Methylated DNA Targeted Immunoprecipitation Kit – KOA0884 and KOA0885

The Methylated DNA IP kit is designed to immunoprecipitate methylated DNA (Methyl DNA IP). This Methyl DNA IP method provides you with methylated DNA (meDNA) and unmethylated DNA (unDNA) controls to be used together with your DNA sample allowing direct CORRELATION between IP'd MATERIAL and METHYLATION STATUS. This methylation analysis is FAST, HIGHLY SPECIFIC and each IP is QUALITY controlled: essential keys for RELIABLE results.

I. KIT COMPONENTS

Methylated DNA IP Kit				
Component	**Catalog Number**	**Size**		
		(X10) KOA0884	**(X48) KOA0885**	**Storage**
Magnetic Beads	KOA0884A	150µl	750µl	4°C Do NOT Freeze
Bead Wash Buffer A	KOA0884B	2mL	10mL	4°C
Bead Wash Buffer B	KOA0884C	100µl	500µl	4°C
Bead Wash Buffer C	KOA0884D	40µl	200µl	-20°C
Anti-5mC Antibody (5-methylcytosine)	KOA0884E	5µl	20µl	-80°C avoid freeze thaw cycles
Positive Control - methylated DNA	KOA0884F	20µl	20µl	-20°C
Negative Control unmethylated DNA	KOA0884G	20µl	20µl	-20°C
Bead Wash Buffer One	KOA0884H	6mL	30mL	4°C
Bead Wash Buffer Two	KOA0884I	4mL	20mL	4°C
DNA isolation Buffer (DIB)	KOA0884J	4mL	20mL	4°C
Proteinase K Solution (100x)	KOA0884K	40µl	200µl	-20°C
Control One methylated DNA primer pair	KOA0884L	50µl	50µl	-20°C
Control Two unmethylated DNA primer pair	KOA0884M	50µl	50µl	-20°C
hTSH2B unmethylated DNA primer pair	KOA0884N	50µl	50µl	-20°C
hGAPDH unmethylated DNA primer pair	KOA0884O	50µl	50µl	-20°C
Water	KOA0884P	2mL	10mL	4°C
200µl tubes	KOA0884Q	2	8	Ambient
Cap strips	KOA0884R	2	8	Ambient

DNA Kit				
Component	**Catalog Number**	**Size**		**Storage**
		(X10) KOA0884	**(X48) KOA0885**	
Digestion Buffer	KOA0884S	3mL	3mL	4°C
Proteinase K (200x)	KOA0884T	300µg/15µl	300µg/15µl	-20°C
Precipitant Solution	KOA0884U	3mL	3mL	4°C
TE Buffer	KOA0884V	3mL	3mL	4°C
RNAse (DNAse free)	KOA0884W	5µg/10µl	5µg/10µl	-20°C

II. STORAGE AND STABILITY CONDITIONS

Store the components at the indicated temperature upon receipt. Store Protein-A Coated Magnetic Beads and Magnetic Beads at 4°C. DO NOT FREEZE magnetic beads because they may become damaged. Keep the beads in liquid suspension during storage as drying will result in reduced performance.

III. INTRODUCTION

The Methylated DNA targeted kit protocol has been improved to allow researchers to work in smaller tubes than traditionally did so far. The kit ensures the use of low amount of reagents per reaction (not only antibodies, but also buffers). The kit also includes fewer buffers in comparison with other kits. This makes this kit simpler and economical. The number of steps is reduced and handling is easier with our Magnetic Methyl DNA IP procedure. Two IP kit formats are available: one for a minimum of 10 IPs and another for a minimum of 48 IPs. In addition, the flexibility of the protocol has also been increased, as the IP'd DNA can be isolated and/or purified in different ways based on the downstream application: an extra-fast and simplified protocol is included in the IP kit (for qPCR analysis); but an additional method, (for subsequent sequencing analysis) is also proposed. Moreover, use of a magnetic rack together with our new IP kit protocol ensures the best IP conditions by working at a constantly cooler temperature. There are Magnetic Racks available that have been designed to be used in IP experiments, keeping samples cool longer and allowing the use of small tubes to reduce the reaction volumes and waste of reagents. The kit protocol also includes more flexibility at the DNA isolation/purification stage, so that the isolated DNA can be analyzed by all sorts of subsequent techniques. In addition, several modules are available for upstream and downstream experiments. The Methylated DNA IP kit protocol has been validated with the Bioruptor®. Nevertheless, DNA can be sheared with any in house protocol and sonication apparatus as long as efficiency is checked before use.

