MagIC Beads

RNA affinity purification protocol

This protocol describes how to perform RNA affinity purification with Magnetic Instant Capture (MagIC) Beads for the capture of RNA-chromatin complexes from chemically cross-linked material.

Product description:
The MagIC Beads RNA affinity purification kit contains:

Beads:
The provided targeting beads carry a pool of DNA hybridization probes covalently attached to the surface of the beads through their 5' ends.

The recommended amount of capture probes for a single reaction capturing the RNA target is 20 pmol. The volume of the original bead suspension carrying 20 pmol of probes is variable.

Consult the MagIC Beads datasheet provided with your order to calculate the volume of the bead suspension carrying 20 pmol of capture probes.

Negative Control beads are also provided with the kit.
Those beads are intended to be incubated with the sample before the incubation with the targeting beads and provide a control for the experimental background of the signal coming from the molecules binding directly to the bead surface in the sample.
The use of those beads is a sufficient experimental control as in the MagIC Beads system the probe binding dependent experimental background is minimal or non-existent.

Buffers:
Four MagIC buffers are supplied with the kit: Lysis Buffer FA, Lysate Dilution Buffer FA, Wash Buffer FA I, and Wash Buffer FA II.
All buffers should be protected from exposure to light and kept at 4°C for long-term storage. The components of the buffers, however, precipitate in low temperatures and need to be carefully re-dissolved before each use.

MagIC Lysis Buffer FA – optimized for cell lysis and solubilization of cross-linked nucleic acid-protein complexes, as well as for RNase inhibition.
MagIC Lysate Dilution Buffer FA – provides conditions optimized for probe hybridization to the cellular lysate.
MagIC Wash Buffer I FA – optimized for washing out the molecules non-specifically attached to the probes after the hybridization, while maintaining the deactivation of RNases.
MagIC Wash Buffer II FA – optimized for washing out the molecules non-specifically attached to the surface of the beads, while maintaining the deactivation of RNases.

RNase I Buffer is also supplied with the kit. The buffer is intended to be used with RNase I for the specific elution of the chromatin segments captured by the beads. The buffer is optimized to provide conditions in which RNase I efficiently and without bias degrades ssRNA, dsRNA, and the RNA strand in the DNA:RNA duplexes.

The buffer has the following composition: 20mM Tris-HCl pH 8.0, 125mM KCl, 0.02% Triton X-100, 4mM CaCl2.

For elutions 50ul portions of the buffer, freshly mixed with 100U of RNase I are supposed to be used.

Other required materials (not provided):
- Magnetic rack.
- Temperature controlled mixer

- Centrifuge
- Reagents for the cross-linking method of choice (glutaraldehyde is recommended)
- 1M Dithiothreitol (DTT)
- Protease inhibitors of choice
- Fresh MiliQ water for final bead wash
- Nuclease-free 10mM Tris pH 7.5 for the elution for the control of the efficiency of the RNA pull-down
- RNases I for the specific elution of the captured chromatin segments (RNase I solutions of high concentration are recommended, like RNase Jf 50U/ul from NEB Cat.# M0243 or Ambion RNase I 100U/ul from Thermo Fisher Cat.# AM2294)
- Phenol:Chloroform:Isoamyl alcohol solution
- 3M Lithium Chloride solution
- Glycogen 5mg/ml
- Isopropanol

Protocol:

Before you start

Buffer preparation:

MagIC Lysis Buffer FA – Remove the bottle from 4°C and incubate at 22-40°C with occasional gentle shaking until all precipitated buffer components dissolve and the buffer is clear. Transfer to a fresh tube the buffer volume appropriate for the planned experiment. Add 5 µl of 1 molar DTT per ml of the buffer and protease inhibitors of choice to a final concentration of 1x (do not exceed the 1x concentration of the inhibitor), and mix well.

MagIC Lysate Dilution Buffer FA – Remove the bottle from 4°C and incubate at 20-40°C with occasional gentle shaking until all precipitated buffer components dissolve and the buffer is clear. Take additional care when looking for any undissolved buffer components. Keep at room temperature until use.

