ChIP protocol;
step-by-step guidelines and troubleshooting

Version 1.5 – CIGENE/NMBU

Important take-home messages

a. This documentation will be improved by the questions of the users about the established protocols for ChIP experiment for the AQUA-FANNG project
b. To each user, we recommend first you go through the entire protocol of ChIP (Day 1, 2 and 3) with liver samples
c. We warn you TO NOT PROCESS ALL YOUR SAMPLES with Day 1 Protocol even after having experienced good recovery of chromatin and good sonication profile. Indeed, a good sonication profile is not always a guarantee of the quality of the epitopes of the proteins bound to the DNA: Therefore, it is MANDATORY TO PURSUE UNTIL THE END OF DAY 3 with at least one sample (i.e. liver) to confirm the efficiency of the immunoprecipitation (i.e. the percentage of recovery)

Day 1

Tissue disruption

a. The recommended amount of tissue has been determined in salmon. It can be adapted to the species and available quantity of tissue. If the initial material is under 50 mg, we recommend reducing the buffer volume to 1 ml to avoid loss and too high dilution of material, thus preventing a correct assessment of nuclei quality and quantity
b. The indicative weight given in the protocols should allow you to recover enough quantity of chromatin to perform all the 5 different immunoprecipitations once, with a common input
c. When a mortar is used, a pre-cooling of the mortar on dry ice helps the tissue disruption. This is especially mandatory for tissues that we can qualify as hard due to their structure. Indeed, a good disruption of the tissue is necessary to have a better recovery of nuclei
d. The use of 2-ml or 15-ml Dounce homogenizers didn't show any effect on the protocol itself. However, in general, the number of strokes by pestle A and/or B can be person dependent. Indeed, the strength and the habit to use these kinds of device makes a difference. The main common rule to respect is to stop as soon as no more resistance is felt
e. The counting step is essential for the sonication step. Please don't avoid it
f. If you use Trypan blue, be aware that the colorant can precipitate. To avoid any problem of visualization and quality check of your nuclei, we recommend you to not shake the Trypan blue bottle before use. Also, you can filter a part of the colorant before use (filter a higher volume of colorant than what you need)
g. Be sure how to use accurately your cell counting chamber. Don't forget your dilution factor and change the calculation if your working solution is less than 5 ml at that step for specific reasons
   For the Thoma cell (16 squares = 1mm3 = 1ul);

nb of nuclei inside the 16 squares* x 10 (dilution factor) x 1000 (conversion in ml) x 5 ml (working volume) = total nb of nuclei

*If nuclei concentration is too high, count 3 squares instead of 16, calculate the mean nb, and multiply it by 16

Crosslinking

a. LoBind tubes are recommended to avoid any loss of material due to binding to the tubes, at all steps
b. Crosslinking can be done with a preparation of formaldehyde from powder or from a dilution of a concentrated liquid solution (37%). To some extent, no difference in our laboratory has been observed between the use of these two types of solutions. Depending of your habit in the lab, you can also add the concentrated solution of 37% directly to your samples in solution in PBS, by respecting a final concentration of 1% (135 µl for a volume of 5 ml).
c. The formaldehyde solution has to be brought to room temperature for optimal penetration
d. No analysis in our laboratory has been done to compare quenching with glycine and with Tris. Nevertheless, the quenching with glycine has been chosen because it is still the most frequently used
e. No experiment has been conducted in our laboratory to know the optimal ratio of nuclei with the volume of formaldehyde solution. However, it is common to find in the literature 10 million of cultured cells by ml of solution of formaldehyde 1 or 2%. Therefore, and based on the mean number of nuclei recovered by tissue, we recommend you split in two tubes if the total number is over 24M of nuclei
f. For smaller pieces of tissues, the centrifugation can be done with higher speed and longer time if loss of material is observed in the supernatant. Furthermore, skipping the second cleaning and staying in a working solution of 1ml can help to reduce loss of material if the starting material is critical
g. After the decrosslinking and quenching step, keep your sample cold at any time, especially during the sonication process (see above in the section “Sonication”)

Sonication

a. The sonication buffer helps the sonication process. The volume of sonication buffer can be adapted compared to the sonicator you use and the number of nuclei you recover. This step is critical to recover the best profile of sonicated chromatin (200-700 bp size, with a mean size of 350-400 bp and a recovery of 70% minimum of the chromatin in that area)
b. The sonication parameters (number of cycles, cycles parameters) must be adapted to the material you use.
   To assess what are the best parameters for your own sample and material, we recommend to follow this step-by-step systematic procedure;
   1. Prepare your material according to the recommendations of your provider and follow the basic instructions for how to use your material and find the best parameters
   2. The recommended dilution of nuclei for sonication is 1 million of nuclei by 100 µl of sonication buffer
   3. Test sonication parameters which allow a balanced proportion of sonication time and rest time (for example, 1 sec ON / 1 sec OFF, 10 sec ON / 10 sec OFF… until 30 sec ON / 30 sec OFF). If possible, execute cycle no longer than 1 minute in total (periods ON and OFF included). Be aware that for some devices, 30 sec ON means 30 sec ON and 30 sec OFF = a total of 1 min by cycle, while, for others, 30 sec ON means a continuous 30 sec sonication, and then a resting time
   4. Test a number of cycles between 12 and 20. For time management, 20 cycles become too much time consuming, and you can address other parameters at the sonication step (for example, intensity). Furthermore, the longer you sonicate, the harder the immunoprecipitation can be (see next comment)
   5. You can test different number of cycles from the same sample, by recovering 20ul aliquot. But you also have to complete the removed material by sonication buffer to respect the same total volume. Be also aware that, in that procedure, you remove 5% of your material
   6. If the sonicator probe width is 2mm, test intensity between 40 and 55%. Over 55%, you might still recover a good sonicated chromatin. However, the epitopes of the proteins you will target at the immunoprecipitation step can be destroyed
c. Recover the maximum of the volume while avoiding any contamination from the pellet containing cellular and nuclear debris, which can alter the immunoprecipitation process

d. Your tubes should be maintained in a cold environment all along the process; stay aware about the temperature of your cold bath if you work with sonicators in cold bath, and about the quality of the water you use for it, or use a metallic eppendorf holder, immersed in ice, to avoid any alteration of the protein epitopes and chromatin in general
e. We recommend you respect the proportion of 1M of nuclei for 100 µl of sonication buffer. Depending of the sonicator machine used, respect the limit amount of volume by tube (in our case, as minimum, 300 ul, applied for muscle tissue, and at maximum 400 ul, applied for the other tissues)
f. In the particular case of the immature gonads, where the size of the tissues is limited, you should perform the sonication in all the nuclei recovered to avoid any lost of material
   Example; you recover 4.5M of nuclei; perform sonication in one tube, 450 ul total
   You recover 6M of nuclei; perform sonication in two tubes of 300 ul
   Don't discard any of the biological material (please refer to the session "tissue-specific challenges: Gonads for more details)
g. ONLY IF NO OTHER OPTIONS; the supplemental Safe Stop Point just before the sonication step of the protocol should be used in good measure. AFTER the last centrifugation and the removal of the supernatant, AND BEFORE the add of the sonication buffer, you can snap-freeze down in dry ice the pellet of nuclei. When you will continue the protocol, take out the frozen pellet, let it defrost and add the corresponding volume of sonication buffer, homogenize correctly, prior to perform the sonication.
   We don't recommend you use this Safe Stop Point in first instance due to the fact that, even after a good resuspension, some aggregates of nuclei will still remain, so you will loose a proportion of possible material
   This Safe Stop Point is anyway applicable for all tissues (tested in NMBU), except for immature gonads tissue (not tested in NMBU). Just stay aware on the possibility that this doesn't end to work in your samples as it did for us, because of specie specificity, and that you will loose a certain proportion of material due to the resuspension step
   Some tests should be performed in your samples

Tissue-specific challenges

Muscle

The protocol released is based on the publication of Joshi et al., "Improved protocol for chromatin immunoprecipitations from skeletal muscle", J Vis Exp (JOVE), 2017, Nov 6.
Observation about the tissue powder obtained after grinding in cold mortar;
Here some pictures to show how the "powder" looks like;

PICTURES AVAILABLE SOON

Gonads (male/female, immature/mature)

For immature gonads (female and male), the protocol is similar to the one applied for the liver tissue; the only difference is in terms of number of cycles for sonication. Whatever the number of nuclei recovered, we recommend to resuspend all of them in the adequate volume of sonication buffer to discard any of this precious biological material
Follow the instructions of your sonicator machine to perform sonication in all of it
Example in the case of EpiShear sonicator from Active Motif;

Number of million of nuclei recovered	Volume of sonication buffer	Process
1	100	! really limited volume ! – perform anyway
2	200	1 tube of 200 µl
3	300	1 tube of 300 µl
4	400	1 tube of 400 µl
5	500	1 tube of 500 µl or 2 tubes of 250 µl
6	600	2 tubes of 300 µl
7	700	2 tubes of 350 µl
8	800	2 tubes of 400 µl

For female mature gonad, we recommend you apply a step of filtration with a 70-µm filter if the gonad contains some eggs if necessary

For mature male gonad, please follow carefully the protocol (similar to the protocol developed for ATAC), to reduce as much as possible the proportion of sperm in your sample. Please contact us for further specific explanations and troubleshooting

Head Kidney

Please refer to the Diagenode kits specifications

Definitions

- Paraformaldehyde (PFA) is the name of the crystals
- Formaldehyde is in solution (in PBS). Some PFA can be formed in that solution, that is why the solution has to be renewed
- Formalin is formaldehyde in solution with methanol, at 36.5%. The methanol is used to reduce the formation of paraformaldehyde

Version history

Version 1.5 – 2021.03.15	Add of explanation about Safe Stop Point before sonication
Version 1.4 – 2021.01.25	Application of the template Comment about Muscle changed Comment about Gonads developed
Version 1.3 – 2020.01.10	Text correction
Version 1.2 – 2020.01.10	Add of a version history section Add of the comments b and d in section “Tissue disruption” Add of the comments b in section “Crosslinking” Add of the section “Tissue-specific challenges” and comments Add of the section “Definitions” and comments Add of a chapter “Important take-home messages and comments

ChIP protocol for liver

Version 3.6 – NMBU/CIGENE

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - tweezers
  - a Douncer with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic Eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 4.5 ml of PBS with PIC in the Douncer, and place it in the ice bucket
  - take the appropriate volume of formaldehyde solution 1% from the fridge and place it at room temperature before use

Tissue disruption

The procedure (step 1 to 3) should take less than 15 min and the samples should stay in cold environment as much as possible. Glass homogenizers must be used in priority to plastic ones, due to better conductivity of cold

1. Transfer pieces of liver (60 mg recommended for liver) into a Douncer containing 4.5 ml [1] of PBS with PIC in ice
2. Use pestles A and B for the homogenization, 5 strokes each or until obtaining a homogenized solution or feel no more strong resistance
3. Transfer the homogenate into a 5-ml Eppendorf tube [2]. You can cut the bottom of the pipet tip to facilitate the aspiration
4. Rinse the Douncer and the pestles with 0.5 ml of PBS with PIC. Add it to the previous solution. You reach 5ml
5. After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)
   Counting: **nuclei/ml** **5ml =** **million nuclei total** (expected: 20 million)
6. Centrifuge 3 min, 2 500 g, 4˚C
7. Remove and discard supernatant

Crosslinking

8. Add to the pellet 2.5 ml* of formaldehyde solution 1% at room temperature (RT) and resuspend the pellet by pipetting
   **if more than 24 million of nuclei, resuspend in 5 ml of formaldehyde solution 1% and split in two tubes*

[1] for Douncer under 5 ml, split the solution accordingly
[2] or a 15-ml canonical Falcon tube

9 Incubate under constant agitation [3] at RT, 5 min
10 Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation
11 Centrifuge 5 min, 800 g, 4˚C
12 Remove and eliminate in appropriate trash the supernatant containing formaldehyde
13 Resuspend with 4 ml* of PBS with PIC by pipetting (quick vortex if necessary)
**if more than 24 million of nuclei, resuspend each pellet in 2 ml of PBS with PIC, and merge them*
14 Centrifuge 5 min, 800 g, 4˚C
15 Remove the supernatant
16 Resuspend in 5 ml of PBS with PIC by pipetting (quick vortex if necessary)
17 Centrifuge 5 min, 800 g, 4˚C
18 Remove the supernatant
19 Resuspend with 1 ml of PBS with PIC and transfer in a clean 1.5-ml Eppendorf tube
20 Centrifuge 10 min, 3 000 g, 4˚C
21 Remove the supernatant
SAFE STOP POINT: Freeze the nuclei pellet in dry ice. Store in -80 ˚C for 2 months maximum. Please refer to the guidelines for more informations

Sonication

22 Resuspend the pellet in a ratio of 100 µl of complete sonication buffer for 1 million of nuclei. Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before the start of the sonication
23 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [4] or Tape Station D1000 kit, and set-up the incubator at 68˚C
24 Place the metal Eppendorf holder from the fridge on ice. Aliquot your solution by 400 µl
25 Keep the tubes on the holder all along the process
26 Sonicate the chromatin with the following parameters [5]

Number of cycles	**16**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [6]
Cycle time	30 sec

27 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C
28 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume
Volume of sonicated chromatin before dilution: µl
If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube
29 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant
30 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer
31 SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in - 80˚C

Test of the sonication

32 To the 20 µl for sonication control from step 29, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M
33 Incubate 1H30, 68˚C, under agitation [7]
34 Purify with a Qiagen DNA Minelute PCR purification kit. Elute with 20 µl of elution buffer
35 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value
Qubit concentration: ng/µl
36 Run 1 µl of the eluate in the Bioanalyzer or Tape Station to assess the size profile
To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp
Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation

[3] Place the tube in the Hula Mixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)
[4] You can wait to have enough sample to run a full Bioanalyzer chip
[5] These parameters have been established for the device “EpiShear Probe Sonicator” from Active Motif
[6] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec
[7] 500 rpm for a Thermomixer from Eppendorf

To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

Version history

Version 3.6 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 3.5 – 2021.03.15	Correction of the dilution factor for counting step Add of a supplemental Safe Stop Point before sonication
Version 3.4 – 2021.01.21	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion
Version 3.3 – 2020.18.01	Comment about sonication profile change
Version 3.2 – 2020.10.01	Add of a version history section Change of the safe stop point explanation

ChIP protocol for gill

Version 3.8 – NMBU/CIGENE

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - a ceramic mortar and its pestle
  - a metallic spoon or spatula
  - dry ice
  - tweezers
  - a Douncer with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic Eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the mortar and pestle in dry ice for 10 min minimum
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 4.5 ml of PBS with PIC in a Douncer, and place it in the ice bucket
  - take the appropriate volume of formaldehyde solution 1% from the fridge and place it at room temperature before use

Tissue disruption

The procedure (step 1 to 3) should take less than 15 min and the samples should stay in cold environment as much as possible. Glass homogenizers must be used in priority to plastic ones, due to better conductivity of cold

1. **The use of appropriate gloves is mandatory.** Transfer pieces of gill (20 mg recommended) into a refrigerated cold mortar
2. Grind the pieces until obtaining powder
3. Transfer this tissue powder into a Douncer containing 4.5 ml [1] of PBS with PIC in ice
4. Use pestles A and B for the homogenization, 5 strokes each or until obtaining a homogenized solution or feel no more strong resistance
5. Transfer the homogenate into a 5-ml Eppendorf tube [2]. You can cut the bottom of the pipet tip to facilitate the aspiration
6. Rinse the Douncer and the pestles with 0.5 ml of PBS with PIC. Add it to the previous solution. You reach 5 ml
7. After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)
   Counting: **nuclei/ml** **5ml =** **million nuclei total** (expected: 20 million)
8. Centrifuge 3 min, 2 500 g, 4˚C
9. Remove and discard supernatant

[1] for Douncer under 5 ml, split the solution accordingly
[2] or a 15-ml canonical Falcon tube