IV. REQUIRED EQUIPMENT AND REAGENTS

Equipment and reagents:

- Gloves to wear at all steps
- Autoclaved tips
- RNase/DNase-free 1.5mL tubes
- PCR tubes and reagents
- Magnetic rack (for 1.5mL microfuge tubes) (part number: **TMS-06** or **TMS-32**)
- Polymethylpentene microfuge tubes (1.5mL)

- Refrigerated centrifuge for 1.5mL, 15mL and 50mL tubes Cell counter
- Rotator (Rotating wheel)
- Sonication device
- Vortex
- Thermomixer
- Quantitative PCR facilities
- Trypsin-EDTA
- Ice cold PBS buffer (part number: **MB-008**)
- phenol/chloroform/isoamyl alcohol
- 100% ethanol
- Reagent and equipment for agarose gel electrophoresis
- Tubes: 1.5mL and 50mL
- Centrifuges: for 1.5mL and 50 mL tubes
- Cell counter
- sonicator

V. KIT METHOD OVERVIEW AND TIME TABLE

IP Protocol Overview			
Step	**Description**	**Time needed**	**Day**
1	Cell collection and lysis	40 min + overnight incubation	1
2	DNA Extraction	Few hours	2
3	Shearing	20 minutes	2
4	Methylated immunoprecipitation	4 hours or overnight	2
5	Washes	30 minutes	2-3
6	DNA isolation	30 minutes or 5 hours	2-3
7	qPCR	3 hours	2-3

VI. SHORT PROTOCOL FOR EXPERIENCED USERS

Step 1. Cell collection and lysis

1. Pellet suspension culture out of its serum-containing medium. Trypsinize adherent cells and collect cells from the flask. Centrifuge at 300 g for 5 minutes at 4°C.
2. Discard the supernatant. Resuspend cells in 5 to 10mL ice-cold PBS. Centrifuge at 300 g for 5 minutes. Discard the supernatant. Repeat this resuspension and centrifugation step once more.
3. Add **Proteinase K (200x) - KOA0884T** (5µl) to the **Digestion Buffer - KOA0884S** (1mL) to make Complete Digestion buffer.

4. Resuspend cells in complete Digestion buffer (1.5 million cells/ 500µl).
5. Cell Lysis: Incubate the samples with shaking at 50°C for 12 to 18 hours in tightly capped tubes.

Step 2. Nucleic acid extraction and DNA purification

1. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol. Incubate samples 10 minutes at room temperature on a rotating wheel.
2. Centrifuge at 1700 g for 10 minutes.
3. Transfer the aqueous (top) layer to a new tube.
4. Thoroughly extract the samples with an equal volume of chloroform/isoamyl alcohol.

   Incubate samples 10 minutes at room temperature on a rotating wheel.
5. Centrifuge at 1700 g for 10 minutes.
6. Transfer the aqueous (top) layer to a new tube.
7. Add 1/2 volume of **Precipitant Solution- KOA0884U** and 2 volumes of 100% ethanol.
8. Recover DNA by centrifugation at 1700 g for 5 minutes.
9. Rinse the pellet with 70% ethanol. Decant ethanol and air-dry the pellet.
10. Resuspend the pellet of DNA at ~1 mg/mL in **TE Buffer- KOA0884V** until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C.
11. Residual RNA has to be removed at this step by adding 2µl of **RNAse (DNAse free) - KOA0884W** per mL of DNA sample and by incubating 1 hour at 37°C, followed by phenol/chloroform extraction and ethanol precipitation (similar to points above).
12. For DNA analysis, run samples in a 1% agarose gel along with DNA size marker to visualize the DNA preparation efficiency.

Step 3. DNA shearing

1. In a 1.5-mL tube, dilute the DNA sample in **TE Buffer- KOA0884V** to reach 0.1g/µl.
2. Use a final volume of 300 l of DNA sample (30 g) in 1.5mL tubes.
3. Shear the DNA using a sonicator:
   - Bioruptor® Pico: 6 cycles of 30 sec ON / 90 sec OFF (1.5mL microtubes with caps)
   - other models: 10 cycles of 15 sec ON / 15 sec OFF; Low Power
4. Sheared DNA can be analyzed on agarose gel.

