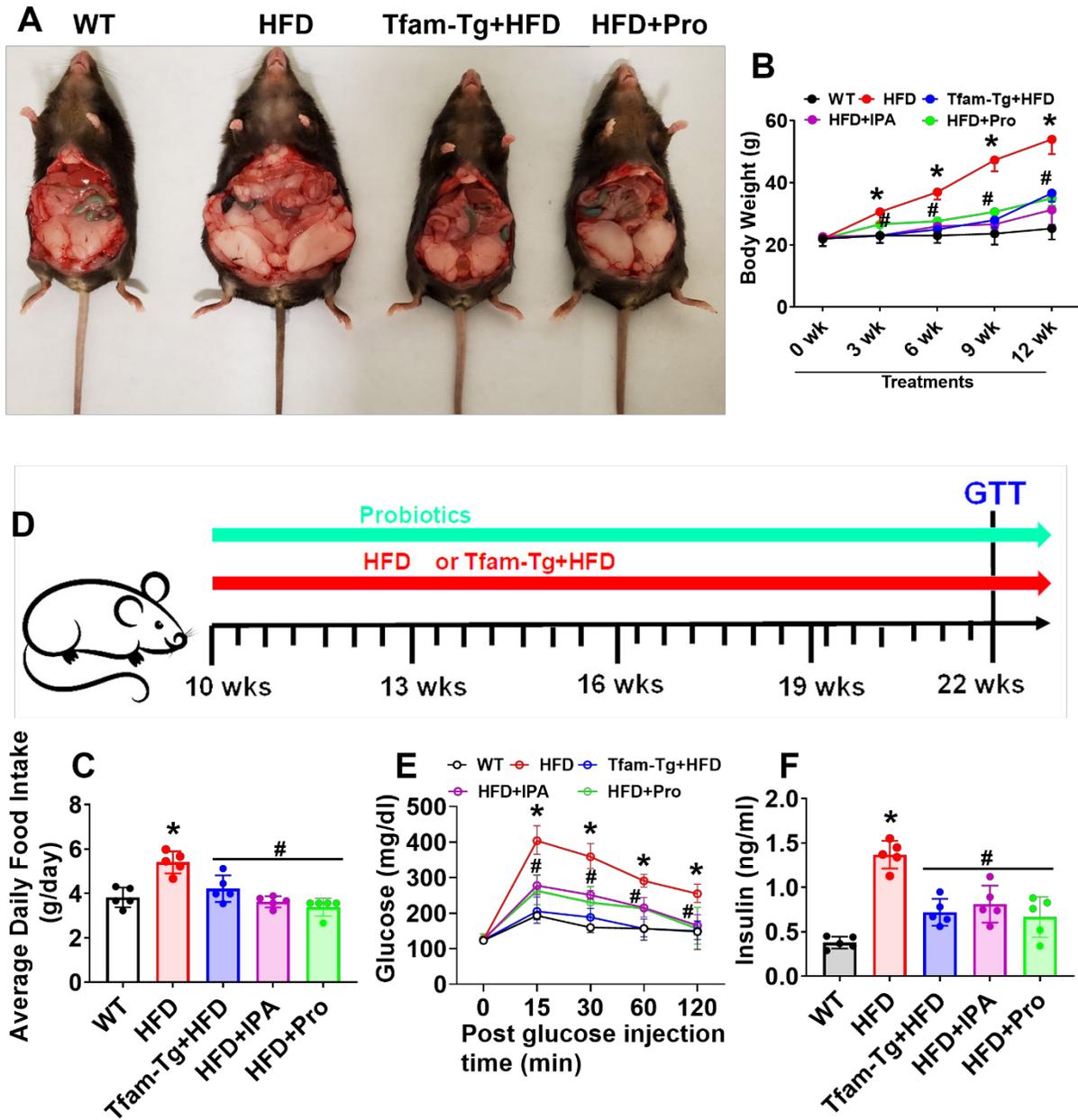
Supplementary Figure S3



Supplementary Figure S3. Effect of probiotics on osteoblast and glycolytic response. (a) 2-NBDG uptake in cultured osteoblast upon JMJD3/Kdm6b overexpression (normalized for DNA content of the well, $n = 6$). * $p < 0.0001$ compared with the WT control, # $p < 0.0001$ compared with the HFD. (b) mRNA transcript expression of Glut 1, 3, 4 expression in femoral tissue of various experimental conditions. * $p < 0.0001$ compared with the WT control and # $p < 0.0109$ (HFD+Pro), # $p < 0.0001$ (Tfam-Tg+HFD) compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. N.s denotes not significant. (c-d) Glucose and lactate level was estimated in osteoblast lysate and culture supernatants, respectively. * $p < 0.0001$ compared with the WT control and # $p < 0.0001$ compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. $n = 6$ mice per group. (e) mRNA transcript expression of glycolytic enzymes (Hk II, Pdk 1) by qPCR assay. * $p < 0.0001$ compared with the WT control and # $p < 0.0001$ compared with the HFD by one-way ANOVA followed by a

Tukey's multiple comparisons test. **(f)** TCA cycle enzyme Cs and Idh2 mRNA transcript expression were analyzed by the qPCR assay. * $p < 0.0001$ compared with the WT control and # $p < 0.0001$ compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. All data are expressed as mean \pm s.e.m. $n = 3$ mice per group. The error bars represent the s.e.m.

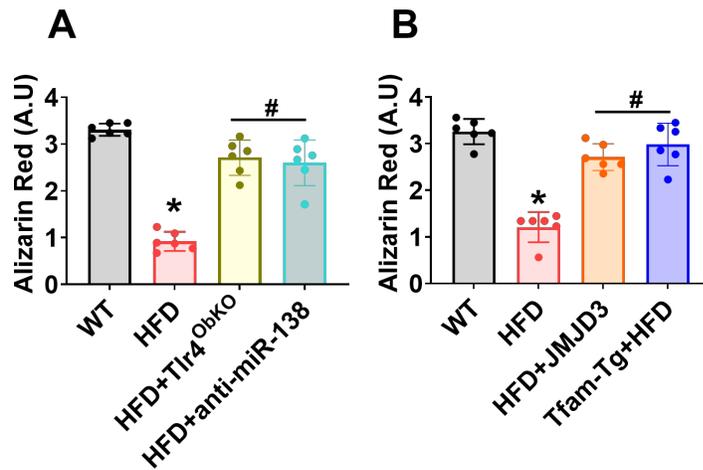
Supplementary Figure S4