Crosslinking

10 Add to the pellet 2.5 ml* of formaldehyde solution 1% at room temperature (RT) and resuspend the pellet by pipetting

**if more than 24 million of nuclei, resuspend in 5 ml of formaldehyde solution 1% and split in two tubes*

11 Incubate under constant agitation [3] at RT, 10 min

12 Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation

13 Centrifuge 5 min, 800 g, 4˚C

14 Remove and eliminate in appropriate trash the supernatant containing formaldehyde

15 Resuspend in 4 ml* of PBS with PIC by pipetting (quick vortex if necessary)

**if more than 24 million of nuclei, resuspend each pellet in 2 ml of PBS with PIC, and merge them*

16 Centrifuge 5 min, 800 g, 4˚C

17 Remove the supernatant

18 Resuspend in 5 ml of PBS with PIC by pipetting (quick vortex if necessary)

19 Centrifuge 5 min, 800 g, 4˚C

20 Remove the supernatant

21 Resuspend with 1 ml of PBS with PIC and transfer in a clean 1.5-ml Eppendorf tube

22 Centrifuge 10 min, 3 000 g, 4˚C

23 Remove the supernatant

SAFE STOP POINT: Freeze the nuclei pellet in dry ice. Store in -80 ˚C for 2 months maximum. Please refer to the guidelines for more informations

Sonication

24 Resuspend the pellet in a ratio of 100 µl of complete sonication buffer for 1 million of nuclei. Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before the start of the sonication

25 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [4] or Tape Station D1000 kit, and set-up the incubator at 68˚C

26 Place the metal Eppendorf holder from the fridge on ice. Aliquot your solution by 400 µl

27 Keep the tubes on the holder all along the process

28 Sonicate the chromatin with the following parameters [5]

Number of cycles	**18**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [6]
Cycle time	30 sec

29 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C

30 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume

Volume of sonicated chromatin before dilution: µl

If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube

31 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant

32 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer

SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in - 80˚C

Test of the sonication

33 To the 20 µl for sonication control from step 31, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl

34 Incubate 1H30, 68˚C, under agitation [7]

35 Purify with a Qiagen DNA Minelute PCR purification kit. Elute with 20 µl of elution buffer

36 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value

Qubit concentration: ng/µl

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[3] Place the tube in the Hula Mixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)

[4] You can wait to have enough sample to run a full Bioanalyzer chip

[5] These parameters have been established for the device “EpiShear Probe Sonicator” from Active Motif

[6] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec

[7] 500 rpm for a Thermomixer from Eppendorf

37 Run 1 µl of the eluate in the Bioanalyzer or Tape Station to assess the size profile

To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp
Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation
To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

Version history

Version 3.8 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 3.7 – 2021.03.15	Correction of the dilution factor for counting step Add of a supplemental Safe Stop Point before sonication
Version 3.6 – 2021.01.21	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion
Version 3.5 – 2020.01.18	Comment of the sonication change Value of nb of nuclei change
Version 3.4 – 2020.10.01	Add of a version history section Change of the safe stop point explanation

ChIP protocol for brain

Version 3.8 – NMBU/CIGENE

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - tweezers
  - a dounce with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic Eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 4.5 ml of PBS with PIC in a Douncer, and place it in the ice bucket
  - take the appropriate volume of formaldehyde solution 1% from the fridge and place it at room temperature before use

Tissue disruption

The procedure (step 1 to 3) should take less than 15 min and the samples should stay in cold environment as much as possible. Glass homogenizers should be preferred to plastic ones, due to better conductivity of cold

1. Transfer pieces of brain (50 mg to 75 mg recommended for brain) into a Douncer containing 4.5 ml [1] of PBS with PIC in ice
2. Use pestles A and B for the homogenization, 5 strokes each or until obtaining a homogenized solution or feel no more strong resistance
3. Transfer the homogenate into a 5-ml Eppendorf tube [2]. You can cut the bottom of the pipet tip to facilitate the aspiration
4. Rinse the Douncer and the pestles with 0.5 ml of PBS with PIC. Add it to the previous solution. You reach 5ml
5. After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)
   Counting: **nuclei/ml** **5ml =** **million nuclei total** (expected: 12 to 16 million)
6. Centrifuge 3 min, 2 500 g, 4˚C
7. Remove and discard supernatant

[1] for Douncer under 5 ml, split the solution accordingly
[2] or a 15-ml canonical Falcon tube

Crosslinking

8 Add to the pellet 2.5 ml* of formaldehyde solution 1% at room temperature (RT) and resuspend the pellet by pipetting

**if more than 24 million of nuclei, resuspend in 5 ml of formaldehyde solution 1% and split in two tubes*

9 Incubate under constant agitation [3] at RT, 5 min

10 Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation

11 Centrifuge 5 min, 2 500 g, 4˚C

12 Remove and eliminate in appropriate trash the supernatant containing formaldehyde

13 Resuspend with 4 ml* of PBS with PIC by pipetting (quick vortex if necessary)

**if more than 24 million of nuclei, resuspend each pellet in 2 ml of PBS with PIC, and merge them*

14 Centrifuge 5 min, 1 500 g, 4˚C

15 Remove the supernatant

16 Resuspend in 5 ml of PBS with PIC by pipetting (quick vortex if necessary)

17 Centrifuge 5 min, 1 500 g, 4˚C

18 Remove the supernatant

19 Resuspend with 1 ml of PBS with PIC and transfer in a clean 1.5-ml Eppendorf tube

20 Centrifuge 10 min, 3 000 g, 4˚C

21 Remove the supernatant

SAFE STOP POINT: Freeze the nuclei pellet in dry ice. Store in -80 ˚C for 2 months maximum. Please refer to the guidelines for more informations

Sonication

22 Resuspend the pellet in a ratio of 100 µl of complete sonication buffer for 1 million of nuclei. Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before the start of the sonication

23 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [4] or Tape Station D1000 kit, and set-up the incubator at 68˚C

24 Place the metal Eppendorf holder from the fridge on ice. Aliquot your solution by 400 µl

25 Keep the tubes on the holder all along the process

26 Sonicate the chromatin with the following parameters [5]

Number of cycles	**15**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [6]
Cycle time	30 sec

27 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C

28 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume

Volume of sonicated chromatin before dilution: µl

If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube

29 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant

30 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer

SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in - 80˚C

Test of the sonication

31 To the 20 µl for sonication control from step 29, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M

32 Incubate 1H30, 68˚C, under agitation [7]

33 Purify with a Qiagen DNA Minelute kit. Elute with 20 µl of elution buffer

34 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value

Qubit concentration: ng/µl

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[3] Place the tube in the Hula Mixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)

[4] You can wait to have enough sample to run a full Bioanalyzer chip

[5] These parameters have been established for the device “EpiShear Probe Sonicator” from Active Motif

[6] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec

[7] 500 rpm for a Thermomixer from Eppendorf

35 Run 1 µl of the eluate in the Bioanalyzer DNA or Tape Station to assess the size profile

To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp
Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation
To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

Version history

Version 3.8 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 3.7 – 2021.03.15	Correction of the dilution factor for counting step Add of a supplemental Safe Stop Point before sonication
Version 3.6 – 2021.01.21	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion
Version 3.5 – 2020.18.01	Comment of the sonication change
Version 3.4 – 2020.10.01	Add of a version history section Change of the safe stop point explanation Change of the number of cycles for sonication

ChIP protocol for muscle

Version 4.4 – NMBU/CIGENE

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - tweezers
  - a Douncer with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic Eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - Add PIC, PMSF and sodium butyrate to the hypotonic buffer (named below complete hypotonic buffer)
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the mortar and pestle in dry ice for 10 min minimum
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 1 ml of complete hypotonic buffer by 200 mg of tissue in a 5-ml tube, and place it in the ice bucket

Tissue disruption

The procedure (step 1 to 2) should take less than 10 min and the samples should stay in cold environment as much as possible. Glass homogenizers must be used in priority to plastic ones, due to better conductivity of cold

1. **The use of appropriate gloves is mandatory.** Transfer pieces of muscle (1 g recommended for muscle) into a mortar which has been kept cold on dry ice
2. Grind the pieces until obtaining powder
3. Transfer this tissue "powder" (looks like corn flakes) to a 5-ml tube containing 1 ml of complete hypotonic buffer by 200 mg of tissue
4. Incubate the tissue into the complete hypotonic buffer 10 min, 4˚C, under agitation [1]
5. Transfer the tissue and the solution into a Douncer
6. Use pestle A for the homogenization, 5 strokes
7. Transfer the homogenate into a 5-ml Eppendorf tube [2]. Avoid transferring the pieces of tissue
8. Complete the solution to 5 ml final

[1] Place the tube in the Hula Mixer, 360˚ rotation, 50 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm) – in a fridge or in a cold room

[2] or a 15-ml canonical Falcon tube

9 After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)
Counting: **nuclei/ml** **5ml =** **million nuclei total** (expected: 12 million)

Crosslinking

10 Add directly to the homogenate 135 µl of formaldehyde solution 37% (1% final) at room temperature (RT)
11 Incubate under constant agitation [1] at RT, 10 min
12 Quench the reaction with 720 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation
13 Transfer the crosslinked tissue back into a Douncer and homogenize with pestle A (5 strokes)
14 Transfer the homogenate into the previous 5-ml Eppendorf tube
15 Centrifuge 10 min, 2 500 g, 4˚C
16 Remove and eliminate in appropriate trash the supernatant containing formaldehyde
17 Resuspend with 4 ml of complete hypotonic buffer by pipetting up and down with a tip cut at the bottom
18 Transfer the solution back into a Douncer and homogenize with pestle A (5 strokes). Make sure the solution is well homogeneous before filtering
19 Filter the lysate through a 40-µm strainer placed on the top of a 50-ml Falcon tube
20 Rinse the homogenizer and the filter with 1 ml of complete hypotonic buffer. You reach 5 ml
21 Transfer the filtered solution into a new 5-ml Eppendorf tube
22 Centrifuge 10 min, 1 000 g, 4˚C
23 Remove the supernatant
24 Resuspend the pellet in 4 ml of complete hypotonic buffer with the help of a tip cut at the bottom. Split the solution by 3 million of nuclei in clean 1.5-ml tubes
25 Centrifuge 10 min, 3 000 g, 4˚C
26 Remove the supernatant
SAFE STOP POINT: Freeze the nuclei pellet in dry ice. Store in -80 ˚C for 2 months maximum. Please refer to the guidelines for more informations

Sonication

27 Resuspend the pellet with 300 µl of complete sonication buffer (for 3 million of nuclei). Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before starting the sonication
28 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [3] or Tape Station D1000 kit, and set-up the incubator at 68˚C
29 Place the metal Eppendorf holder from the fridge on ice
30 Keep the tubes on the holder all along the process
31 Sonicate the chromatin with the following parameters [4]

Number of cycles	**18**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [5]
Cycle time	30 sec

32 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C
33 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume
Volume of sonicated chromatin before dilution: **µl**
If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube
34 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant
35 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer
SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in -80 ˚C

[3] You can wait to have enough sample to run a full Bioanalyzer chip
[4] These parameters have been established for the device “EpiShear Probe Sonicator” from Active Motif
[5] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec

Test of the sonication

36 To the 20 µl for sonication control from step 34, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M

37 Incubate 1H30, 68˚C, under agitation [6]

38 Purify with a Qiagen DNA Minelute PCR purification kit. Elute with 20 µl of elution buffer

39 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value

Qubit concentration: **ng/µl**

40 Run 1 µl of the eluate in the Bioanalyzer DNA 1000 ChIP or Tape Station to assess the size profile

To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp

Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation

To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

[6] 500 rpm for a Thermomixer from Eppendorf

Version history

Version 4.4 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 4.3 – 2021.03.15	Correction of the dilution factor for counting step Add of a supplemental Safe Stop Point before sonication
Version 4.2 – 2021.03.03	Correction in the volume explanation by amount of tissue
Version 4.1 – 2021.01.18	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion
Version 4.0 – 2021.01.08	Complete new version of the protocol Start of the “version history” section

ChIP protocol for immature gonads

Version 2.3 – NMBU/CIGENE

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - tweezers
  - a Douncer with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic Eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 4.5 ml of PBS with PIC in the Douncer, and place it in the ice bucket
  - take the appropriate volume of formaldehyde solution 1% from the fridge and place it at room temperature before use

Tissue disruption

The procedure (step 1 to 3) should take less than 15 min and the samples should stay in cold environment as much as possible. Glass homogenizers must be used in priority to plastic ones, due to better conductivity of cold

1. Transfer pieces of gonads (30 mg recommended for immature male gonad; 100 mg recommended for immature female gonad) into a Douncer containing 4.5 ml [1] of PBS with PIC in ice
2. Use pestles A and B for the homogenization, 5 strokes each or until obtaining a homogenized solution or feeling no more strong resistance
3. Transfer the homogenate into a 5-ml Eppendorf tube [2]. You can cut the bottom of the pipet tip to facilitate the aspiration
4. Rinse the Douncer and the pestles with 0.5 ml of PBS with PIC. Add it to the previous solution. You reach 5ml
5. After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)

   Counting: **nuclei/ml** **5ml =** **million nuclei total** (expected: 15 million for male, 8 million for female)
6. Centrifuge 3 min, 2 500 g, 4˚C
7. Remove and discard supernatant

[1] for Douncer under 5 ml, split the solution accordingly
[2] or a 15-ml canonical Falcon tube

Crosslinking

8 Add to the pellet 2.5 ml* of formaldehyde solution 1% at room temperature (RT) and resuspend the pellet by pipetting

**if more than 24 million of nuclei, resuspend in 5 ml of formaldehyde solution 1% and split in two tubes*

9 Incubate under constant agitation [3] at RT, 5 min

10 Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation

11 Centrifuge 5 min, 800 g, 4˚C

12 Remove and eliminate in appropriate trash the supernatant containing formaldehyde

13 Resuspend with 4 ml* of PBS with PIC by pipetting (quick vortex if necessary)

**if more than 24 million of nuclei, resuspend each pellet in 2 ml of PBS with PIC, and merge them*

14 Centrifuge 5 min, 800 g, 4˚C

15 Remove the supernatant

16 Resuspend in 5 ml of PBS with PIC by pipetting (quick vortex if necessary)

17 Centrifuge 5 min, 800 g, 4˚C

18 Remove the supernatant

19 Resuspend with 1 ml of PBS with PIC and transfer in a clean 1.5-ml Eppendorf tube

20 Centrifuge 10 min, 3 000 g, 4˚C

21 Remove the supernatant

Sonication

22 Resuspend the pellet in a ratio of 100 µl of complete sonication buffer for 1 million of nuclei. Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before the start of the sonication

23 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [4] or Tape Station D1000 kit, and set-up the incubator at 68˚C

24 Place the metal Eppendorf holder from the fridge on ice. Aliquot your solution by 200 µl minimum, 500 µl maximum. Process all the material

25 Keep the tubes on the holder all along the process

26 Sonicate the chromatin with the following parameters [5]

Number of cycles	**18**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [6]
Cycle time	30 sec

27 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C

28 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume

Volume of sonicated chromatin before dilution: µl

If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube

29 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant

30 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer

SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in - 80˚C

Test of the sonication

31 To the 20 µl for sonication control from step 29, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M

32 Incubate 1H30, 68˚C, under agitation [7]

33 Purify with a Qiagen DNA Minelute PCR purification kit. Elute with 20 µl of elution buffer

34 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value

Qubit concentration: ng/µl

35 Run 1 µl of the eluate in the Bioanalyzer or Tape Station to assess the size profile

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[3] Place the tube in the Hula Mixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)

[4] You can wait to have enough sample to run a full Bioanalyzer chip

[5] These parameters have been established for the device “EpiShear Probe Sonicator” from Active Motif

[6] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec

[7] 500 rpm for a Thermomixer from Eppendorf

To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp
Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation
To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

Version history

Version 2.3 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 2.2 – 2021.03.15	Correction of the dilution factor for counting step
Version 2.1 – 2021.03.03	Term “liver” in step 1 replace by “gonads” Volume by sonication tube and number of sonication cycles corrected
Version 2.0 – 2021.01.24	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion
Version 1.0 – 2021.01.04	Add of a version history section Change of the safe stop point explanation

ChIP protocol for mature female gonad

Version 1.2 – NMBU/CIGENE

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - tweezers
  - a Douncer with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic Eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 4.5 ml of PBS with PIC in the Douncer, and place it in the ice bucket
  - take the appropriate volume of formaldehyde solution 1% from the fridge and place it at room temperature before use

Tissue disruption

The procedure (step 1 to 3) should take less than 15 min and the samples should stay in cold environment as much as possible. Glass homogenizers must be used in priority to plastic ones, due to better conductivity of cold

1. Transfer pieces of gonad (200 mg recommended for mature female gonad) into a Douncer containing 4.5 ml [1] of PBS with PIC in ice