Step 4. Methylated DNA immunoprecipitation

Bead preparation

1. Prepare the bead wash Buffer by diluting 1:5 the **Bead Wash Buffer A - KOA0884B**. (100µl/ IP).
2. Resuspend provided **Magnetic Beads - KOA0884A**, transfer the amount of **Magnetic Beads - KOA0884A** needed into a new tube (11µl beads / IP).
3. Place the tube on a magnet or centrifuge to remove of the supernatant. Keep the beads.
4. Wash **Magnetic Beads - KOA0884A** with ice-cold bead wash Buffer as follows: add Buffer (22µl / IP), and resuspend the beads. Centrifuge for 5 minutes at 1,300 rpm (If volume is small, use the magnetic rack instead), discard the supernatant and keep the bead pellet.

5. After washing, resuspend the beads in 22µl / IP of bead wash Buffer. Keep on ice.
6. Prepare IP incubation mix (1 sample + 1 input) as described in the table.

IP mix preparation

IP Incubation Mix		
Components	**Volume per 1 IP + input**	**Volume per 2 IP + input**
Water – KOA0884P	45µl	90µl
Bead Wash Buffer A - KOA0884B	24µl	48µl
Bead Wash Buffer B - KOA0884C	6µl	12µl
Positive Control - methylated DNA - KOA0884F	1.5µl	3µl
Negative Control unmethylated DNA - KOA0884G	1.5µl	3µl
DNA Sample	12µl	24µl
Total Volume	90µl	180µl

7. Incubate the IP mix at 95ºC for 3 minutes.
8. Quickly chill on ice (it is best to use ice-water).
9. Quickly perform a short spin at 4°C.
10. Aliquot per tube (200µl - 8 tube strip) 75µl IP incubation mix per IP. Add 7.5µl of IP incubation mix (input) to a new tube. Input samples will be stored at 4°C until DNA isolation (STEP 6).
11. In a new tube, dilute the provided antibody 1:2 using water. With 2µl you can perform up to 6 IPs.
12. Then use the 1:2 freshly diluted antibody to prepare the Diluted Antibody mix as shown in the table below.
    - Add to the antibody, the **Bead Wash Buffer A - KOA0884B** and water first. Add the **Wash Buffer C - KOA0884D** afterwards.

Diluted Antibody Mix					
Components	**1 IP**	**2 IPs**	**4 IPs**	**6 IPs**	**8 IPs**
Antibody 1:2	0.30µl	0.75µl	1.50µl	2.0µl	3.0µl
Bead Wash Buffer A - KOA0884B	0.60µl	1.5µl	3.0µl	4.0µl	6.0µl
Water – KOA0884P	2.1µl	5.25µl	10.5µl	14.0µl	21.0µl
Wash Buffer C - KOA0884D	2.0µl	5.0µl	10.0µl	13.0µl	20.0µl
Final Volume	5.0µl	12.5µl	25.0µl	33.0µl	50.0µl

13. Add 5µl of Diluted Antibody Mix per IP tube.
14. Mix and add the 20µl of washed beads to each 200µl-IP tubes.
15. Place on a rotating wheel at 4ºC for 4 hours or overnight.

Step 5. Washes

1. Place the Magnetic Bead Wash buffers and Magnetic Rack on ice.
2. Spin down and place the IP tubes in the ice-cold Magnetic Rack and work on ice, wait 1 minute and discard the buffer.
3. Wash the DNA IP Samples three times with 100µl ice-cold **Bead Wash Buffer One - KOA0884H**.
4. Wash the beads with 100µl ice-cold **Bead Wash Buffer Two - KOA0884I**.
5. After the last wash, discard the last traces of Wash buffer (using a P200 pipet). Keep the bead pellets on ice and proceed to the next step.
    - Avoid drying of beads!