MagIC Wash Buffer I FA - Remove the bottle from 4°C and incubate at 20-40°C with occasional gentle shaking until all precipitated buffer components dissolve and the buffer is clear. Keep it at room temperature until needed.

MagIC Wash Buffer II FA - Remove the bottle from 4°C and incubate at 20-40°C with occasional gentle shaking until all precipitated buffer components dissolve and the buffer is clear. Take additional care when looking for any undissolved buffer components. Keep it at room temperature until needed.

RNase I Buffer - Remove the bottle from 4°C and let the buffer equilibrate to room temperature before use.

Thermo blocks:

Prepare thermo blocks, which will be used at 40°C for hybridization and washes, 37°C, and 92°C for the elution of captured molecules.

Cross-linking cells

Cells have to be chemically cross-linked to make the capture of RNA-chromatin complexes possible. Typically formaldehyde or glutaraldehyde are preferred in this type of experiment (glutaraldehyde is further preferred over formaldehyde due to the superior stability of the cross-links). The MagIC Beads capture is compatible with various chemical cross-linking protocols and a wide array of different ones might be employed. Below, an example of a well-performing cross-linking procedure with 1% glutaraldehyde for cultured cells is described.

1. Prepare 1% solution of glutaraldehyde in PBS (1 ml of the solution per every million cells is required) freshly before use.
2. Take cellular pellets freshly harvested into 50 ml tubes (free of any excess liquid) and resuspend the cells in a small volume of 1% glutaraldehyde until no chunks are visible.
3. Add the remaining glutaraldehyde solution to the tube to the final required amount of 1 ml per million cells. Mix by inverting and cross-link for **10 min** on a rotator or in a shaker.
4. Add glycine to 125 mM final concentration and continue incubating for another **5 min**.
5. Pellet the cells at **2000 g** for **5 min** at **4 °C**.
6. Remove the supernatant from the cells and resuspend them in 20 ml of cold PBS, pellet them again at **2000 g** for **5 min** at **4°C**.
7. Remove the supernatant and resuspend the pellet in 1ml of cold PBS per 20 mln cells.
8. Transfer the cells to a 2 ml Eppendorf tube, pellet them at **2000 g** for **3 min** at **4 °C** and **remove as much PBS as possible** with a pipette.
9. Use cells directly for the following steps of the protocol or flash-freeze the pellets in liquid nitrogen and store them at -80 °C.

Preparation of the lysates for the enrichment

1. Take desired amount of cells/tissues free of the collection buffer (recommended ~20 mln cells per 20 pmol of capture probes per sample) collected freshly or previously flash-frozen in liquid nitrogen and stored at -80°C. If using frozen cells, place tubes on ice and allow them to thaw partially.
2. After the cells have partially thawed add to them 3 cell pellet volumes of room temperature MagIC Lysis Buffer FA containing the protease inhibitors and 5mM DTT. Resuspend the cells in the buffer by pipetting up and down until the pellet is evenly spread in the buffer.
3. Sonicate the cells to obtain a desired DNA size range.

The obtained sizes of the fragments of the nucleic acids in the sample will determine the resolution of the target-DNA binding maps obtained in the downstream sequencing as well as the efficiency of the target enrichment. It is recommended to keep the DNA size range within the frame not shorter than 2-3x the average distance between the binding sites of the hybridization probes specific to the target RNA. If you need help choosing the optimal DNA size range, contact customer support at info@elementzero.bio

The sonication procedure required to obtain the desired DNA size range is variable as it is heavily dependent on the model of sonicator used as well as the type of biological material, the cross-linking protocol and the number of cells used. The condition of sonication has to be established in advance for each specific setup before performing the MagIC Beads enrichment procedure.