   An initial step of washing/filtering may be necessary to remove eggs depending of their stage of development
2. Use pestles A and B for the homogenization, 5 strokes each or until obtaining a homogenized solution or feel no more strong resistance
3. Transfer the homogenate into a 5-ml Eppendorf tube [2]. You can cut the bottom of the pipet tip to facilitate the aspiration
4. Rinse the Douncer and the pestles with 0.5 ml of PBS with PIC. Add it to the previous solution. You reach 5ml
5. After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)

   Counting: **nuclei/ml** **5ml =** **million nuclei total** (expected: 8 million)
6. Centrifuge 3 min, 2 500 g, 4˚C
7. Remove and discard supernatant

[1] for Douncer under 5 ml, split the solution accordingly
[2] or a 15-ml canonical Falcon tube

Crosslinking

8 Add to the pellet 2.5 ml* of formaldehyde solution 1% at room temperature (RT) and resuspend the pellet by pipetting

**if more than 24 million of nuclei, resuspend in 5 ml of formaldehyde solution 1% and split in two tubes*

9 Incubate under constant agitation [3] at RT, 5 min

10 Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation

11 Centrifuge 5 min, 800 g, 4˚C

12 Remove and eliminate in appropriate trash the supernatant containing formaldehyde

13 Resuspend with 4 ml* of PBS with PIC by pipetting (quick vortex if necessary)

**if more than 24 million of nuclei, resuspend each pellet in 2 ml of PBS with PIC, and merge them*

14 Centrifuge 5 min, 800 g, 4˚C

15 Remove the supernatant

16 Resuspend in 5 ml of PBS with PIC by pipetting (quick vortex if necessary)

17 Centrifuge 5 min, 800 g, 4˚C

18 Remove the supernatant

19 Resuspend with 1 ml of PBS with PIC and transfer in a clean 1.5-ml Eppendorf tube

20 Centrifuge 10 min, 3 000 g, 4˚C

21 Remove the supernatant

SAFE STOP POINT: Freeze the nuclei pellet in dry ice. Store in -80 ˚C for 2 months maximum. Please refer to the guidelines for more informations

Sonication

22 Resuspend the pellet in a ratio of 100 µl of complete sonication buffer for 1 million of nuclei. Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before the start of the sonication

23 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [4] or Tape Station D1000 kit, and set-up the incubator at 68˚C

24 Place the metal Eppendorf holder from the fridge on ice. Aliquot your solution by 400 µl

25 Keep the tubes on the holder all along the process

26 Sonicate the chromatin with the following parameters [5]

Number of cycles	**18**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [6]
Cycle time	30 sec

27 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C

28 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume

Volume of sonicated chromatin before dilution: µl

If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube

29 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant

30 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer

31 SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in - 80˚C

Test of the sonication

32 To the 20 µl for sonication control from step 29, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M

33 Incubate 1H30, 68˚C, under agitation [7]

34 Purify with a Qiagen DNA Minelute PCR purification kit. Elute with 20 µl of elution buffer

35 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value

Qubit concentration: ng/µl

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[3] Place the tube in the Hula Mixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)

[4] You can wait to have enough sample to run a full Bioanalyzer chip

[5] These parameters have been established for the device “EpiShear Probe Sonicator” from Active Motif

[6] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec

[7] 500 rpm for a Thermomixer from Eppendorf

36 Run 1 µl of the eluate in the Bioanalyzer or Tape Station to assess the size profile

To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp
Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation
To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

Version history

Version 1.2 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 1.1 – 2021.03.15	Correction of the dilution factor for counting step Add of a supplemental Safe Stop Point before sonication Determination of the number of sonication cycle
Version 1.0 – 2021.01.25	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion Add of a version history section Change of the safe stop point explanation

ChIP protocol for mature male gonad

Version 5.4 – CIGENE/NMBU

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ Equipment:
  - microscope, cell counting chamber, Trypan Blue/Hoescht
  - tweezers
  - a Douncer with pestles
  - a probe sonicator / sonication device
  - an ice bucket
  - a 15-ml centrifuge set-up at 4˚C
  - a 1.5/2-ml centrifuge set-up at 4˚C
  - a metallic eppendorf tubes holder
  - an incubator set-up at 68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - Add PIC, PMSF and sodium butyrate to PBS (named below PBS*plus*)
  - PBS with PIC
  - Add PIC, PMSF and sodium butyrate to the sonication buffer (named below complete sonication buffer)
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - refrigerate the metallic Eppendorf tubes holder in the fridge at 4˚C
  - pour 4 ml of PBS*plus* in a Douncer, and place it in the ice bucket
  - take the appropriate volume of formaldehyde solution 1% from the fridge and place it at room temperature before use
  - Prepare one Iodixanol gradient 1 in a 5-mL tube and two Iodixanol gradient 2 in 2-mL tubes

Tissue disruption

The procedure (step 1 to 10) should take less than 40 min (step 1 to 6 = 30 min, step 7 to 10 = 10 min) and the samples should stay in cold environment as much as possible. Glass homogenizers must be used in priority to plastic ones, due to better conductivity of cold

1. Transfer pieces of gonad (400 mg to 600 mg recommended for mature male gonad [1]) into a 5-ml Eppendorf tube containing 1 ml of PBS*plus*
2. Squeeze the tissue pieces with the help of a plastic device or else to release the sperm. Vortex full speed for 30 seconds
3. Filtrate the solution through a 40-µm strainer* placed on the top of a 50-ml Falcon tube
   ** change the strainer every 2 washings*
4. Rinse the tissue pieces with 500 µl of PBS*plus*
5. Discard the supernatant and transfer the tissue pieces from the filter to the initial 5-ml Eppendorf tube. Add 1 ml of PBS*plus*
6. Repeat steps 2 to 5 two to six times, or more if necessary, to release sperm from the tissue as much as possible

[1] This protocol will have to be performed 2 to 4 times to have a good amount of chromatin generated from the gonad and not from the gametes. This point is important for successful immunoprecipitation

7 Transfer the pieces of tissue into a Douncer containing 4 ml [2] of PBS*plus*
8 Use pestles A and B for the homogenization, 5 strokes each or until you obtain a homogenized solution or feel no more strong resistance
9 Recover the solution into a clean 5-ml Eppendorf tube [3]. Avoid transferring pieces of tissue
10 Rinse the Douncer and the pestles with 1 ml of PBS*plus*. Add it to the previous solution. You reach 5 ml
11 Centrifuge 10 min, 3 000 g, 4˚C
12 Discard the maximum of the supernatant by avoiding contact with the pellet and add 800 µl of fresh PBS*plus*
13 Add 560 µl of iodixanol 60% to the solution to obtain 1400 µl of 25% iodixanol solution with filtrate
14 Homogenize the 25% iodixanol solution with filtrate and carefully, drop by drop, place this solution on the top of the Iodixanol gradient 1
15 Centrifuge 30 min, 3 200 g, 4˚C, centrifuge breaks OFF
A band should be visible between the 29% and 40% iodixanol layers
16 Pipet and discard the first two layers (25% and 29% iodixanol layers)
17 Carefully pipet the remaining 29% iodixanol solution before reaching the nuclear layer
18 Recover the nuclear layer into a clean 1.5-ml Eppendorf tube (200-400 µl)
19 To the volume recovered in step 19, add 0.7 volume of PBS*plus* to obtain a 15-20% iodixanol solution with nuclei
20 Homogenize the 15-20% iodixanol solution with nuclei
21 Let sediment the solution for 5 to 10 min in ice
22 Transfer only the supernatant of the solution into another clean 1.5-ml Eppendorf tube. Avoid transferring pieces of tissue or big aggregates
23 Transfer half of this solution, drop by drop, on the top of the layer of each 2-ml tube containing the Iodixanol gradient 2
24 Centrifuge 40 min, 12 000 g, 4˚C, centrifuge breaks OFF
A band should be visible between the 15-20% and 50% iodixanol layers
25 For **each tube**, pipet and discard the first gradient solution containing 15-20% iodixanol solution
26 For **each tube**, carefully pipet the remaining 15-20% iodixanol solution before reaching the nuclear layer
27 Recover the nuclear layer from **each tube** into the **same** clean 1.5-ml LoBind Eppendorf tube (100-200 µl)
28 Dilute 10 times the nuclear layer with PBS*plus* to obtain a clean solution with nuclei
29 Centrifuge 10 min, 5 000 g, 4˚C
30 Discard the supernatant and resuspend the nuclei pellet with 2.5 ml of clean PBS*plus*
31 After homogenization of the tube, take an aliquot of 10 µl to assess the number of nuclei (dilute 2x with Trypan blue or add Hoescht for microscopy observation)

Counting: **nuclei/ml**
Counting: **sperm/ml**
Ratio nuclei/sperm:
2.5 ml = **million nuclei (not sperm) total** (expected: 4 to 6 million)

NB: Count only the "normal" nuclei. We expect a ratio "nuclei from cells/sperm" from 1/2 to 1/5. If you observe a ratio over 1/10, please contact us

Crosslinking

32 To the 2.5 ml* solution of PBS*plus* with nuclei, add 67.5 µl of formaldehyde solution 37% at room temperature (RT) and mix well
**if more than 24 million of nuclei, resuspend in 5 ml of formaldehyde solution 1% and split in two tubes*
33 Incubate under constant agitation [4] at RT, 5 min
34 Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 min at RT, under constant agitation
35 Centrifuge 5 min, 800 g, 4˚C
36 Remove and eliminate in appropriate trash the supernatant containing formaldehyde
37 Resuspend with 4 ml* of PBS with PIC by pipetting (quick vortex if necessary)
**if more than 24 million of nuclei were crosslinked in two tubes, resuspend each pellet in 2 ml of PBS with PIC, and merge them*
38 Centrifuge 5 min, 800 g, 4˚C

[2] for Douncer under 5 ml, split the solution accordingly
[3] or a 15-ml canonical Falcon tube
[4] Place the tube in the Hula Mixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)

39 Remove the supernatant
40 Resuspend with 1 ml of PBS with PIC and transfer in a clean 1.5-ml Eppendorf tube
41 Centrifuge 10 min, 3 000 g, 4˚C
42 Remove the supernatant
SAFE STOP POINT: Freeze the nuclei pellet in dry ice. Store in -80 ˚C for 2 months maximum. Please refer to the guidelines for more informations

Sonication

43 Resuspend the pellet by following the table;

Ratio nuclei/sperm	Number of million of nuclei by 300 µl of sonication buffer	Number of sonication cycles
1/5	1.5	18
1/4	2	18
1/3	2.5	20
1/2	3	20

Pipet up and down until the pellet is completely dissolved. Keep the solution in ice 5 min before the start of the sonication

44 Take out from the fridge the Qubit BR kit and Bioanalyzer DNA D1000 kit [5] or Tape Station D1000 kit, and set-up the incubator at 68˚C
45 Place the metal Eppendorf holder from the fridge on ice. Aliquot your solution by 300 µl
46 Keep the tubes on the holder all along the process
47 Sonicate the chromatin with the following parameters [6]

Number of cycles	**See table above**
Intensity	55%
Cycle rhythm (On/Off period)	1 sec/1 sec [7]
Cycle time	30 sec

48 Centrifuge the tubes of sonicated chromatin 10 min, 8 000 g, 4˚C
49 Transfer the supernatant into a new LoBind 1.5-ml Eppendorf tube. Note the volume
Volume of sonicated chromatin before dilution: µl
If sonication done in multiple tubes, keep them separated until the quality control of the sonication for each tube
50 Take out an aliquot of 20 µl for sonication control by tube of transferred supernatant
51 Dilute the remaining chromatin by adding 3 volumes of complete IP buffer
SAFE STOP POINT: Place the tubes of diluted sonicated chromatin for storage in -80 ˚C

Test of the sonication

52 To the 20 µl for sonication control from step 50, add 67 µl of elution buffer and 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M
53 Incubate 1H30, 68˚C, under agitation [8]
54 Purify with a Qiagen DNA Minelute PCR purification kit. Elute with 20 µl of elution buffer
55 Quantify by Qubit the chromatin concentration of 1 µl of eluate. Report the value
Qubit concentration: ng/µl
56 Run 1 µl of the eluate in the Bioanalyzer DNA 1000 ChIP or Tape Station to assess the size profile
To be qualified as good for ChIP, minimum 70% of the sonicated chromatin should have a size distribution of 100-600 bp, centered around 350 bp
Later, you can pool together the tubes with this expected distribution of size of fragmented chromatin to run the immunoprecipitation
To calculate the average concentration of your final pool, don't forget to take into account the difference of volume of chromatin you did recover

[5] You can wait to have enough sample to run a full Bioanalyzer chip
[6] These parameters have been established for the device "EpiShear Probe Sonicator" from Active Motif
[7] 1 cycle is 30 sec total, composed by a repetition of 1 sec ON/ 1 sec OFF. The total ON period is 15 sec
[8] 500 rpm for a Thermomixer from Eppendorf

Version history

Version 5.4 – 2021.03.25	Precision in the conclusion of the protocol about the range of size and the calcul of the average concentration
Version 5.3 – 2021.03.15	Avoid one centrifugation Definition of number of cycles of sonication Definition of the number of nuclei by tube of 300 µl of sonication buffer depending of the ratio nuclei/sperm Correction of the dilution factor for counting step Add of a supplemental Safe Stop Point before sonication
Version 5.2 – 2021.02.18	Avoid some steps of washing to reduce loss of nuclei
Version 5.1 – 2021.01.18	Improvement explanation iodixanol gradient
Version 5.0 – 2021.01.08	Complete new version of the protocol Start of the “version history” section

Day2 ChIP protocol – all tissues

Version 4.1 – NMBU/CIGENE

Day 2 – Beads preparation and immunoprecipitation

Preparations

- ☐ Equipment:
  - Hula mixer or wheel in a fridge or refrigerated room (4°C) for rotation of the samples
  - Protein A and protein G beads
  - Antibodies
  - an ice bucket
  - a 1.5/2-ml centrifuge
  - an incubator
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
- ☐ To do:
  - Calculate the volume of chromatin to pre-clear to perform the desired amount of immunoprecipitation (5 µg by IP recommended and 50 ng input)
  - Calculate the amount of beads to prepare by immunoprecipitation and for pre-clearing

(you can follow the table given at the end of this Day2 protocol for help)

Washing of the beads, antibody coupling and for pre-clearing of the chromatin

1 Prepare a total mix of beads as followed (by IP)[1];
- 33 µl of Protein A beads
- 33 µl of Protein G beads
- 286 µl of complete IP buffer

2 Mix by pipetting and/or vortexing and centrifuge shortly to collect beads on the lid

3 Put on magnet 1-2 mins

4 **Discard** supernatant

5 Remove the tube from the magnet

6 Add same volume of complete IP buffer

7 Repeat 2 more times the steps 2 to 6

8 Add same volume of complete IP buffer

The coupling of antibodies to the beads and the pre-clearing can be performed in parallel

I - COUPLING OF THE ANTIBODIES TO THE BEADS

8.1. Mix 130 µl of beads with the appropriate amount of antibodies

II – PRE-CLEARING OF THE CHROMATIN

8.2. To each 5 µg of diluted chromatin used for immunoprecipitation, add 130 µl of beads

9 Put in rotation the tubes from steps 8.1 and 8.2 at room temperature for 30 min

[1] These values take into account a loss of material due to pipetting

10 Place the tubes containing the beads coupled with antibodies (I) on the magnetic rack
11 **Discard** the supernatant
12 Place the tubes containing the pre-cleared sonicated chromatin (II) on the magnetic rack
13 Take out the volume equivalent to 50 ng for input. Store in 4°C
14 **Transfer** the equivalent of 5 µg to the tubes containing the beads couples with antibodies
15 To each final tube (chromatin + beads coupled with anitbodies), add 0.5 volume of complete IP buffer
16 Rotate the tubes overnight (12-16H) at 4°C

Day 3 ChIP protocol – all tissues

Version 3.5 – CIGENE/NMBU

Day 3 – Wash beads, decrosslinking and DNA recovery

Preparations

- ☐ Equipment:
  - a 1.5/2ml centrifuge set-up at 4˚C
  - a magnetic rack
  - a mini-centrifuge
  - an incubator set-up at 65˚C/68˚C
- ☐ Solutions to prepare (see **Buffers for ChIP**):
  - Add PIC, PMSF and sodium butyrate to the IP buffer (named below complete IP buffer)
  - Add PIC, PMSF and sodium butyrate to the low salt wash buffer (named below complete low salt wash buffer)
  - Add PIC, PMSF and sodium butyrate to the high salt wash buffer (named below complete high salt wash buffer)
  - Add PMSF to recovery buffer

Wash of the beads

1. Pulse spin the tubes, put on magnet and discard supernatant
2. Resuspend the beads in 200 µl of complete IP buffer
3. Pulse spin the tubes, put on magnet and discard supernatant
4. Resuspend the beads in 200 µl of complete low salt wash buffer, put in the magnet, discard supernatant
5. Repeat step 4
6. Resuspend the beads in 200 µl of complete high salt wash buffer, put in the magnet, discard supernatant
7. Repeat step 6
8. Resuspend the beads in 57 µl of recovery buffer
9. Elute the DNA from the beads for 1H30 at 65˚C, under agitation [1]
10. Pulse spin the tubes and place on magnetic rack
11. Transfer the eluted chromatin to new clean LoBind 1.5-ml Eppendorf tubes
12. Wash the beads with 30 µl of recovery buffer. Mix and pulse spin the tubes
13. Place the tubes on magnetic rack. Transfer this volume to the eluted chromatin from step 11 (87 µl final)
14. Take out the "input control" tube stocked in the fridge
15. If the volume of input is under 82 µl, complete with recovery buffer until 82 µl

    The input tube and the eluted chromatin from IP tubes are ready for DNA extraction

One-step decrosslinking

16. For the tubes containing the IPs of step 13 (87 µl), add 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M
17. For the input of step 15, **by 82 µl**, add 5 µl of SDS 20%, 2 µl of RNase A, 5 µl of proteinase K and 6 µl of NaCl 5M
18. Incubate 1H30, 68˚C, under agitation [2]
19. Take out from the fridge the Qubit HS kit

[1] 1000 rpm into an Eppendorf Thermomixer
[2] 500 rpm into an Eppendorf Thermomixer

Purification and quantification

20 Purify the samples with MinElute PCR purification kit. Elute from the column with 32 µl of low TE buffer

21 Quantify the samples with Qubit HS kit as followed;

Input	H3K4me1	H3K4me3	H3K27ac	H3K27me3	CTCF
1 µl of a 1/10 dilution	**6 µl**	**6 µl**	**2 µl**	**2 µl**	**4 µl**

The DNA from the input and immunoprecipitated chromatin can be stored at – 20˚C until library preparation

Library preparation

Kit

The kit which has been used is Microplex v3 (Diagenode).
See kit specifications to process to library preparations.