Step 6. DNA isolation

1. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.
2. Prepare 100µl complete buffer DNA Isolation Buffer (DIB) per sample as follows. Add 1µl of **Proteinase K Solution - KOA0884K** per 100µl of **DNA Isolation Buffer - KOA0884J**. Scale accordingly knowing that 100µl are needed per IP'd DNA sample and 92.5µl, per input DNA sample.
3. Remove the tubes from the Magnetic Rack and add 100µl of Complete DIB per IP'd DNA sample. Resuspend the beads and transfer the suspension into 1.5mL tubes.
4. Add 92.5µl of Complete DIB to 7.5µl of input DNA sample.
5. Incubate at 55°C for 15 minutes both IP'd DNA sample and input DNA sample.
6. Incubate at 100°C for 15 minutes all the samples.
7. Spin down and place the 1.5mL tubes into a magnetic rack for 1.5mL tubes or centrifuge at 14,000 rpm for 5 minutes at 4°C.
8. Transfer the supernatants into new labeled tubes. That is the DNA ready for qPCR analysis. Make aliquots if necessary and store at -20°C. For alternative purification methods refer to page 22.

Step 7. qPCR analysis of IP'd DNA

1. Use the purified DNA from Methyl DNA IPs and DNA input. From the 100µl of isolated DNA, use 5µl per PCR.
2. Prepare your qPCR mix using SYBR PCR Green master mix. qPCR mix (total reaction volume is 25µl: 1.00µl of provided primer pair (stock: 10µM each: reverse and forward), 12.50µl of master mix (e.g.: iQ SYBR Green supermix), 5.00µl of diluted purified DNA sample (see above for DNA dilutions) and 6.50µl of water.
3. PCR cycles: amplification:

qPCR Cycles			
	Temperature	**Time**	**Cycles**
PCR Amplification	95°C	7 minutes	X1
	95°C	15 seconds	X40
	60°C	60 seconds	X40
	95°C	1 minute	X1
Melting Curve	65°C and increment of 0.5°C per cycle	1 minute	X60

VII. DETAILED PROTOCOL

Starting material cell culture

Each Methyl DNA IP assay requires 1µg of DNA; scale accordingly

- The DNA module provides you with an excess of buffer for the preparation of DNA. Sufficient buffer is given for the preparation of several genomic DNA batches, each obtained from 1 to 3x 10e6 cultured cells (see DNA STEP 1, and scale accordingly based on your starting material).
- From about 3 million cells, 20 to 30µg of DNA can be expected.
- It is also possible to start with less cells, keeping in mind that 1µg of DNA is needed per IP.
- Scale volumes accordingly based on the starting material that is available. Then, the number of IPs that can be done will also depend on the amount of prepared DNA that is available.
- After DNA preparation, most of the DNA is then sheared to be used in the IP experiment, but remember that some of the DNA and sheared DNA are needed as control for:
  1. DNA preparation efficiency
  2. Shearing efficiency
  3. The IP experiment efficiency: input sample.

DNA preparation	Cell Number needed	DNA amount expected	DNA to be used in IP
For 1 Methyl DNA IP	0.3x 10^e6	2.0-3.0µg	1µg
For 10 Methyl DNA IP	1-1.5x 10^e6	8-12µg	10µg/10 IPs
For 20 Methyl DNA IP	3x 10^e6	20-30µg	20µg/20 IPs

Step 1. Cell collection and lysis

Starting material: Culture cells

1. Pellet suspension culture out of its serum-containing medium or trypsinize adherent cells and collect cells from the flask. Centrifuge at 300× g for 5 minutes at 4°C.
2. Discard the supernatant. Resuspend cells in 5 to 10mL ice-cold PBS. Centrifuge at 300× g for 5 minutes. Discard the supernatant. Repeat this resuspension and centrifugation step once more. This step is to wash the cells.

- Meanwhile, place the **Digestion Buffer - KOA0884S** at room temperature (RT) and the **Proteinase K (200x) - KOA0884T** on ice.

3. Prepare the complete Digestion Buffer by adding 5µl **Proteinase K (200x) - KOA0884T** to 1mL **Digestion Buffer - KOA0884S**.
4. Resuspend cells in Complete Digestion buffer.
    - For 1.0 to 1.5 million cells, use up to 500µl Complete Digestion buffer.
    - It might be necessary to use more buffer to avoid viscosity when performing the extractions.
5. Incubate the samples with shaking at 50°C for 12 to 18 hours in tightly capped tubes.