4. Centrifuge the lysates at **22°C**, **18 000 - 20 000 g** for **15 min** to pellet the insoluble cellular components.
5. Transfer the supernatant to a fresh tube, while avoiding the disruption of the pellet.
6. Centrifuge the lysates again at **22°C**, **18 000 - 20 000 g** for **15 min** to pellet any remaining insoluble cellular components.
7. Transfer the supernatant to a fresh tube, while avoiding the disruption of the pellet, and add to them room temperature MagIC Lysate Dilution Buffer FA at a volume equal to that of MagIC Lysis Buffer FA added previously.
8. Mix lysates well by pipetting or vortexing. The MagIC Lysate Dilution Buffer FA is viscous and thorough mixing of the sample after adding it is critical.
9. At this point, it is recommended to take aside 1% of the lysate volume as input control for later analysis of the efficiency of the enrichment.

Preparation of magnetic beads for the enrichment

1. Place the containers with targeting and control magnetic beads on the bench and allow the content to equilibrate to room temperature.

2. After the bead suspension is warmed up resuspend the particles thoroughly.
3. Transfer desired amount of targeting (typically equivalent to 20 pmol of probes on the beads per capture reaction) and control beads (typically 20 µl of 5mg/ml bead suspension) to fresh 2ml Eppendorf tubes.
4. Keep the beads in the storage buffer at room temperature, until needed.

Preparation of the experimental background control bead samples

1. Concentrate the prepared control beads on a magnet and remove the storage buffer.
2. Add the processed lysate to the beads, remove the tube from a magnet and resuspend the beads in the lysate thoroughly by pipetting or vortexing. Avoid excess foaming of the sample.
3. Place the tube into a thermo block preheated to **40°C** and incubate with **1200-1400 RPM** with **5sec on/30 sec off** cycles. **The optimal RPM value will vary depending on the volume of the sample, and the beads used. It may have to be adjusted empirically. It is critical to provide shaking conditions, which prevent the particles from accumulating at the bottom of the tube.**
4. Incubate the sample for **10 min** at **40°C** with **5sec on/30 sec off** shaking cycles.
5. After the incubation, place the tube on the magnetic rack and let the beads concentrate for at least **5 min**.

Enrichment of the target RNA-protein complexes

1. Concentrate the prepared targeting beads on a magnet and remove the storage buffer.
2. Transfer all of the sample volume from the concentrated negative control beads to the concentrated targeting beads, remove the tube from a magnet and resuspend the targeting beads in the lysate thoroughly by pipetting or vortexing. Avoid excess foaming of the sample.
3. Add 0.5 ml of MagIC Wash Buffer I FA to the control beads. Leave them on the bench top at room temperature. Later, perform all wash steps on the control beads simultaneously with washes on the targeting beads (step 8 of this section of the protocol).
4. Place the tube with targeting beads into a thermo block preheated to **40°C** and incubate with **1200-1400 RPM** with **5sec on/30 sec off** cycles. **The optimal RPM value will vary depending on the volume of the sample, and the beads used. It may have to be adjusted empirically. It is critical to provide shaking conditions, which prevent the particles from accumulating at the bottom of the tube.**
5. Incubate the sample for **30 min** at **40°C** with **5sec on/30 sec off** shaking cycles.
6. After the incubation, place the tube on the magnetic rack and let the beads concentrate for at least **5 min**.
7. Remove the sample from the beads (the lysates after the incubation with the beads may be saved and used for subsequent rounds of target captures from the same sample).
8. Add 0.5 ml of MagIC Wash Buffer I FA to the targeting beads, resuspend them and the negative control beads (from step 3 of this section of the protocol) by gentle pipetting, and wash by incubating for 10 min at **40°C** with interval mixing with **5sec on/30sec** off cycles at **1200 RPM**.
9. After the wash, concentrate the beads on a magnet for at least **5 min** and discard the buffer.
10. Steps 8-9 should be repeated for a total of **2 washes**.
11. Add 0.5 ml of MagIC Wash Buffer II FA to all bead samples, resuspend them by gentle pipetting and wash by incubating for 10 min at **40°C** with interval mixing with **5sec on/30sec** off cycles at **900 RPM**.
12. After the wash, concentrate the beads on a magnet for at least **2 min** and discard the buffer.
13. Steps 11-12 should be repeated for a total of **3 washes**.
14. Add 0.5 ml of MagIC Wash Buffer II FA to the beads, resuspend them by gentle pipetting and carefully transfer all of the sample volume to a fresh 2 ml Eppendorf tube.
15. Perform the last wash of the beads well resuspended in the buffer in fresh tubes by incubating for 10 min at **40°C** with interval mixing with **5sec on/30sec** off cycles at **900 RPM.**
16. Concentrate the beads on a magnet for at least **2 min** and discard the buffer.
17. Resuspend the beads evenly in 1 ml of fresh MiliQ water by gently pipetting.