Supplementary Figure S4. Tfam overexpression or probiotics supplementation prevents HFD-induced obesity and impaired glucose tolerance. (a) Photograph of representative mice of various experimental conditions. (b) Bodyweight monitored throughout the treatments (starting from 0 wks to 12 wks). * $p < 0.001$ compared with the WT control and # $p < 0.001$ compared with the HFD by two-way ANOVA followed by a Tukey's multiple comparisons test. (c) Average daily food intake by mice during 8 weeks of treatments. * $p < 0.0002$ compared with

the WT control and #p < 0.0001 (HFD+IPA or HFD+Pro), #p < 0.0041 (Tfam-Tg+HFD) compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. **(d-e)** Glucose tolerance test (GTT) after 8 weeks of diet or probiotics or IPA supplementation by using an i.p. the dose of 1 g of glucose per kg of body weight. *p < 0.01 compared with the WT control and #p < 0.01 compared with the HFD by two-way ANOVA followed by a Tukey's multiple comparisons test. **(f)** Plasma insulin level was measured in the experimental mice. *p < 0.0001 compared with the WT control and #p < 0.0001 (Tfam-Tg+HFD), #p = 0.0004 (HFD+IPA) compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. All data are expressed as mean ± s.e.m. The error bars represent the s.e.m.

Supplementary Figure S5



Supplementary Figure S5. Restoring the osteoblast mineralization using Tfam overexpression. (a) Effect of Tlr4 knockout and miR-138 inhibition (anti-miR-138) on Osteoblast mineralization, were performed using alizarin red assay (ARS). * $p < 0.0001$ compared with the WT control and # $p < 0.0001$ compared with the HFD by two-way ANOVA followed by a Tukey's multiple comparisons test. All data are expressed as mean \pm s.e.m. (b) Effect of JMJD3 and Tfam overexpression on Osteoblast mineralization was demonstrated using ARS assay. * $p < 0.0001$ compared with the WT control and # $p < 0.0001$ compared with the HFD by one-way ANOVA followed by a Tukey's multiple comparisons test. All data are expressed as mean \pm s.e.m. The error bars represent the s.e.m.