Supplemental informations – number of cycles of amplification applied;

DNA Input (ng)	Number of Cycles
50	5
20	6
10	7
5	8
3	**9**
2	10
1	11
0.5	**12**
0.3	**13**
0.2	14
0.08	**15**
0.05	16

If possible, please start from minimum 0.2 ng. You should start from a volume of 10 µl.

Version history

Version 3.5 – 2021.03.29	Change of the name of the elution buffer from beads to recover the chromatin which has been captured. From ChIP elution buffer to recovery buffer
Version 3.4 – 2021.03.19	Explanations added for the treatment of the input
Version 3.3 – 2021.01.25	Application of the template Simplification of the comments for sonication part Correction of the volume of elution buffer for aliquot digestion
Version 3.2 – 2020.10.01	Add of a version history section

diagenode

Innovating Epigenetics Solutions

MicroPlex Library Preparation Kit v3

High Performance Library Preparation for Illumina® NGS Platforms

Cat. No. C05010001 (48 rxns)
C05010002 (96 rxns)

USER GUIDE

Version 1 01_07_2019

Please read this manual carefully
before starting your experiment

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Introduction

The MicroPlex Library Preparation Kit v3 combined with Diagenode's Dual Indexes for MicroPlex v3 (available separately) generates indexed libraries with multiplexing for up to 384 samples for sequencing on Illumina NGS platforms. Once purified and quantified, the resulting dual indexed libraries are ready for Illumina NGS instruments using standard Illumina sequencing reagents and protocols.

Generated libraries are compatible with **single-end or paired-end** sequencing. MicroPlex chemistry is specifically developed and optimized to generate DNA libraries with high molecular complexity from the lowest input amounts. Only **50 pg to 50 ng** of fragmented **double-stranded DNA** is required for library preparation. The entire **three-step workflow** takes place in a **single tube or well** in about **2 hours**. **No intermediate purification steps and no sample transfers** are necessary to prevent handling errors and loss of valuable samples. The kit provides excellent results for

- DNA-seq (high-coverage, deep sequencing: de novo sequencing, whole genome resequencing, whole exome sequencing, cell-free plasma DNA sequencing)
- Enrichment techniques: ChIP-seq, MethylCap-seq
- RNA-seq (cDNA)

The MicroPlex Library Preparation Kit v3 can be used manually or with automation using the IP-Star Compact Automated System. The corresponding protocols are included in this manual.

Kit method overview

The MicroPlex Library Preparation Kit v3 is based on patented MicroPlex technology (Figure 1). Unlike other NGS library preparation kits, which are based on ligation of Y-adapters, MicroPlex uses stem-loop adapters to construct high quality libraries with a fast and efficient workflow:

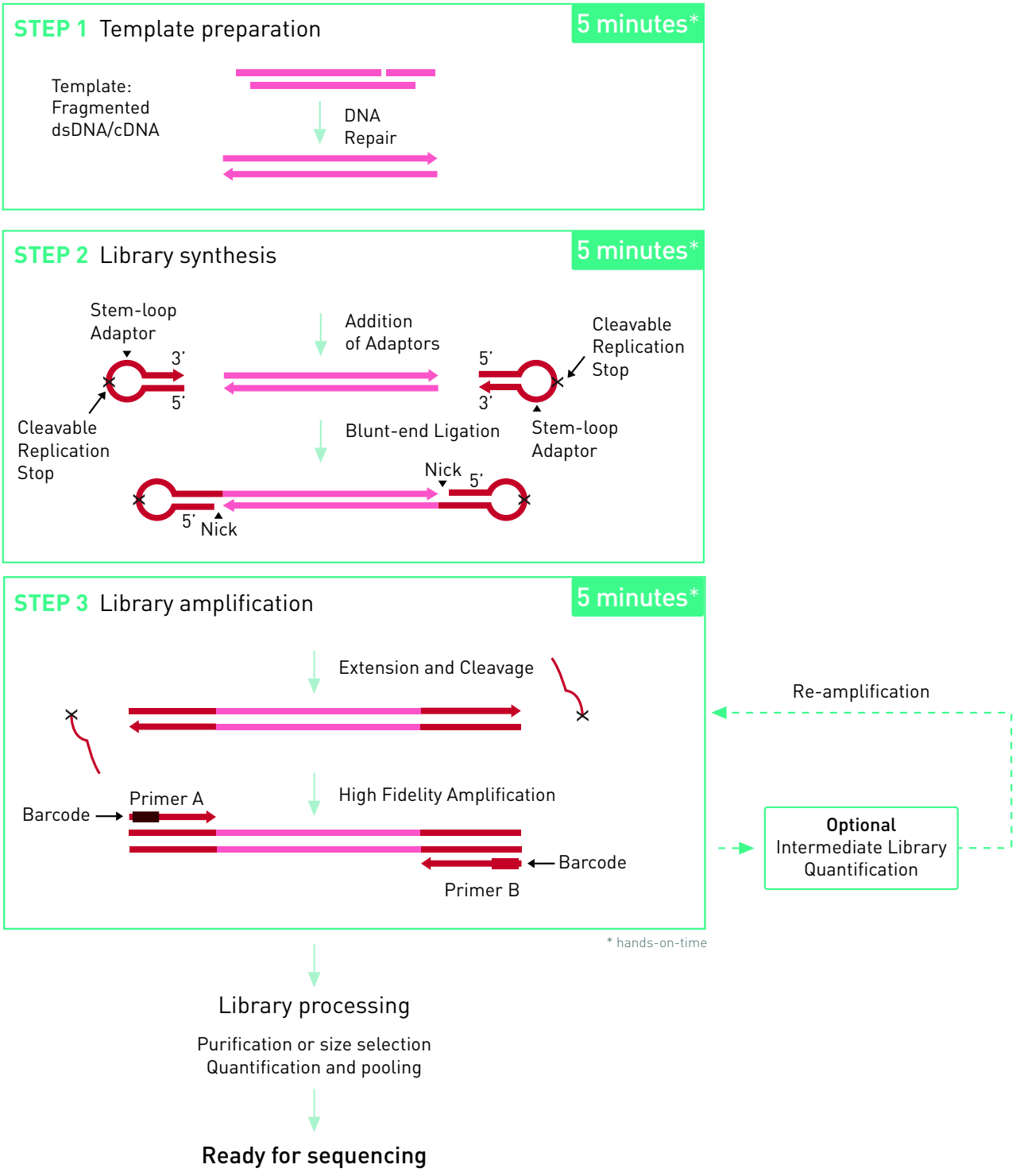


Figure 1. MicroPlex technology.

Step 1. Template Preparation provides efficient repair of the fragmented double-stranded DNA input.

In this step, the DNA is repaired and yields molecules with blunt ends.

Step 2. Library Synthesis enables ligation of MicroPlex patented stem-loop adapters.

In the next step, stem-loop adaptors with blocked 5' ends are ligated with high efficiency to the 5' end of the genomic DNA, leaving a nick at the 3' end. The adaptors cannot ligate to each other and do not have single-strand tails, both of which contribute to non-specific background found with many other NGS preparations.

Step 3. Library Amplification enables extension of the template, cleavage of the stem-loop adaptors, and amplification of the library. Illumina-compatible dual indexes are also introduced using a high-fidelity, highly-processive, low-bias DNA polymerase.

In the final step, the 3' ends of the genomic DNA are extended to complete library synthesis and Illumina-compatible dual indexes are added through a high-fidelity amplification. Any remaining free adaptors are destroyed. Hands-on time and the risk of contamination are minimized by using a single tube and eliminating intermediate purifications.

Obtained libraries are purified, quantified and sized. The libraries pooling can be performed as well before sequencing (Figure 1).

Kit materials

The MicroPlex Library Preparation Kit v3 contains sufficient reagents to prepare up to the specified number of reactions. Enough buffers and enzymes are provided for four uses or freeze-thaw cycles. Contents of the MicroPlex Library Preparation Kit v3 are not interchangeable with other Diagenode products. The dual indexes compatible with this kit have to be purchased separately (listed in the paragraph: Required materials not provided).

Table 1. MicroPlex Library Preparation kit v3

Name	Cap colour	µl/rxn	48 rxns	96 rxns
Dual Template Preparation Buffer	Red	2	105 µl	205 µl
Dual Template Preparation Enzyme	Red	1	50 µl	105 µl
Dual Library Synthesis Buffer	Yellow	1	50 µl	105 µl
Dual Library Synthesis Enzyme	Yellow	1	50 µl	105 µl
Dual Library Amplification Buffer	Green	25	1260 µl	2 x1275 µl
Dual Library Amplification Enzyme	Green	1	50 µl	105 µl
Nuclease-Free Water	Clear	4	500 µl	500 µl

SHIPPING AND STORAGE: The MicroPlex Library Preparation Kit v3 is shipped on dry ice. The kit should be stored at –20°C upon arrival.

The volumes of buffers and enzymes mentioned above are the minimum volumes required to complete 48 or 96 reactions. However, an excess is included in each tube to cover pipetting loss.

Required materials not provided

- Barcoded primers

The following validated barcoded primers (sold separately) should be used with the Microplex Library Preparation Kit v3:

Product	Cat. No.	Number of dual indexes	Number of rxns	Format
24 Dual indexes for MicroPlex Kit v3	C05010003	24	48 rxns	Tubes
96 Dual indexes for MicroPlex Kit v3 – Set I	C05010004	96	96 rxns	Plate
96 Dual indexes for MicroPlex Kit v3 – Set II	C05010005	96	96 rxns	Plate
96 Dual indexes for MicroPlex Kit v3 – Set III	C05010006	96	96 rxns	Plate
96 Dual indexes for MicroPlex Kit v3 – Set IV	C05010007	96	96 rxns	Plate

Barcoded primers can be used not only for high-level multiplexing (up to 384 samples) but also for low-level multiplexing of a small number of samples. It is important to select unique index combinations that meet Illumina-recommended compatibility requirements. Please refer to Illumina's technical manuals (Index Adapters Pooling Guide, Illumina Document # 1000000041074 v06) for additional information.

- Low binding aerosol barrier tips
- Freshly prepared 80% (v/v) ethanol
- Agencourt® AMPure® XP beads (Beckman Coulter, Cat. No. A63880)
- Magnetic rack:
  - Compatible with 0.2 ml PCR tubes if working with tubes or strips (eg. Diagenode DiaMag 0.2ml , Cat. No. B04000001)
  - Alternatively: 96 well plate magnetic rack – if working with PCR plates
- Low TE buffer pH 8.0 (10 mM Tris, 0.1 mM EDTA, pH 8.0), molecular grade

- Centrifuge
- Thermal cycler

***NOTE**: Use a thermal cycler equipped with a heated lid that can handle 50 µl reaction volumes. Set the temperature of the heated lid to 101°C – 105°C to avoid sample evaporation during incubation and cycling. We recommend a ramp rate of 3°C/s – 5°C/s; higher ramp rates are not recommended and could impact the quality of the library.*

- 0.2 ml PCR tubes or 96-well PCR plates and seals

***NOTE**: Select appropriate tubes or plates that are compatible with the thermal cyclers used. Use appropriate caps or sealing films and seal thoroughly to eliminate evaporation during cycling conditions. Evaporation could reduce robustness and reproducibility of the reactions.*

- BioAnalyzer® (Agilent) or Fragment Analyzer® (Advanced Analytical) (for library quantification and sizing)
- High sensitivity DNA kit from Agilent or High Sensitivity NGS Fragment Analysis kit (35 bp-6000 bp) from Advanced Analytical
- Qubit 2.0 Fluorometer® (Life Technologies) or Quant-iT™PicoGreen® dsDNA Assay Kit (Life Technologies) (for library quantification). Optional: KAPA® Library Quantification Kit – Illumina (Kapa Biosystems), specific to a real time PCR system used) and real time PCR system can be also used.

Additional supplies if working with IP-Star Compact:

- IP-Star Compact Automated System (Diagenode, Cat. No. B03000002)
- 200 µl tube strips (8 tubes/strip) + cap strips (Diagenode, Cat. No. C30020002)
- Tips (box) (Diagenode, Cat. No. C30040021)
- Tips (bulk) (Diagenode, Cat. No. C30040020)
- 2 ml microtube (Diagenode, Cat. No. C30010014)
- Medium reagent container (Diagenode, Cat. No. C30020003)
- 96 well microplates (Diagenode, Cat. No. C30080030)

Quality Control

The MicroPlex Library Preparation Kit v3 is functionally tested using Next Generation Sequencing (NGS) to ensure product quality and consistency.

Safety Information

Follow standard laboratory safety procedures and wear a suitable lab coat, protective goggles and disposable gloves to ensure personal safety as well as to limit potential cross contamination during the sample preparation and subsequent amplification reactions.

Remarks before starting

DNA format

Fragmented **double-stranded DNA** (gDNA or cDNA), chromatin immunoprecipitates (ChIP), degraded DNA from sources such as FFPE, plasma, or other biofluids are suitable. This kit is **not** for use with single-stranded DNA (ssDNA) or RNA.

Input DNA amount

Input DNA in the range of **50 pg to 50 ng** can be used as starting material. However, for the highest sequencing data quality it is recommended to use as much DNA as possible. For deep whole genome sequencing (WGS) and whole exome sequencing (WES) using human gDNA, FFPE, or plasma DNA, greater than 10 ng of input DNA is recommended to achieve a highly diverse library. For sequencing samples with reduced complexity, such as cDNA, immunoprecipitated DNA from ChIP, bacterial DNA, or targeted genomic regions, lower input amounts (picogram levels) can be used.

Fragment Size

The optimal DNA fragment size is **less than 1,000 bp**. The MicroPlex Library Preparation Kit v3 is a ligation-based technology and **adapters** added during the process result in an approximately **140 bp** increase in the size of each DNA template fragment. Library molecules with shorter inserts (200 – 300 bp) tend to cluster and amplify more efficiently on the Illumina flow cell. Depending on the application and requirements, the AMPure purification step following the final step (library amplification) can be replaced with AMPure size-selection step to remove unwanted large fragments.

Input Volume

The maximum input sample volume is **10 µl**. If a sample is in a larger volume, the DNA must be concentrated into 10 µl or less. Alternatively, the sample may be split into 10 µl aliquots, processed in separate tubes,

and the corresponding products pooled prior to the purification step preceding sequencing.