Step 2. Nucleic Acid extraction and DNA purification

6. Thoroughly extract the samples with an equal volume of phenol/chloroform/isoamyl alcohol (Work under a fume-hood).
    - Add 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1).
    - One volume is 500µl (see 4. above).
    - Incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation. Use gentle rotation and do not vortex.
7. Centrifuge at 1,700× g for 10 minutes.
    - If the phases do not resolve properly, add another volume of DNA Digestion buffer omitting proteinase K, and repeat the centrifugation.
    - If there is a thick layer of white material at the interface between the phases, repeat the extraction.
8. Transfer the aqueous (top) layer to a new tube.
    - Increase volume to avoid viscosity if necessary and pipette slowly.
9. Thoroughly extract the samples with an equal volume of chloroform/isoamyl alcohol.
10. Incubate for 10 minutes at room temperature.
11. Centrifuge at 1,700 g for 10 minutes.
12. Transfer aqueous layer to a new tube.
13. Add 1/2 volume of **Precipitant Solution- KOA0884U** and 2 volumes of 100% ethanol.
    - One volume corresponds to the original amount of top layer.
14. Recover DNA by centrifugation at 1700× g for 5 minutes.
    - Do not use higher speed to avoid genomic DNA fragmentation.
    - This brief precipitation in the presence of an optimized high salt precipitant (DNA precipitant) reduces the amount of RNA in the DNA sample. For long-term storage it is convenient to leave the DNA in the presence of ethanol.
15. Rinse the pellet with 70% ethanol. Decant ethanol and air-dry the pellet.
    - It is important to rinse extensively to remove any residual of salt and phenol.
16. Resuspend the pellet of DNA at ~1 mg/mL in **TE Buffer- KOA0884V** until dissolved. Shake gently at room temperature or at 65°C for several hours to facilitate solubilization. Store at 4°C.
    - From 1-1.5 million cells, ~8 to 12µg of DNA can be expected (in a volume of 8 to 12µl).

- From 3 million cells, ~20 to 30µg of DNA can be expected (in a volume of 20 to 30µl).
- If possible, it is recommended to get at least 30µg of DNA (when enough material is available) to be able to work with 30µg of DNA: see 2/ DNA shearing protocol).

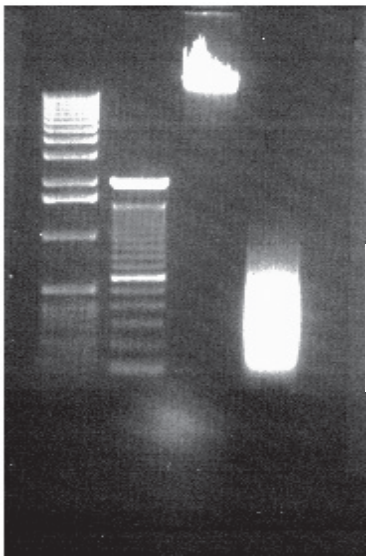
17. Residual RNA has to be removed at this step by adding 2µl of **RNAse (DNAse free) - KOA0884W** per mL of DNA sample and incubating 1 hour at 37°C, followed by phenol/chloroform extraction and ethanol precipitation.

Step 3. DNA shearing

Genomic DNA must to be randomly sheared by sonication to generate fragments between 100-600 bp.

18. In a 1.5mL tube, dissolve the DNA sample in **TE Buffer- KOA0884V** to reach 0.1µg/µl
19. Use a final volume of 300µl of DNA sample (30µg DNA) in 1.5mL tubes
    - e.g. for one IP done in duplicate and the input (corresponding to 10% of one IP), you will need 24µl of sheared DNA sample.
    - Scale accordingly based on your sample size and number of IPs to do.
20. Shear the DNA using a sonicator:
    - Bioruptor® Pico: 6 cycles of 30 sec ON / 90 sec OFF (1.5mL microtubes with caps)
    - other models: 10 cycles of 15 sec ON / 15 sec OFF; Low Power
21. Sheared DNA has to be analyzed on agarose gel as shown in the picture below.

    Note: If your shearing conditions (e.g. DNA concentration, number of tubes) differ from the conditions mentioned in this manual, optimization of these parameters will be needed.



Shearing of the genomic DNA for 10 minutes at Low power using cycles of 15 seconds “on” and 15 seconds “off”, volume sample of 300µl (in 1.5mL tube), and DNA concentration of 0.1µg/µl.