Supplementary Table S1: Sequences of primers used for real-time quantitative PCR

| Gene Name | Primer sequence 5'-3' |
|---------------------|--|
| Runx2 | FP: TTTAGGGCGCATTCTCATC RP: TGCCTTGTGGATTGAAAGGAC |
| Bglap | FP:GCGCTCTGTCTCTCTGACCT RP: ACCTTATTGCCCTCCTGCTT |
| Alpl | FP: CCAGAAAGACACCTTGACTGTGG RP: TCTTGTCCGTGTCGCTCACCAT |
| Col1a1 | FP: CCTCAGGGTATTGCTGGACAAC RP: CAGAAGGACCTTGTTTGCCAGG |
| Spp1 | FP: GACAACAACGGAAAGGGCAG RP: GATCGGCACTCTCCTGGCT |
| Ctsb | FP:GGATGAAATCTCTCGGCGTTT RP:GGTTATGGGCAGAGATTTGCTT |
| Nfatc1 | FP:GAGACAGACATCCGGAGGAAGA RP:GTGGGATGTGAACACGGAAGA |
| RANK | FP:ACTGAGGAGGCCACCCAAGGA RP:TGAAGAGGACCAGAACGATGAG |
| Hk 2 | FP: TGATCGCCTGCTTATTCACGG RP: AACCGCCTAGAAATCTCCAGA |
| Pdk1 | FP: CCCCATTTCAGGTTTACAG RP: CCCGGTCACTCATCTTCACA |
| Pgk1 | FP: GGAGCGGGTTCGTGATGA RP: GCCTTGATCCTTTGGTTGTTTG |
| Glut 4 | FP: CCAGCCACGTTGCATTGTA RP: AACTGGTCCTAGCTGTATTCT |
| Glut 3 | FP:TGGTAGCTCAGATCTTTGGTTTGG RP:GATCTCTGTAGCTTGGTCTTCCTC |
| Glut 1 | FP: GGGCATGTGCTTCCAGTATGT RP: ACGAGGAGCACCGTGAAGAT |
| mtTFA | FP: GGAGGCAAAGGATGATTCCG RP: TCGTCCAACCTCAGCCATCT |
| Sdha | FP: GAGATACGCACCTGTTGCCAAG RP: GGTAGACGTGATCTTTCTCAGGG |
| GAPDH | FP: TGCACCACCAACTGCTTGC RP: GGCATGGACTGTAGTCAGAG |
| miRNA-138-3p | FP: AGCTGGTGTGTGAATCAGGCCG RP: GCCTGATTCACAACACCAGCTTT |
| U6snRNA | FP:CTCGCTTCGGCAGCACA RP:AACGCTTCACGAATTTGCGT |
| ChIP Primers | |
| mtTFA | FP:CAGTCCATAGGCACCGTATTG RP:CAAGGCAGAAGGAGAGCG |

Footnotes: Runt-related transcription factor 2 (RUNX2), bone gamma-carboxyglutamic acid-containing protein (Bglap), Mitochondrial transcription factor A (mtTFA or Tfam).

Supplementary Table S2: Primary antibodies and mitochondrial specific probes used

| Antibody | Clone | Dilution | Source | Cat. No |
|--|------------------------|-----------------|-----------------------------|----------------|
| Western blot | | | | |
| Rabbit Anti-RUNX2 antibody | polyclonal | 1:250 | Abcam | ab23981 |
| Rabbit Anti-Osteocalcin antibody | polyclonal | 1:250 | Abcam | ab93876 |
| Rabbit anti-Kdm6b/Jmjd3 Antibody | polyclonal | 1:500 | Abcam | ab169197 |
| Rabbit anti-Tfam Antibody | polyclonal | 1:250 | Abcam | ab131607 |
| Mouse anti-GAPDH Antibody | Monoclonal, clone 6C5 | 1:500 | Millipore | CB1001 |
| Mouse anti-H3K27me3 Antibody | Monoclonal, ChIP grade | 1:250 | Abcam | ab6002 |
| Rabbit anti-H3 Antibody | polyclonal | 1:500 | Abcam | ab18521 |
| Flow cytometry and immunofluorescence | Indicator | Dilution | Source | Cat. No |
| MitoTracker™ Green FM | Mitochondria imaging | 100 nM | Invitrogen Molecular Probes | M7514 |