Input Buffer

Input DNA must be eluted or resuspended in a **low-salt and low-EDTA buffered solution**. The preferred buffer is low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0). The concentrations of Tris and EDTA must not exceed 10 mM and 0.1 mM, respectively. Avoid phosphate containing buffers.

Positive and Negative Controls

If necessary, include appropriate positive and negative controls in the experimental design to help verify that reactions proceed as expected. If the experimental samples contain any carryover contaminant(s) in the buffer, the downstream reactions may be impacted, and inclusion of controls would help elucidate such problems. A suitable positive control (reference DNA) is Bioruptor-sheared purified genomic DNA (200 – 300 bp) of comparable input amount. Always prepare fresh dilutions of reference DNA. Include a negative control (No Template Control, NTC) with low TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0) or nuclease-free water. The positive control and experimental samples should perform equivalently, while the NTC should not amplify.

Preparation of Master Mixes

A master mix with appropriate buffers and enzymes must be prepared at each workflow step based on the number of reactions to be performed. Transfer the enzymes to ice just prior to use and centrifuge briefly to collect contents at the bottom of the tube. Thaw the buffers, vortex briefly and centrifuge prior to use. Keep all the components and master mixes on ice. Once the master mix is prepared, thoroughly mix the contents several times with a pipette while avoiding introduction of excessive air bubbles and briefly centrifuge prior to dispensing into the PCR plate or tube(s).

Indexing Reagents

The Indexing Reagents consist of amplification primers containing Illumina-compatible indexes. Index sequences, index plate handling instructions, multiplexing and index pooling guidelines can be found in the manual of the Dual Indexes for MicroPlex v3.

Library yield and selection of the optimal number of cycles for library amplification

The requirements for a final library concentration depend on a sequencer and may vary between different sequencing service providers. The usual range is between **5-20 nM** in a final volume **10-15 µl** but we recommend inquiring with your sequencing platform.

The number of PCR cycles required at Step 3 of the protocol (Library Amplification) is dependent on the amount of input DNA, quality, fragmentation size and a thermal cycler. Note that an over-amplification could result in higher rate of PCR duplicates in the library.

When working with DNA sample of known quantity or quality, use the **Amplification Guide** for selecting the number of PCR cycles.

When working with DNA samples of unknown quantity and/or quality (such as DNA from FFPE tissue or environmental sample, low input samples below quantification limit etc.), amplify samples for **12 cycles** and perform an intermediate quantification of unpurified libraries as described at OPTIONAL INTERMEDIATE LIBRARY QUANTIFICATION STEP. Depending on the yield, libraries can be re-amplified for few additional cycles to achieve a desired yield or purified.

Library purification or size selection

Purification using Agencourt AMPure® XP (Beckman Coulter) is the preferred method because sequence complexity is conserved. Do not use silica-based filters for purification. The optimal library size is dictated by the sequencing application and a sequencer specification. The final library size corresponds to the initial size of DNA fragments plus approximately 140 bp due to the ligated adapters. Most part of applications will not require

a size selection if the initial DNA fragment size is less than 1,000 bp. In this case, amplified products should be purified by Agencourt AMPure XP (Beckman Coulter) to get rid of primers and adaptors (See section "Library processing, option A"). If the size selection is required, follow the protocol described in the section "Library processing, option B" to get rid of primers/adaptor and fragments above 600 bp.

Library Quantification

There are several approaches available for library quantification including real-time PCR, UV absorption, fluorescence detection, or sizing and quantification using the Agilent Bioanalyzer or Fragment Analyzer (Advanced Analytical). It is important to understand the benefits and limitations of each approach.

Real-time PCR-based approaches (such as the KAPA Library Quantification Kit from Kapa Biosystems) quantify the library molecules that carry the Illumina adapter sequences on both ends and, therefore reflect the quantity of the clustering competent library molecules. This approach assumes a relatively uniform size of sheared or fragmented starting gDNA inserts used for library construction. Quantification by PCR can be done on unpurified libraries.

The Agilent Bioanalyzer system or Fragment Analyzer (Advanced Analytical) provide sizing and quantification information about the library analysed, but not about the clustering competency. Quantification can be done both on unpurified or purified samples. In a case of unpurified samples, a region corresponding to libraries should be limited in order to discriminate between primers/adaptors and a library itself.

UV absorption/fluorescence detection-based methods (i.e., Nanodrop® (Thermo Scientific), Qubit®2.0 Fluorometer (Life Technologies), or Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) simply quantify total nucleic acid concentration. These methods do not discriminate adapter presence and offer no information about the size of the library molecules. They can be used only on purified libraries. We better recommend fluorescence-based assays than spectrophotometric measurements (e.g. NanoDrop) due to higher specificity and sensitivity.

PROTOCOL

MANUAL PROCESSING

PROTOCOL FOR LIBRARY PREPARATION

STEP 1

Template Preparation

1.1 Dispense **10 µl** of fragmented doubled-stranded DNA into each PCR tube or well of a PCR plate compatible with your thermal cycler.

***NOTE**: Positive control reactions using reference DNA: If necessary, assemble reactions using 10 µl of a reference gDNA (e.g., Bioruptor-fragmented DNA, 200-300 bp average size) at an input amount comparable to that of the samples.*

***NOTE**: Negative control reactions (NTCs): If necessary, assemble NTCs with 10 µl of nuclease-free water or TE buffer (e.g., 10 mM Tris, 0.1 mM EDTA, pH 8.0).*

1.2 Prepare **Template Preparation Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Component	Volume for 1 sample
Dual Template Preparation Buffer (red cap)	2.0 µl
Dual Template preparation Enzyme (red cap)	1.0 µl

1.3 Add **3 µl** of the **Template Preparation Master Mix** to each 10 µl sample from step 1.1 above. Mix thoroughly with a pipette.

1.4 Tightly cap the tube(s) or seal the PCR plate using an appropriate sealing film.

1.5 Centrifuge briefly to ensure the entire volume of the reaction is collected at the bottom of each tube or well.

1.6 Place the tube(s) or plate in a thermal cycler with heated lid set to 101°C – 105°C. Perform the **Template Preparation Reaction** using the following settings:

Template Preparation Reaction	
Temperature	Time
22°C	25 min
55°C	20 min
4°C	Hold for ≤ 2 hours

1.7 After the thermal cycler reaches 4°C, remove the plate or tube(s) and centrifuge briefly.

1.8 Continue to Library Synthesis Step in the same tube(s) or plate.

STEP 2

Library Synthesis

2.1 Prepare **Library Synthesis Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until used.

Component	Volume for 1 sample
Dual Library Synthesis Buffer (yellow cap)	1.0 µl
Dual Library Synthesis Enzyme (yellow cap)	1.0 µl

2.2 Add **2 µl** of the **Library Synthesis Master Mix** to each tube or well. Mix thoroughly with a pipette.

2.3 Tightly cap the tube(s) or seal the PCR plate using an appropriate sealing film.

2.4 Centrifuge briefly to collect the contents to the bottom of each well or tube.

2.5 Return the tube(s) or plate to the thermal cycler with heated lid set to 101°C – 105°C.

2.6 Perform **Library Synthesis Reaction** using the following settings:

Library Synthesis Reaction	
Temperature	Time
22°C	40 min
4°C	Hold for ≤ 30 min

2.7 After the thermal cycler reaches 4°C, remove the tube(s) or plate and centrifuge briefly.

2.8 Continue to the Library Amplification Step in the same tube(s) or plate maintained at 4°C.

STEP 3

Library Amplification

***NOTE**: The dual indexes compatible with this kit have to be purchased separately (see: Required materials not provided) . Select the right dual index for MicroPlex kit v3 and follow the guidelines from this kit regarding index handling.*

3.1 Prepare the **Dual Indexing Reagents** (available separately) as follows:

- Remove the Dual Indexing Reagents from freezer and thaw for 10 minutes on the bench top.
- Spin the Dual Indexing Reagents in a table top centrifuge to collect contents at the bottom of the well.
- Thoroughly wipe the Dual Indexing Reagent Tubes or Dual Indexing Reagent Plate foil seal with 70% ethanol and allow it to dry.

3.2 Prepare the **Library Amplification Master Mix** as described in the table below for the desired number of reactions. Mix thoroughly with a pipette. Keep on ice until use.

Component	Volume for 1 sample
Dual Library Amplification Buffer (green cap)	25.0 µl
Dual Library Amplification Enzyme (green cap)	1.0 µl
Nuclease-Free Water (clear cap)	4.0 µl

3.3 Add **30 µl** of the **Library Amplification Master Mix** to each tube or well containing 15 µl of Library Synthesis Reaction Product.

3.4 Add **5 µl** of the appropriate **Dual Indexing Reagent** (available separately) to each well or tube.

***NOTE:** if using 96 Dual Indexes for MicroPlex Kit v3 (Set 1-4):*

- Thoroughly wipe the Dual Indexes Plate foil seal with 70% ethanol and allow it to dry to prevent cross-contamination.
- Make sure the two corner notches of the Dual Indexing Reagent Plate are on the left, and the barcode label on the long side of the index plate is facing you.
- Use a clean pipet tip to pierce the seal above the specific indexing reagent on the Dual Indexing Reagent Plate; discard the tip used for piercing.
- Use a clean pipet tip to collect 5 µl of the indexing reagent and add to the reaction mixture.

3.5 Mix thoroughly with a pipette. Avoid introducing excessive air bubbles.

3.6 Seal the PCR plate or tube(s) tightly and centrifuge briefly to collect the contents to the bottom of each well or tube.

3.7 Return the tube(s) or plate to thermal cycler with heated lid set to 101°C – 105°C.

3.8 Perform **Library Amplification Reaction** using the cycling conditions in the tables below.

***CAUTION**: Ensure that the thermal cycler does not have a denaturing step programmed until Stage 3.*

	Stage	Temperature	Time	Number of Cycles
Extension & Cleavage	1	72°C	3 min	1
	2	85°C	2 min	1
Denaturation	3	98°C	2 min	1
Addition of Indexes	4	98°C	20 s	4
		67°C	20 s	
		72°C	40 s	
Library Amplification	5	98°C	20 s	5 to16*
		72°C*	50 s	
Hold	6	4°C	Hold	1

** If working with **DNA samples of known quantity or quality**, please use the **Amplification Guide** below to select the required number of amplification cycles.*

Amplification guide (for stage 5)	
DNA Input (ng)	**Number of Cycles**
50 ng	6 - 8
20 ng	7 - 8
10 ng	7 - 8
5 ng	7 - 9
2 ng	8 - 10
1 ng	11 - 12
0.2 ng	14 - 15
0.05 ng	15 - 16

*If working with **DNA samples of unknown quantity** and/or low quality (eg. FFPE extracted DNA), amplify for **12 cycles** and perform an **INTERMEDIATE LIBRARY QUANTIFICATION OF UNPURIFIED LIBRARY** to estimate the library yield . If the desired yield is not achieved, the libraries can be re-amplified for few additional cycles.*

3.9 Remove the tube(s) or PCR plate from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.

3.10 Proceed to the **Library Purification by Agencourt AMPure XP** in the section "Library processing" (recommended for samples of known quantity or quality) or to the **Intermediate Library Quantification** option shown below (recommended for samples with unknown quantity and/or low quality).

Optional Intermediate Library Quantification

(recommended for samples with unknown quantity and/or a low quality)

Quantify **unpurified** libraries using the BioAnalyzer, the Fragment Analyzer or similar devices. This intermediate quantification allows estimating the library yield and enabling an additional re-amplification if needed. You will need a High Sensitivity NGS Fragment Analysis kit (1 bp-6,000 bp) from Advanced Analytical if using Fragment Analyzer or High Sensitivity DNA kit from Agilent if using BioAnalyzer. Follow the manufacturer's instruction for kit handling and protocol.

> ***NOTE**: The quantification of unpurified library by qPCR (e.g. using KAPA® Library Quantification Kit – Illumina (Kapa Biosystems), is also possible at this step while it will increase the total protocol duration.*

1. Take an aliquot of unpurified libraries: **1 µl** if the BioAnalyzer will be used or **2 µl** if the Fragment Analyzer will be used. Keep remaining libraries at 4°C .

2. Load the aliquot of unpurified library on the Fragment Analyzer or BioAnalyzer and run the analysis.

3. Estimate the library yield using a region selection option to discriminate between unincorporated primers/adaptors and libraries themself as it is shown in the Figure 2 below.

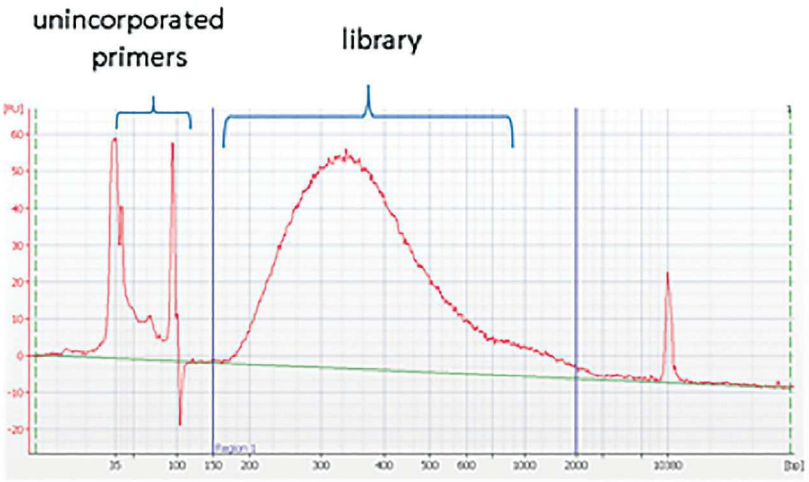


Figure 2. BioAnalyzer trace of unpurified library. A region corresponding to the amplified library is selected (blue bars) to estimate the yield.

1. If the library has a sufficient yield, proceed to the **Library Purification or size selection step** in the section: **Library processing**. If the library has a low yield, the remaining library can be further **re-amplified** for 2 to 3 additional cycles to achieve the desired yield. Spin down the tube or plate containing the library and return the plate or tube(s) to a thermal cycler.

2. Use the following cycling settings:

Number of cycles	Temperature	Time
2-3 cycles	98°C	20 s
	72°C	50 s
1 cycle	4°C	hold

***NOTE**: Higher yields may come at the expense of reduced sequencing quality, therefore it is important to avoid an over-amplification.*

***NOTE**: MicroPlex libraries can be further amplified with no extra reagents added after storage at 4°C for up to 6 hours or -20°C for up to 7 days.*

PROTOCOL

MANUAL PROCESSING

LIBRARY PROCESSING

Library Purification or Size Selection

Most part of applications will not require a size selection if an initial DNA fragments size is less than 1,000 bp. Amplified products should be purified by Agencourt AMPure XP beads (Beckman Coulter) as described at **Option A**. Mixing the sample and AMPure XP beads at a **1:1 ratio** is critical to get rid of primers and adaptors.

If your application requires a size-selection, please refer to the **Option B**.

You will need

- Freshly prepared 80% (v/v) ethanol
- DiaMag 0.2ml magnetic rack compatible with 0.2 ml tubes (Diagenode, Cat. No. B04000001) or 96 well plate magnetic rack
- Low TE buffer pH 8.0

Option A: Library Purification using AMPure® XP beads

1. Carefully resuspend the AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.

2. Precisely estimate a library volume and add an equal volume of AMPure XP beads to get a final 1:1 volume ratio. Mix by pipette 8 – 10 times until the mixture is homogeneous.

3. Incubate at room temperature for 5 minutes.

4. Place the tube (or the plate) on a magnetic rack. Wait until the beads are completely bound to the magnet (~ 2 minutes).

5. Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.

6. Wash the beads pellet 2 times as follows:

   - With the tubes on the magnet, add **150 µl** of **80% ethanol** without disturbing the bead pellet and wait for 30 seconds.

- Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
- Perform a brief low speed spin (~2000 g) to collect all droplets remaining on the tube walls.

Repeat the wash as described above 1 time and proceed to the next step.

7. Leaving the tube cap open, let dry the beads on the magnet for 5 minutes.

8. Elute DNA by resuspending the beads in **20 µl of 1x Low TE buffer**, pH 8.0.

9. Place the tube (or a plate) on a magnetic rack. Wait until the beads are completely bound to the magnet (~ 2 minutes).

10. Without disturbing the pellet, carefully aspirate and transfer the **supernatant containing purified libraries** to a new tube.