1: molecular weight marker (1 kb)
2: molecular weight marker (100 pb)
3: genomic DNA preparation (not sheared, 10µg)
4: sheared genomic DNA (10µg): average size of 300 bp

Step 4. Methylated DNA immunoprecipitation

The sonicated DNA is then immunoprecipitated with a 5-methylcytosine antibody. 1µg of DNA is required for one IP. A portion of sheared DNA (10% of IP sample) is kept as Input control.

Bead preparation

22. Prepare the bead wash Buffer by diluting 1:5 the **Bead Wash Buffer A - KOA0884B** with water. The volume of bead wash Buffer needed per IP reaction is 100µl.

23. Resuspend the provided **Magnetic Beads - KOA0884A** and transfer 11µl of **Magnetic Beads - KOA0884A** per IP into a new tube.
    - Keep beads in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.
24. Place the tube on a magnet or centrifuge to get rid of the supernatant. Keep the beads.
25. Wash the provided **Magnetic Beads - KOA0884A** twice with ice-cold bead wash Buffer as follows: resuspend the beads in Buffer, then centrifuge for 5 minutes at 1,300 rpm (If volume is small, use the magnetic rack instead), discard the supernatant and keep the bead pellet.
    - For 2 IPs: add a volume of 55µl Buffer to 22µl stock solution of beads.
    - For 8 IPs: add a volume of 220µl Buffer to 88µl stock solution of beads.
    - For 16 IPs: add a volume of 440µl Buffer to 176µl stock solution of beads.

    Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility.
26. After washing, resuspend the beads in wash Buffer as indicated below, keep on ice.
    - For 1 IPs: add 22µl of bead wash Buffer.
    - Do not freeze the beads.

    Resuspend the beads before each use.

IP mix preparation

27. Prepare the IP incubation mix as described in the table.
    - When preparing the IP incubation mix, take into account the following:
        - the input sample must also be used as control next to the IP
        - when possible it is best to perform the IP at least in duplicate

    Note that this mix includes the 2 DNA internal IP controls (unmethylated and methylated DNA).

IP Incubation Mix		
Components	**Volume per 1 IP + input**	**Volume per 2 IP + input**
Water - KOA0884P	45µl*	90µl*
Bead Wash Buffer A - KOA0884B**	24µl	48µl
Bead Wash Buffer B - KOA0884C	6µl	12µl
Positive Control - methylated DNA - KOA0884F	1.5µl	3µl
Negative Control unmethylated DNA - KOA0884G	1.5µl	3µl
DNA Sample	12µl	24µl
Total Volume	90µl	180µl

* If the DNA sample is at a concentration of 0.1µg/µl, use 45µl water per IP. If the concentration of the DNA sample is not at 0.1µg/µl, adjust the volumes of water to add. Keep the volume of the Incubation Mix without DNA sample per IP and input at 78µl (see next point).

** Contains detergent; if its appearance is cloudy and crystalized please warm gently prior to use.

If performing 1 IP and input, please proceed as follows:

Add to a new tube: 78µl of IP Incubation Mix and 12µl of DNA sample (1.2µg; concentration 0.1µg/µl). The total volume is 90µl corresponding to 1 IP (75µl), 10% input (7.5µl) and excess (7.5µl).

If performing 2 IPs and input, please proceed as follows:

Add to a new tube: 156µl of IP Incubation Mix and 24µl of DNA sample (2.4µg; concentration 0.1µg/µl). The total volume is 180µl corresponding to 2 IPs (150µl), 10% input (7.5µl) and excess (22.5µl).

28. Incubate at 95ºC for three minutes.
29. Quickly chill sample on ice (it is best to use ice-water).
30. Perform a pulse spin to consolidate your sample.
31. Take out 7.5µl (that is 10% input) and transfer to a new labeled tube. Keep the input samples at 4°C. The input sample is to be used as a control of starting material and it is therefore not to be used in IP.
32. Then, transfer from what is left: 75µl per tube into two 200µl tubes using the provided 200µl tube strips (or individual 200µl tubes that can fit in our Magnetic Rack). Keep at 4°C.
33. In a new tube, dilute the antibody 1:2 with water.
34. Then use the 1:2 freshly diluted antibody, to prepare the Diluted Antibody Mix as shown in the table below.
    - Add to the antibody, the **Bead Wash Buffer A - KOA0884B** and **Water - KOA0884P** first.
    - Add the **Wash Buffer C - KOA0884D** afterwards.

Diluted Antibody Mix					
Components	**1 IP**	**2 IPs**	**4 IPs**	**6 IPs**	**8 IPs**
Antibody 1:2	0.30µl	0.75µl	1.50µl	2.0µl	3.0µl
Bead Wash Buffer A - KOA0884B	0.60µl	1.5µl	3.0µl	4.0µl	6.0µl
Water - KOA0884P	2.1µl	5.25µl	10.5µl	14.0µl	21.0µl
Wash Buffer C - KOA0884D	2.0µl	5.0µl	10.0µl	13.0µl	20.0µl
Final Volume	5.0µl	12.5µl	25.0µl	33.0µl	50.0µl

    - In the table, final volumes include excess. (Note that 5µl of Diluted Antibody mix is required per IP).
    - Scale the volumes accordingly, based on the number of IPs that are performed on the day.
    - Discard what is not used on the day.
    - Do not omit the dilution step as the amount of antibody to be used is critical.
35. Add 5µl of Diluted Antibody Mix per 200µl IP tube (from Point 23. above).
    - Diluted Antibody Mix is added to the IPs, which contain IP incubation mix and DNA sample
    - The Diluted Antibody mix that is left must be discarded.
36. Mix and add 20µl of washed beads to each 200µl IP tubes (final volume: 100µl).

37. Place on a rotating wheel at 4ºC for 4 hours or overnight.

Step 5. Washes

38. Place the Magnetic Bead Wash buffers and Magnetic Rack on ice. It is best to perform the washes on ice or in a cold room.
39. Spin down and place the IP tubes in the ice-cold Magnetic Rack and work on ice, wait 1 minute and discard the buffer.
40. Wash the DNA IP Samples three times as follows. Add per tube, 100µl ice-cold **Bead Wash Buffer One - KOA0884H**, close the tube caps, invert the 8-tube strip to resuspend the beads, incubate for 4 minutes at 4°C on a rotating wheel (40 rpm), spin, place in the Magnetic Rack, wait 1 minute and discard the buffer. Keep the captured beads.
    - Do not disturb the captured beads attached to the tube wall.
    - Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the Magnetic Rack.
    - Use 150µl Wash Buffer for each wash if working in a 1.5mL tube Magnetic rack.
41. Wash the beads once with 100µl ice-cold **Bead Wash Buffer Two - KOA0884I**.
42. After the last wash, discard the last traces of Wash buffer (using a P200 pipet). Keep the bead pellets on ice and proceed to the next step.

    From the washed beads, the bound DNA can now be purified (proceed to the next point).

Step 6. DNA isolation

43. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.
44. Prepare 100µl Complete DNA Isolation Buffer (DIB) per sample as follows. Add 1µl of **Proteinase K Solution - KOA0884K** per 100µl of **DNA Isolation Buffer - KOA0884J**. Scale accordingly knowing that 100µl are needed per IP'd DNA sample and 92.5µl, per input DNA sample.
45. Remove the tubes from the Magnetic Rack and add 100µl of complete DIB per IP'd DNA sample. Resuspend the beads and transfer the suspension into 1.5mL tubes.
46. Add 92.5µl of complete DIB to 7.5µl of input DNA sample.
47. Incubate at 55°C for 15 minutes both IP'd DNA sample and input DNA sample.
48. Next, incubate at 100°C for 15 minutes all the samples.
49. Spin down and place the 1.5mL tubes into a magnetic rack for 1.5mL tubes or centrifuge at 14,000 rpm for 5 minutes at 4°C.
50. Transfer the supernatants in new labeled tubes. That is the DNA ready for qPCR analysis. Make aliquots and store at -20°C.

Step 7. qPCR analysis of IP'd DNA

The Methylated DNA IP module includes four validated primer pairs specific to four types of DNA:

- methylated DNA (Control One methylated DNA primer pair) - **KOA0884L**
- unmethylated DNA (Control Two unmethylated DNA primer pair) - **KOA0884M**
- methylated human DNA region (testis-specific H2B, TSH2B) - **KOA0884N**
- unmethylated human DNA region (GAPDH promoter) – **KOA0884O**

51. Prepare your qPCR mix using SYBR Green PCR master mix and start out qPCR.

    qPCR mix (total volume of 25µl/reaction:

    - 1.00µl of provided primer pair (stock: 10µM each: reverse and forward)
    - 12.50µl of master mix (e.g.: iQ SYBR Green supermix)
    - 5.00µl of isolated DNA or diluted purified DNA sample (see above for DNA dilutions)
    - 6.50µl of water

DNA Preparation	Cell Number Needed	DNA Amount Expected	DNA to be Used in IP
For 1 Methyl DNA IP	0.3x 10^e6	2.0-3.0µg	1µg
For 10 Methyl DNA IP	1-1.5x 10^e6	8-12µg	10µg/10 IPs
For 20 Methyl DNA IP	3x 10^e6	20-30µg	20µg/20 IPs

When the PCR is done, analyze the results. Some major advices are given below.