11. Quantify purified libraries using the method of your choice (for more information refer to Remarks before starting).

Option B: Library size selection using AMPure XP beads

1. Carefully resuspend the AMPure XP beads by shaking and light vortexing until no pellet is visible at the bottom of the container.

2. Precisely estimate library volume and adjust it to **50 µl** using nuclease-free water.

3. Add **32.5 µl** of **AMPure XP beads** to each sample (to get a final volume ratio of AMPure beads: library of 0.65). Mix by pipette 8 – 10 times until the mixture is homogeneous.

4. Incubate at room temperature for 10 minutes.

5. Place the tube (or a plate) on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes). **Transfer the supernatant** to new tube for further processing.

6. Add to the supernatant **12.5 µl** of **room temperature AMPureXP beads** each. Mix by pipette 8 – 10 times until the mixture is homogeneous.

7. Incubate at room temperature for 10 minutes.

8. Place the tube (or a plate) on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes). Carefully aspirate by pipette and **discard the supernatant** without disturbing the pellet.

9. Wash the beads pellet 2 x times as follows:

   - With the tubes on the magnet, add **150 µl** of **80% ethanol** without disturbing the bead pellet and wait for 30 seconds.
   - Carefully aspirate by pipette and discard the supernatant without disturbing the pellet.
   - Perform a brief low speed spin (~2000 g) to collect all droplets remaining on the tube walls.

   Repeat the wash as described above 1 time and proceed to the next step.

10. Leaving the tube cap open, let dry the beads on the magnet for 5 minutes.

11. Elute DNA by resuspending the beads in **15 µl** of **1x Low TE buffer, pH 8.0**.

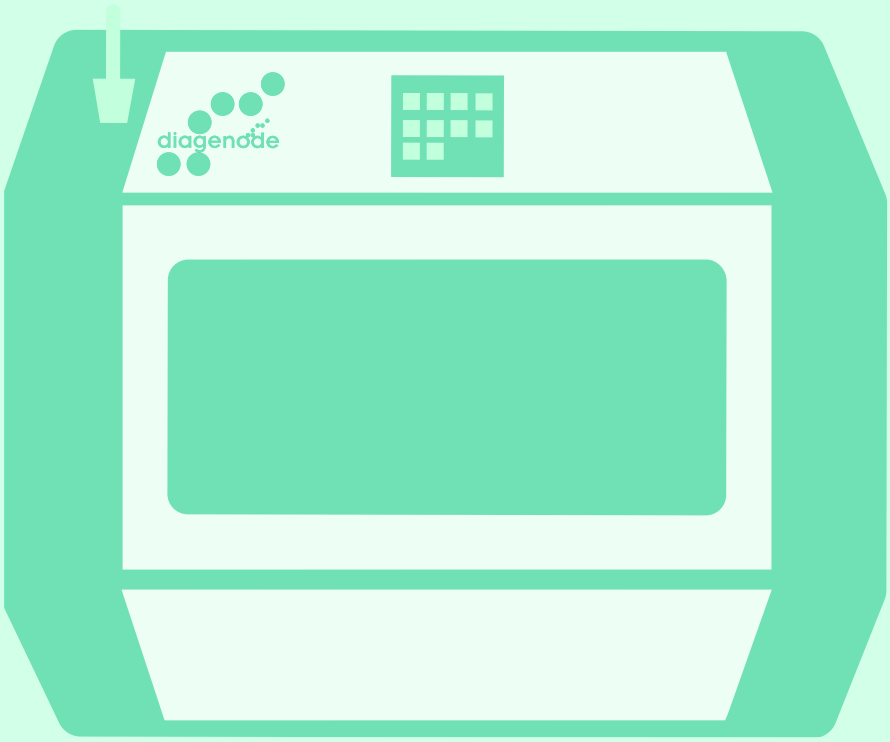
12. Place the tube (or a plate) on a magnetic rack. Wait until the beads are completely bound to the magnet (~2 minutes).

13. Without disturbing the pellet, carefully aspirate and transfer the **supernatant containing purified libraries** to a new tube.

14. Quantify purified libraries using the method of your choice (for more information refer to Remarks before starting).

Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer to Appendix). The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform. The total molarity is the sum of all the individual libraries' molarities in the final volume, e.g. if you add 5 µl of a 10 nM library to 5 µl of a 20 nM library, you have 10 µl of a 15 nM pool. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of each library.

AUTO PROTOCOL



AUTOMATED PROCESSING

PROTOCOL FOR LIBRARY PREPARATION

Protocol for library preparation using IP-Star Compact Automated System

The MicroPlex Library Preparation Kit v3 has been validated on IP-Star Compact Automated System (Diagenode, Cat. No. B03000002). The below protocol provides flexibility to prepare 1 to 48 libraries in one run starting with **50 pg-50 ng** of DNA. The whole protocol of library preparation takes approximately 1h 30 minutes. It allows you to prepare up to 96 libraries per day with 2 runs. At the end, you will recover ligated products ready for amplification. Amplified libraries can be further processed on IP-Star Compact - for libraries purification or size selection.

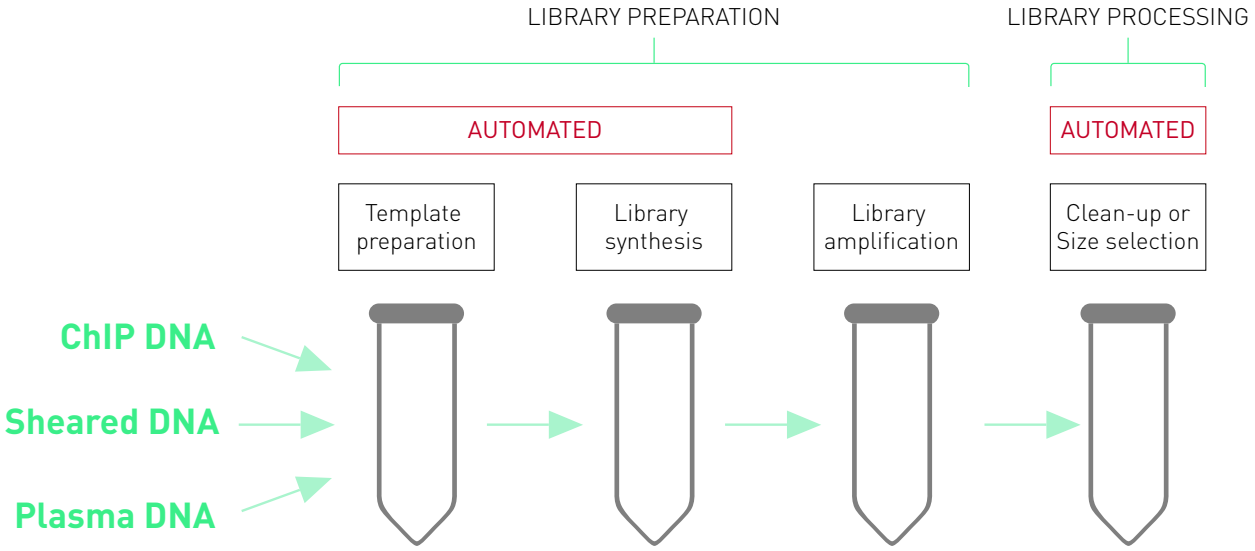


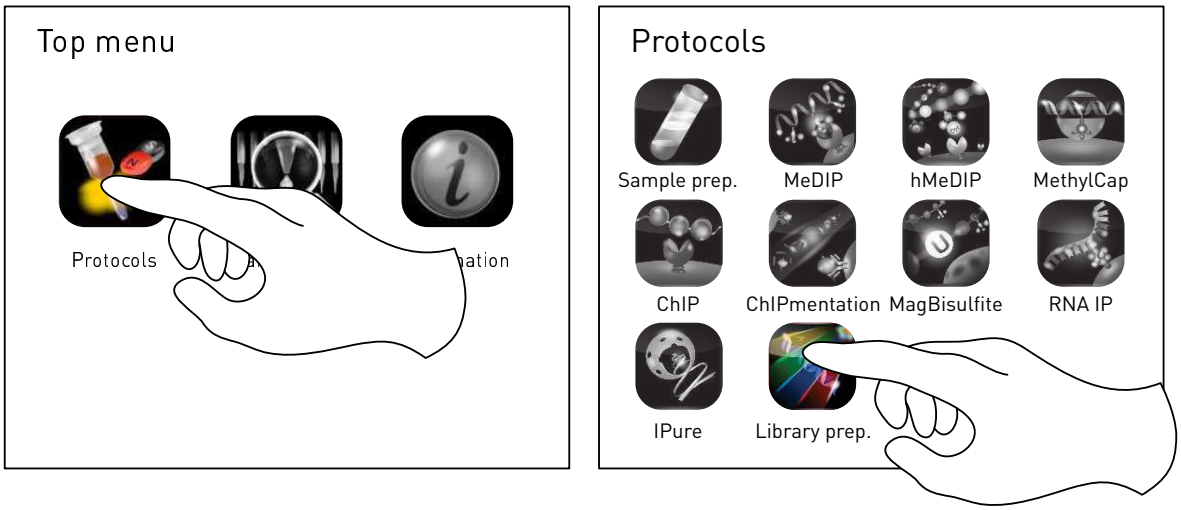
Figure 3. Microplex Library preparation workflow using IP-Star Compact Automated System.

IP-Star setup

1.1 Switch ON the IP-Star Compact.

1.2 Select "**Protocols**" icon and then "**Library prep**" category.

1.3 Select "**MicroPlex_Library_Preparation**" protocol:



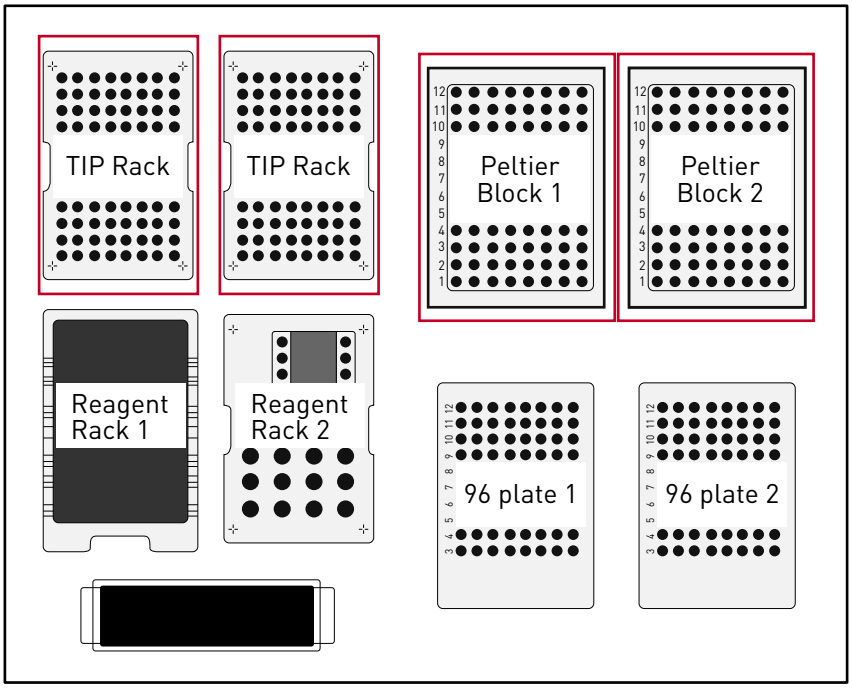
1.4 Setup the exact number of samples that you want to process by pressing the black box.

***NOTE**: The **Peltier Block 1** is now cooling down to 4°C to keep the enzymes and reagents cold.*

If you plan to run between

- **1** and **8** samples → "**MicroPlex_Library_Preparation_08**"
- **9** and **16** samples → "**MicroPlex_Library_Preparation_16**"
- **17** and **24** samples → "**MicroPlex_Library_Preparation_24**"
- **25** and **32** samples → "**MicroPlex_Library_Preparation_32**"
- **33** and **40** samples → "**MicroPlex_Library_Preparation_40**"
- **41** and **48** samples → "**MicroPlex_Library_Preparation_48**"

1.5 Setup all the plastics on the platform according to the screen layout (press the relevant module to see detailed information).



1.6 Fill **TIP Rack 1** (and 2 if processing more than 8 samples) with tips according to the screen.

1.7 Fill **Peltier Blocks 1 and 2** with 200 µl tube strips according to the screen.

STEP 1&2

Template Preparation & Library Synthesis

***NOTE**: Allow the reagent from MicroPlex Library Preparation kit v3 to come at 4°C. Work on ice from this point.*

2.1 Prepare the following mixes.

Template Preparation pre-mix:

	Number of samples						
	1	**8**	**16**	**24**	**32**	**40**	**48**
Dual Template Preparation Buffer (red cap)	2 µl	16 µl	32 µl	48 µl	64 µl	80 µl	96 µl
Dual Template Preparation Enzyme (red cap)	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
TOTAL	**3 µl**	**24 µl**	**48 µl**	**72 µl**	**96 µl**	**120 µl**	**144 µl**

***NOTE**: 10 µl of DNA will be added later for each sample.*

Library Synthesis pre-mix:

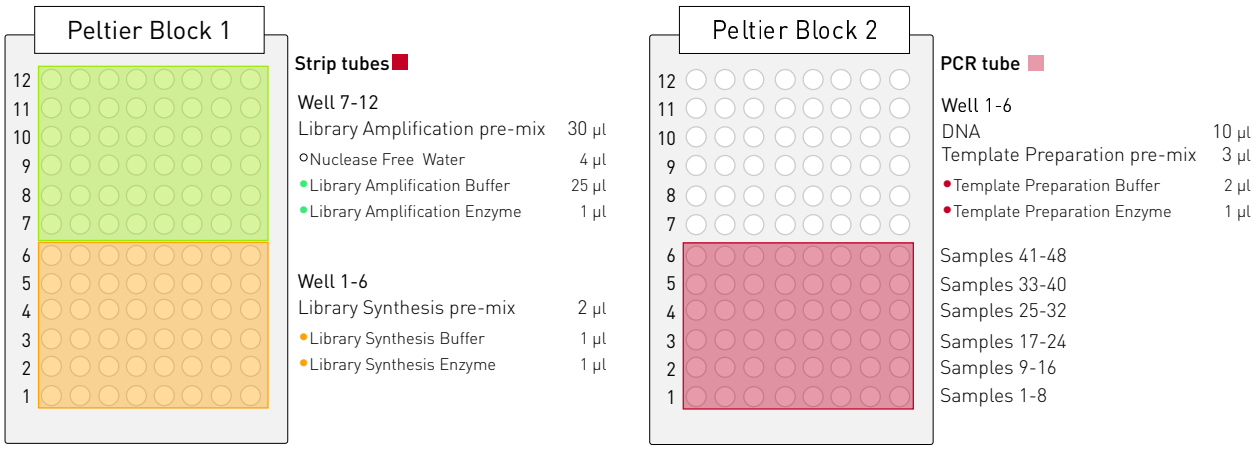
	Number of samples						
	1	**8**	**16**	**24**	**32**	**40**	**48**
Dual Library Synthesis Buffer (yellow cap)	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
Dual Library Synthesis Enzyme (yellow cap)	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
TOTAL	**2 µl**	**16 µl**	**32 µl**	**48 µl**	**64 µl**	**80 µl**	**96 µl**

Library Amplification pre-mix:

	Number of samples						
	1	**8**	**16**	**24**	**32**	**40**	**48**
Nuclease-Free Water (clear cap)	4 µl	32 µl	64 µl	96 µl	128 µl	160 µl	192 µl
Dual Library Amplification Buffer (green cap)	25 µl	200 µl	400 µl	600 µl	800 µl	1,000 µl	1,200 µl
Dual Library Amplification Enzyme (green cap)	1 µl	8 µl	16 µl	24 µl	32 µl	40 µl	48 µl
TOTAL	**30 µl**	**240 µl**	**480 µl**	**720 µl**	**960 µl**	**1,200 µl**	**1,440 µl**

2.2 Fill the **Peltier Block 1** with **Library Synthesis pre-mix** and **Library Amplification pre-mix** according to the screen layout.

2.3 Fill the **Peltier Block 2** with the **Template Preparation pre-mix** according to the screen layout.



2.4 Add **10 µl of DNA** to each tube containing the Template Preparation pre-mix (according to the screen layout).

2.5 Check the proper insertion of the racks and consumables.

2.6 Close the door and press "Run" to start.

2.7 MicroPlex is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.

STEP 3

Library Amplification

***NOTE**: The dual indexes compatible with this kit have to be purchased separately (see: Required materials not provided). Select the right Dual index for MicroPlex kit and follow the guidelines from this kit regarding index handling.*

Prepare the Dual Indexing Reagents (available separately) as follows:

- Remove the Dual Indexing Reagents from freezer and thaw for 10 minutes on the bench top.
- Spin the Dual Indexing Reagents in a table top centrifuge to collect contents at the bottom of the well.
- Thoroughly wipe the Dual Indexing Reagent Tubes or Indexing Reagent Plate foil seal with 70% ethanol and allow it to dry.