Data interpretation

The efficiency of methyl DNA immunoprecipitation of particular genomic locus can be calculated from qPCR data and reported as a recovery of starting material: % (meDNA-IP/ Total input).

% (meDNA-IP/ Total input) = 2^[(Ct(10%input) - 3.32) - Ct(meDNA-IP)]x 100%

Here 2 is the AE (amplification efficiency), Ct (meDNA-IP) and Ct (10%input) are threshold values obtained from exponential phase of qPCR for the methyl DNA sample and input sample respectively; the compensatory factor (3.32) is used to take into account the dilution 1:10 of the input. The recovery is the % (meDNA-IP/ Total input).

VIII. TROUBLESHOOTING

Critical steps	Trouble	Solution
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol.
Cell number	The amount of cells required for one preparation of genomic DNA is important.	It is important to keep in mind that enough cells must be available to start with, to be able to use then one µg of DNA per IP.
Picture of Sheared DNA	The migration of large quantities of DNA on agarose gel can lead to poor quality pictures, which do not reflect the real DNA fragmentation.	Do not load too much on a gel. Do not load more than 10µg/lane. Also treat the sample with RNAse.
	Agarose concentration.	Use 1% agarose gel and run slowly
	Running buffer concentration.	1X TAE or TBE is preferred to 0.5X TAE which can lead to smears on gel.
Beads in the IP	Beads centrifugation.	Don't spin the beads at high speed. Use gentle centrifugation (500x g for 2-3 minutes) as described in the manual protocol. g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of

		rotation in mm. It is possible to centrifuge the 1.5mL tubes at 1,000 – 2,000 x g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze.
	Bead binding capacity	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Antibody in IP	Can I use another antibody?	This kit has been validated with the provided antibody. It is essential to use the provided antibody together with buffers. Follow the kit instructions.
	Amount of antibody per IP to use?	To ensure efficient IP it is important to use the diluted antibody as described in the protocol. The lack of antibody can result in low IP efficiency whereas large excess of antibody might lead to lower specificity.
PCR	Your own primers.	- length: 18 to 24 nucleotides - Tm: 60°C (+/- 3.0°C) - % GC: 50% (+/- 4%)
	PCR controls: –ve and +ve	-ve PCR control: PCR with primers specific for a DNA region that is not methylated (kit control and human) +ve PCR control: PCR with primers specific for a DNA region that is methylated (kit control and human) +ve PCR control: PCR on input DNA.
	Methyl DNA IP: qPCR primer pairs.	The provided qPCR primers are targeting human genomic loci (unmethylated and methylated) as well as control DNA (unmethymated and methylated).
	qPCR primers are provided for rapid checking of the Methyl DNA IP efficiency.	The provided qPCR primers are targeting human genomic loci (unmethylated and methylated) as well as control DNA (unmethymated and methylated).
Freezing	Samples can be frozen at several steps of the protocol.	- genomic DNA - sheared DNA - input DNA - IP'd DNA
	To Avoid freeze/thawing	Snap freeze cells and thaw on ice.

IX. RELATED PRODUCTS

Catalog #	Product Name	Size
600-401-GV0	Anti-GAPDH (RABBIT) Antibody	100µg
MB-064-1000	ELISA Microwell Blocking Buffer with Stabilizer (Azide and Mercury Free)	1L
TMBE-100	TMB ELISA PEROXIDASE SUBSTRATE	100mL
TMS-06	6 position Microcentrifuge Tube TrueBlot® Magnetic Separator	1x6
TMS-15-50	15/50 Tube TrueBlot® Magnetic Separator	1x15, 1x50
TMS-32	32 position Microcentrifuge Tube TrueBlot® Magnetic Separator	1x32