3.1 Recover your samples on the **Peltier Block 1** in lane 7 to 12 according to the screen layout.

3.2 Press "OK" and "Back" until the homepage appears on the screen.

3.3 Add **5 µl** of the appropriate **Dual Indexing Reagent** (available separately) to each sample. Final volume of your sample is 50 µl.

NOTE: if using 96 Dual Indexes for MicroPlex Kit v3 (Set I - IV):

- *Thoroughly wipe the Dual Indexes Plate foil seal with 70% ethanol and allow it to dry to prevent cross-contamination.*
- *Make sure the two corner notches of the Dual Indexing Reagent Plate are on the left, and the barcode label on the long side of the Index Plate is facing you.*
- *Use a clean pipet tip to pierce the seal above the specific indexing reagent on the Dual Indexing Reagent Plate; discard the tip used for piercing.*
- *Use a clean pipet tip to collect 5 µl of the indexing reagent and add to the reaction mixture.*

3.4 Mix 4x with a pipette set to 50 µl. Avoid introducing excessive air bubbles.

3.5 Seal the tube(s) tightly and centrifuge briefly to collect the contents to the bottom of each well or tube.

3.6 Return the tube(s) to thermal cycler with heated lid set to 101°C – 105°C.

3.7 Perform **Library Amplification Reaction** using the cycling conditions in the tables below.

Caution: Ensure that the thermal cycler does not have a denaturing step programmed until Stage 3.

	Stage	Temperature	Time	Number of Cycles
Extension & Cleavage	1	72°C	3 min	1
	2	85°C	2 min	1
Denaturation	3	98°C	2 min	1
Addition of Indexes	4	98°C	20 s	4
		67°C	20 s	
		72°C	40 s	
Library Amplification	5	98°C	20 s	5 to16*
		72°C*	50 s	
Hold	6	4°C	Hold	1

* If working with **DNA samples of known quantity or quality**, please use the **Amplification Guide** below to select the required number of amplification cycles.

Amplification guide (for stage 5)	
DNA Input (ng)	**Number of Cycles**
50 ng	6 - 8
20 ng	7 - 8
10 ng	7 - 8
5 ng	7 - 9
2 ng	8 - 10
1 ng	11 - 12
0.2 ng	14 - 15
0.05 ng	15 - 16

*If working with **DNA samples of unknown quantity** and/or low quality (eg. FFPE extracted DNA), amplify for **12 cycles** and perform an **INTERMEDIATE LIBRARY QUANTIFICATION OF UNPURIFIED LIBRARY** to estimate the library yield . If the desired yield is not achieved, the libraries can be re-amplified for few additional cycles.*

3.8 Remove the tube(s) from the thermal cycler and centrifuge briefly to collect the contents to the bottom of each well.

3.9 Proceed to the **Library processing section** (recommended for samples of known quantity or quality) or to the **Intermediate Library Quantification** option shown below (recommended for samples with unknown quantity and/or low quality).

Optional Intermediate Library Quantification

(recommended for samples with unknown quantity and/or a low quality)

Quantify **unpurified** libraries using the BioAnalyzer, the Fragment Analyzer or similar devices. This intermediate quantification allows estimating the library yield and enabling an additional re-amplification if needed. You will need a High Sensitivity NGS Fragment Analysis kit (1 bp-6,000 bp) from Advanced Analytical if using Fragment Analyzer or High Sensitivity DNA kit from Agilent if using BioAnalyzer. Follow the manufacturer's instruction for kit handling and protocol.

***NOTE**: The quantification of unpurified library by qPCR (e.g. using KAPA® Library Quantification Kit – Illumina (Kapa Biosystems), is also possible at this step while it will increase the total protocol duration.*

1. Take an aliquot of unpurified libraries: **1 µl** if the BioAnalyzer will be used or **2 µl** if the Fragment Analyzer will be used. Keep remaining libraries at 4°C .

2. Load the aliquot of unpurified library on the Fragment Analyzer or BioAnalyzer and run the analysis.

3. Estimate the library yield using a region selection option to discriminate between unincorporated primers/adaptors and libraries themself as it is shown in the Figure 2 below.

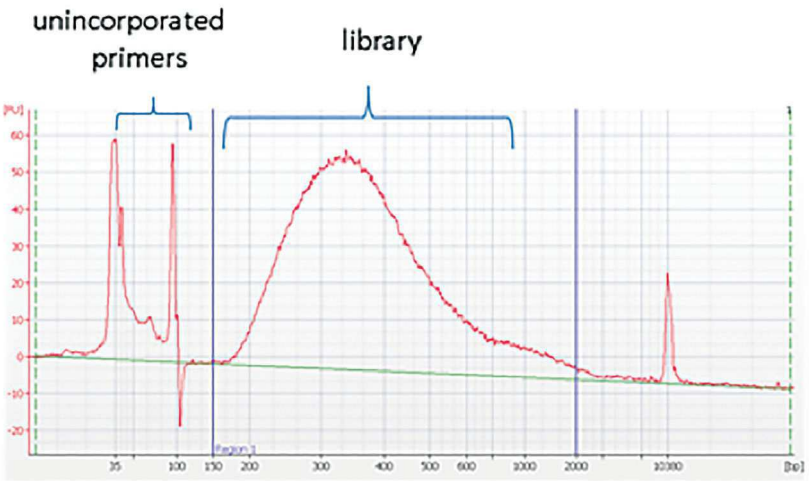


Figure 2. BioAnalyzer trace of unpurified library. A region corresponding to the amplified library is selected (blue bars) to estimate the yield.

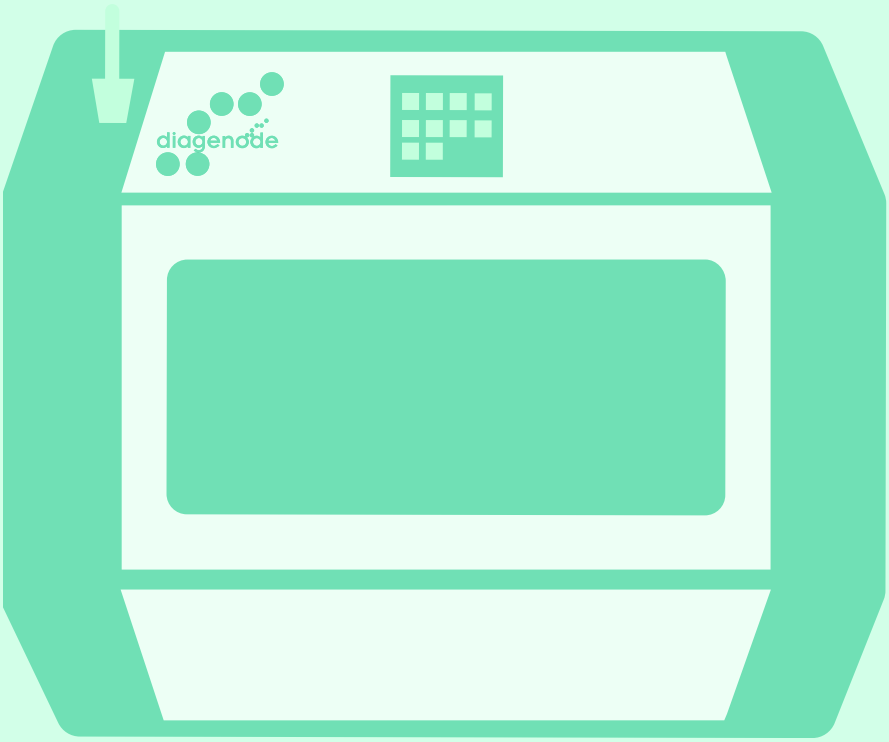
4. If the library has a sufficient yield, proceed to the **Library Purification or size selection step** in the section: **Library processing**. If the library has a low yield, the remaining library can be further **re-amplified** for 2 to 3 additional cycles to achieve the desired yield. Spin down the tube or plate containing the library and return the plate or tube(s) to a thermal cycler.

5. Use the following cycling settings:

Number of cycles	Temperature	Time
2-3 cycles	98°C	20 s
	72°C	50 s
1 cycle	4°C	hold

***NOTE**: Higher yields may come at the expense of reduced sequencing quality, therefore it is important to avoid an over-amplification.*

AUTO PROTOCOL



AUTOMATED PROCESSING
LIBRARY PROCESSING

Library processing using IP-Star Compact Automated System

Library Purification or Size Selection

Most part of applications will not require size selection if initial DNA fragment size is less than 1,000 bp. Amplified products should be **purified** by Agencourt AMPure XP beads (Beckman Coulter) as described at **Option A**. If your application requires a size-selection, please refer to the **Option B**, which allows to perform **size selection** from 250bp to 500bp just by changing the amount of beads.

You will need

- Freshly prepared 80% (v/v) ethanol
- Low TE buffer pH 8.0
- Agencourt AMPure XP beads (Beckman Coulter)

Option A: Library Purification using AMPure XP beads

Use the room temperature AMPure XP beads for the clean-up.

1. Select "**Protocols**" icon and then "**IPure**" category.

2. Select "**AMPure_XP_Purification**" protocol:

3. Setup the exact number of samples that you want to process by

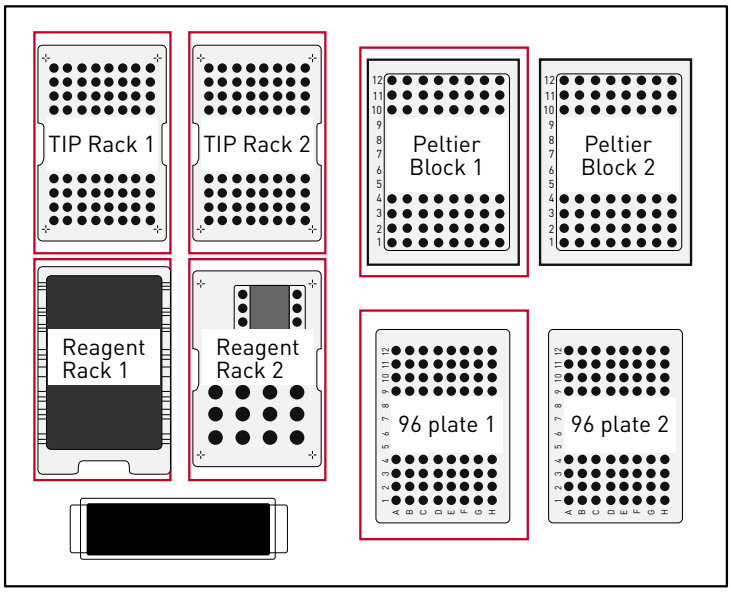
If you plan to run between
- **1** and **8** samples → "**AMPure_XP_Purification_08**"
- **9** and **16** samples → "**AMPure_XP_Purification_16**"
- **17** and **24** samples → "**AMPure_XP_Purification_24**"

pressing the black box.

***NOTE**: The Peltier Block 1 is now cooling down to 4°C to keep your samples cold.*

4. Set up all the plastics on the platform according to the screen layout.

   - Fill **TIP Rack 1** (and 2 if processing more than 8 samples) with tips according to the screen.
   - Fill **Reagent Rack 1 & 2** with reagent containers according to the screen.
   - Fill **96 plate 1** with a 96-well microplate.
   - Fill **Peltier Block 1** with 200 µl tube strips according to the screen



5. Fill the robot with all reagents.

   - **Peltier Block 1**:
     Put your **samples** in row 1 - if processing 1 to 8 samples
     in row 2 - if processing 9 to 16 samples
     in row 3 – if processing 17 to 27 samples
   - 96-well microplate:
     Distribute **room temperature AMPure XP Beads**:

     - in row 1 - if processing 1 to 8 samples
     - in row 5 - if processing 9 to 16 samples
     - in row 9 - if processing 17 to 24 samples

   The amount of beads must be 1x the volume of sample (e.g. for 45 µl of samples use 45 µl of beads)

   ***NOTE**: Resuspend the beads with pipetting up and down several times before dispense them.*

- Fill the container of the **Reagent Rack 1** with freshly prepared 80% ethanol according to the screen.
- Fill the container of **Reagent Rack 2** with Low TE buffer pH 8.0 ("Resuspension Buffer" on the screen) according to the screen.
- Check the proper insertion of the racks and the consumables.

6. Close the door and press "Run" to start.

7. Purification is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.

8. After the run, recover your samples on the upper row of the Peltier Block 1 as shown on the screen layout. The final volume of each sample is 20 µl.

9. Press "OK" and "Back" until the homepage appears on the screen. Press "Shutdown" and wait until the screen is black before switching off the IP-Star.

***NOTE**: Remove all the plastics from the IP-Star platform, empty the waste shuttle, and clean the inner side of the IP-Star with 70% ethanol.*

Option B: Library size selection using AMPure XP beads

***NOTE**: Use room temperature AMPure XP beads for the size selection.*

1. Select "**Protocols**" icon and then "**Library prep.**" category.

2. Select "**AMPure_XP_Size_Selection**" protocol:

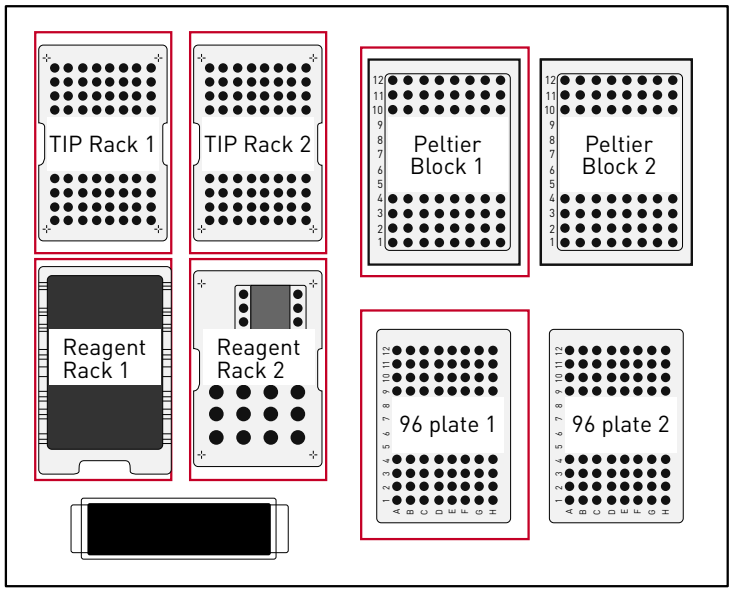
If you plan to run between
- **1** and **8** samples → "**AMPure_XP_ Size_Selection_08**"
- **9** and **16** samples → "**AMPure_XP_ Size_Selection_16**"

3. Setup the exact number of samples that you want to process by pressing the black box.

***NOTE**: The Peltier Block 1 is now cooling down to 4°C to keep your samples cold.*

4. Setup all the plastics on the platform according to the screen layout.

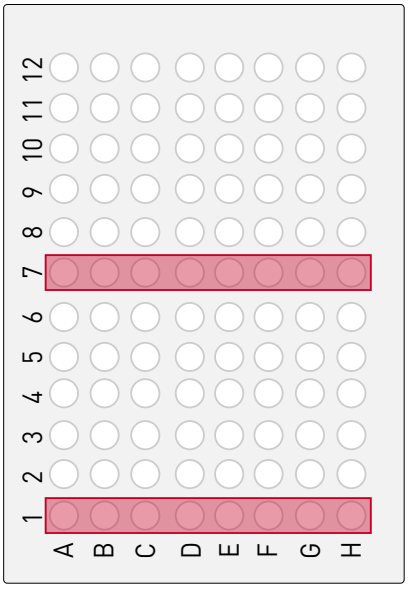
   - Fill **TIP Rack 1** (and 2 if processing more than 8 samples) with tips according to the screen.
   - Fill **Reagent Rack 1 & 2** with reagent containers according to the screen.
   - Fill **96 plate 1** with a 96 well microplate.
   - Fill **Peltier Block 1** with 200 µl tube strips according to the screen.



5. Fill the robot with all reagents.

   - Fill **20 µl of samples** in lane 1 (and 2 if processing more than 8 samples) of the **Peltier Block 1**.
   - Add **80 µl of nuclease-free water** ("Resuspension buffer" on the screen) to each sample to have a final volume of 100 µl.
   - Distribute **AMPure XP Beads** in lane 1 on 96 **Plate 1** (and 7 if processing more than 8 samples) according to the required size following recommendations from the table shown below (and on the screen).

   ***NOTE**: Resuspend the beads with pipetting up and down several times before dispensing them.*



Ampure XP	Final size
110 µl	250 bp
100 µl	280 bp
90 µl	325 bp
80 µl	400 bp
70 µl	500 bp

- Fill the container of the **Reagent Rack 1** with freshly prepared 80% ethanol according to the screen.
- Fill the container of **Reagent Rack 2** with Low TE buffer pH 8.0 ("Resuspension Buffer" on the screen) according to the screen.

6. Check the proper insertion of the racks and the consumables.

7. Close the door and press "Run" to start.

8. Library size selection is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.

9. After the run, recover your samples on the upper row of the Peltier Block 1. The final volume is 20 µl for each sample.

10. Press "OK" and "Back" until the homepage appears on the screen. Press "Shutdown" and wait until the screen is black before switching off the IP-Star.

***NOTE**: Remove all the plastics from the platform, empty the waste shuttle and clean the inner side of the IP-Star with 70% ethanol.*

Individual libraries, quantified and purified according to the above protocol, can be pooled at desired molar ratios to allow multiplex sequencing. Libraries that are being pooled must have been prepared with different indexes (for multiplexing and index pooling guidelines refer

to the Appendix). The minimal molar concentration needed to sequence the pool depends on the requirements of the sequencing platform. The total molarity is the sum of all the individual libraries' molarities in the final volume, e.g. if you add 5 µl of a 10 nM library to 5 µl of a 20 nM library, you have 10 µl of a 15 nM pool. If libraries are prepared from similar input amounts, they can be pooled by combining equal volume aliquots of each library.

FAQs

Can I use the available Illumina primers and validate them with the MicroPlex Kit v3?

Although the final flanking sequences of MicroPlex are the same as those used by Illumina, the PCR primers are not identical and part of them is supplied with the buffer. For this reason Illumina primers will not work as substitute.

The BioAnalyzer profile of purified library shows the presence of low molecular weight peaks (primers/adaptors) in the samples. Should I re-purify the samples or they can be used directly to the sequencing? If the second purification is recommended, which ratio sample/AMPure beads should I use?

You can do a second round of purification using 1:1 ratio of AMPure beads to sample and this should get rid of the majority of the dimers.

I am going to use the MicroPlex Library Preparation Kit v3 on ChIP samples . Our thermocycler has ramp rate 1.5°/s max while the protocol recommends using a ramp rate 3 to 5°/s. How would this affect the library prep?

We have not used a thermocycler with a ramp rate of 1.5 °C, which seems faster than most of thermocyclers. Too fast of a ramp rate may affect the primer annealing and ligation steps.

What is the function of the replication stop site in the adapter loops?

The replication stop site in the adaptor loops function to stop the polymerase from continuing to copy the rest of the stem loop.

I want to do ChIP-seq. Which ChIP-seq kit can I use for sample preparation prior to Microplex Library Preparation Kit v3?

In our portfolio there are several ChIP-seq kits compatible with Microplex

Library Preparation Kit v3. Depending on your sample type and target studied you can use the following kits: iDeal ChIP-seq Kit for Transcription Factors (Cat. No. C01010055), iDeal ChIP-seq Kit for Histones (Cat. No. C01010051), True MicroChIP kit (Cat. No. C01010130), Universal Plant ChIP-seq Kit (Cat. No. C01010152). All these kits exist in manual and automated versions.

Is Microplex Library Preparation Kit v3 compatible with exome enrichment methods?

Microplex Library Preparation Kit v3 is compatible with major exome and target enrichment products, including Agilent SureSelect®, Roche NimbleGen® SeqCap® EZ and custom panels.

What is the nick that is mentioned in the kit method overview?

The nick is simply a gap between a stem adaptor and 3' DNA end, as shown on the schema in the kit method overview.

Related products

Product	Cat. No.
iDeal ChIP-seq kit for Transcription Factors	C01010055
Auto iDeal ChIP-seq Kit for Transcription Factors	C01010172
iDeal ChIP-Seq Kit for Histones	C01010051
Auto iDeal ChIP-seq Kit for Histones	C01010171
True MicroChIP Kit	C01010130
Auto True MicroChIP Kit	C01010140
iDeal FFPE Kit	C01010190
Universal Plant ChIP-seq Kit	C01010152
Auto Universal Plant ChIP-seq kit	C01010153
Bioruptor Pico	B01060010
IP-Star Compact Automated System	B03000002
24 Dual indexes for MicroPlex Kit v3	C05010003
96 Dual indexes for MicroPlex Kit v3 – Set I	C05010004
96 Dual indexes for MicroPlex Kit v3 – Set II	C05010005
96 Dual indexes for MicroPlex Kit v3 – Set III	C05010006
96 Dual indexes for MicroPlex Kit v3 – Set IV	C05010007

Technical support

For technical support contact custsupport@diagenode.com

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PBS 1X with PIC (10 ml)	**stock**	**mix**	**final concentration**
PBS 1X	1X	10 ml	1X
PIC tablets - format 10 ml	tablets	1	

for the day of use
conservation in ice

PIC 7X (1.5 ml)	**stock**	**mix**	**final concentration**
PIC tablets - format 10 ml	tablets	1	7X
dH_2O		1.5 ml	

conservation -20˚C 3 months
no more than 3 thaw/freeze cycles

PIC 70X (1.5 ml)	**stock**	**mix**	**final concentration**
PIC tablets - format 50 ml	tablets	2	70X
dH_2O		1.5 ml	

conservation -20˚C 3 months
no more than 3 thaw/freeze cycles

Formaldehyde 1% stock (100 ml)	**stock**	**mix**	**final concentration**
Formaldehyde	37%	2.7 ml	1%
PBS	1X	97.3 ml	

conservation 4˚C 1 month

Glycine 1M (40 ml)	**stock**	**mix**	**final concentration**
Glycine (75.07 g/mol)	powder	3 g	1M
dH_2O		40 ml	

conservation 4°C 1 month

NaCl 5M (10 ml)	**stock**	**mix**	**final concentration**
NaCl (58.44g/mol)	powder	2.92 g	5M
dH_2O		10 ml	

conservation RT 1 year
to redo if lot of salt appear around the bottleneck

KCl 3M (10 ml)	**stock**	**mix**	**final concentration**
KCl (74.55g/mol)	powder	2.24 g	3M
dH_2O		10 ml	

conservation RT 1 year

Ethanol 70% (2 ml)	**stock**	**mix**	**final concentration**
Absolute ethanol	100%	1.4 ml	70%
dH_2O		0.6 ml	

conservation RT 1 week

Sodium butyrate	**stock**	**mix**	**final concentration**
sodium butyrate (110.09 g/mol)	powder	5 g	2.5M
dH_2O		18.167 ml	add the volume to the recipient

conservation 4˚C

Hypotonic buffer (50 ml) (for muscle protocol only)	**stock**	**mix**	**final concentration**	**conservation 4˚C 1 month**
HEPES pH 7.4	1M	500 ul	10mM	
KCl	3M	165 ul	10mM	
$MgCl_2$	1M	250 ul	5mM	
Nonidet-P40	10%	500 ul	0.1%	
dH_2O		48.585 ml		

Add PIC, PMSF and sodium butyrate the day of use

For 10 ml of complete hypotonic buffer: 9985 ul of hypotonic buffer, 1 tablet of PIC, 10 ul of 0.1M PMSF and 5 ul of 2.5M Na-butyrate

PBSplus (25ml) (for mature male gonad only)	**stock**	**mix**	**final concentration**	**for the day of use** **conservation in ice**
PBS	1X	25 ml	1X	
PIC tablet for 50 ml		1/2 tablet	1X	
PMSF	0.1M	25 ul	0.1mM	
sodium butyrate	2.5M	50 ul	5mM	

Sonication buffer (100 ml)	**stock**	**mix**	**final concentration**	**conservation RT 1 month**
Tris-HCl pH 8.0	1M	5 ml	50mM	
EDTA	0.5M	2 ml	10mM	*If precipitation of the SDS,*
SDS	20%	5 ml	1.0%	*warm and agitate a little*
dH_2O		88 ml		

Add PIC, PMSF and sodium butyrate the day of use

For 1ml of complete sonication buffer: 845 ul of sonication buffer, 140 ul of PIC solution 7X, 10 ul of 0.1M PMSF and 5 ul of 2.5M Na-butyrate

Iodixanol gradient 1 (for mature male gonad only)	**stock**	**mix**	**final concentration**	**for the day of use**
Bottom layer (1200 ul)				
iodixanol	60%	800 ul	40%	*First, prepare the bottom layer*
PBSplus		400 ul		*Then, you can carefully pour the middle layer*
Middle layer (1200 ul)				*on the top of the bottom layer, drop by drop*
iodixanol	60%	580 ul	29%	
PBSplus		606.2 ul		
Nonidet-P40	10%	12 ul	0.10%	
spermine	0.15M	1.2 ul	0.15mM	
spermidine	1M	0.6 ul	0.5mM	

Iodixanol gradient 2 (for mature male gonad only)	**stock**	**mix**	**final concentration**	**for the day of use**
Bottom layer (600 ul) - to prepare 2 times				
iodixanol	60%	417 ul	50%	
PBSplus		183 ul		

IP buffer (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4˚C 3 months**
Tris-HCl pH 8.0	1M	2 ml	20mM	
EDTA	0.5M	400 ul	2mM	
Triton X-100	100%	100 ul	0.1%	
NaCl	5M	3 ml	150mM	
dH_2O		94.5 ml		

Add PIC, PMSF and sodium butyrate the day of use

For 10 ml of complete IP buffer: 9.896 ml of IP buffer, 1 tablet of PIC, 100 ul of 0.1M PMSF and 4 ul of 2.5M Na-butyrate

recovery buffer (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4˚C 3 months**
Tris-HCl pH 7.5	1M	5 ml	50mM	
EDTA	0.5M	1 ml	5mM	
SDS	20%	5 ml	1%	
NaCl	5M	1 ml	50mM	
dH_2O		88 ml		

Add PMSF the day of use

By 2 ml of ChIP elution buffer, add 2 ul of 0.1M PMSF

Low salt wash solution (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4˚C 3 months**
Tris-HCl pH 8.0	1M	2 ml	20mM	
EDTA	0.5M	400 ul	2mM	
NaCl	5M	3 ml	150mM	
Triton X-100	100%	1 ml	1%	
SDS	20%	500 ul	0.1%	
dH_2O		93.1 ml		

Add sodium butyrate the day of use

By 5 ml of low salt wash solution, add 2 ul of 2.5M Na-butyrate

High salt wash solution (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4˚C 3 months**
Tris-HCl pH 8.0	1M	2 ml	20mM	
EDTA	0.5M	400 ul	2mM	
NaCl	5M	10 ml	500mM	
Triton X-100	100%	1 ml	1%	
SDS	20%	500 ul	0.1%	
dH_2O		86.1 ml		

Add sodium butyrate the day of use

By 5 ml of high salt wash solution, add 2 ul of 2.5M Na-butyrate

Elution buffer (10 ml)	**stock**	**mix**	**final concentration**	**conservation RT indefinite**
Tris-HCl pH 8.0	1M	100 ul	10mM	
dH_2O		9.9 ml		

Low TE buffer (10 ml)	**stock**	**mix**	**final concentration**	**conservation RT indefinite**
Tris-HCl pH 8.0	1M	100 ul	10mM	
EDTA	0.5M	20 ul	1mM	
dH_2O		9.88 ml		

References enzymes and reagents

Product	Company	Reference	Form	Comments	checklist
Common reagents :					
Tris-HCl 1M pH 8	Sigma-Aldrich	T2694-100ML	liquid		
EDTA 0.5M	Sigma-Aldrich	E7889-100ML	liquid		
NaCl	Sigma-Aldrich	S3014-500G	powder	prepare 5M solution	
KCl	Sigma-Aldrich	P9541-500G	powder	prepare 3M solution	
PBS tablets	Sigma-Aldrich	P4417-50TAB	tablets	prepare 1 or 10X solution	
SDS 20%	Fluka Analytical	05030-1L-F	liquid		
Triton X-100 100%	Sigma-Aldrich	T8787-50ML	liquid		
nuclease free water					
Specific reagents:					
Formaldehyde at 36.5-38% v/v	Sigma-Aldrich	F8775-25ML	liquid	add 135 ul directly to 5 ml PBS 1X	
glycine	Sigma-Aldrich	50048-50G	powder	prepare 1M solution	
sodium butyrate	Sigma-Aldrich	303410-5G	powder	prepare 2.5M solution	
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	4693159001	tablets	prepare 7X solution (can be kept frozen in -20°C) or add 1 tablet by 10 ml solution	
cOmplete EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	5056489001	tablets	3 glass vials with 20 tablets each - to add to 50 ml solution	
Protein A Dynabeads	Life Technologies	10004D	in solution		
Protein G Dynabeads	Life Technologies	10002D	in solution		
PMSF 0.1M	Sigma-Aldrich	93482-50ML-F	liquid		
Bioanalyzer Broad Range kit and DNA Chips	Agilent Technologies	5067-1504			
OR Tape Station material:					
D1000 ScreenTape	Agilent Technologies	5067-5582			
D1000 Reagents	Agilent Technologies	5067-5583			
Loading Tips, 10 Pk (TapeStation)	Agilent Technologies	5067-5599			
Optical tube strip caps (8x Strip)	Agilent Technologies	401425			
Optical tube strips (8x Strip)	Agilent Technologies	401428			
Qubit High Sensitivity kit (DNA)	ThermoFisher	Q32854			
Qubit Broad Range kit (DNA)	ThermoFisher	Q32853			
Qubit assays tubes	ThermoFisher	Q32856			
MinElute PCR Purification kit	QIAGEN	28006			
HEPES solution 1M	ThermoFisher	15630049	liquid	*or prepare it from powder*	FOR MUSCLE
$MgCl_2$ 1M	Sigma-Aldrich	M1028-100ML	liquid	*or prepare it from powder*	FOR MUSCLE
KCl 3M	Sigma-Aldrich	60135-250ML	liquid	*or prepare it from powder*	FOR MUSCLE
Nonidet P40 Substitute 10%	Sigma-Aldrich	11332473001	liquid		FOR MUSCLE AND MATURE MALE GONAD
OptiPrep (iodixanol 60%)	Sigma-Aldrich	D1556-250ML	liquid	protect with aluminium foil from the light	FOR MATURE MALE GONAD
spermine	Sigma-Aldrich	S3256	powder	prepare 1M solution and aliquot to freeze in -20°C	FOR MATURE MALE GONAD
spermidine	Sigma-Aldrich	S2626	powder	prepare 0.15M solution and aliquot to freeze in -20°C	FOR MATURE MALE GONAD
Enzymes:					
Proteinase K (20mg/ml)	Qiagen	19133 (10ML)	liquid		
RNase A (100mg/ml)	Qiagen	19101 (2.5ML)	liquid		
Others:					
Eppendorf tubes 1.5 ml LoBind					
Eppendorf tubes 2 ml LoBind					
Eppendorf tubes 5 ml (LoBind if desired)					
Rack and magnetic rack (1.5 ml and 0.2 ml)					
Ice bucket					
douncer and pestles					
Ceramic mortar and pestles					
Dry ice					
Balance or pocket balance;					
Kern pocket balance, capacity 60 g, resolution: 0.01 g	Sigma-Aldrich	Z662658-1EA		example	
Tweezers					
Thermomixer, incubator, water bath or dry bath					

Timer					
Cool centrifuges 1.5/2 ml and 15 ml tubes					
Vortexer					
Mini-centrifuge					
Sonicator device; example of our device in NMBU:	Active Motif	"EpiShear Probe Sonicator", with a 2mm probe			
Qubit machine					
Bioanalyzer machine (or Tape Station)					
Hula, rotating wheel or orbital shaker / tube roller					
microscope, cell counting chamber, Trypan Blue/Hoescht					
40-µm filter	Corning	431750			FOR MUSCLE AND MATURE MALE GONAD
Antibodies and library preparation kits:					
SEE NEXT TABLE SHEET FOR DETAILS					
Agencourt Ampure XP beads	Beckman Coulter	A63880			
Ethanol 80%					
PCR machine and tubes					