18. Take 100 µl of the bead suspension to a fresh tube for the later analysis of the RNA pull-down efficiency.
19. Concentrate all bead portions on a magnet for at least **4 min**.
20. In the meantime prepare the RNase I solution for the elution by taking to a fresh tube 50 µl of the provided 1x RNase I Buffer, adding 100U of RNase I, and mixing well.
21. Discard the water from all bead portions.
22. For the RNase-mediated elution of captured chromatin segments add to the main portion of the beads the buffer-RNAse I mixture, resuspend the beads well by pipetting and incubate in the thermo block at **37°C** with constant shaking at **800 RPM** for **30 min**.
23. For the elution of all captured molecules from the control 10% fraction of the beads add 100 µl of 10mM Tris pH 7.5, resuspend the beads well and incubate in the thermo block for **2 min at 92°C**. Concentrate the beads immediately on the magnet and transfer the eluates to fresh tubes.
24. After the 30 min incubation of the main bead portion with the RNase, concentrate the beads on the magnet for at least **2 min** and transfer the eluate to a fresh tube.
25. While beads are concentrating prepare another portion of the 50 µl RNase I Buffer well mixed with 100U of RNase I and add it to the beads after the first elution is finished, resuspend the beads well incubate again in the thermo block at **37°C** with constant shaking at **800 RPM** for **30 min**.
26. After the second 30 min incubation, concentrate the beads on a magnet for at least **2 min** and transfer the second eluate to the tube already containing the first one, combining both eluates.

Isolation of the nucleic acids

1. To the 1% of input sample add an equal volume of MiliQ water and 1/10 of the resulting sample volume of 20 mg/ml Proteinase K solution.
2. To all bead eluates add an equal volume of the MagIC Wash Buffer I FA and 1/10 of the resulting sample volume of 20mg/ml Proteinase K solution.
3. Incubate the samples with proteinase in the thermo block at **55°C** with constant shaking at **900 RPM** for **90 min**.

-----Nucleic acids should not be purified with any other method than the Phenol/Chloroform extraction!-----

4. Add to the samples volumes of Phenol/Chloroform/Isoamyl alcohol equal to the sample volumes. Vortex thoroughly and centrifuge at **22°C** for **15 min**.
5. Transfer the aqueous phases from the sample to fresh tubes without disrupting the interphase of water-chloroform.
6. Add 1ul of 5mg/ul Glycogen and 1/10 of 3M lithium chloride to the recovered samples.
7. Mix well, centrifuge briefly, and add 1 sample volume of Isopropanol to each tube.
8. Mix samples well and centrifuge at **20 000 g** for **30 min** at **4°C**.
9. Discard the alcohol from the nucleic acids pellet and centrifuge them again for 1-3 seconds at **20 000 g**.
10. Discard any remaining liquid from the pellets and air dry them for **5-10 min**.
11. Resuspend the nucleic acid pellets in desired volumes of fresh MiliQ water.
12. The samples may be used immediately for downstream processing or analysis or stored at -80°C for later use.

The eluates obtained from the RNase I treatments are intended for library preparations and downstream analysis with sequencing. The heat eluted samples are intended for the evaluation of the pull-down efficiency to control for the success of the experiment before the sequencing.

13. Proceed to cDNA synthesis with random primers on the nucleic acids isolated from the 1% of input sample and heat eluted samples according to the protocol recommended for Reverse Transcriptase of choice and analyze the efficiency of the RNA capture using RT-qPCR with primer pairs of choice.
14. Calculate the efficiency and specificity of the target capture before preparing the samples for sequencing.
15. Proceed to the preparation of the sequencing library with a chosen kit according to the manufacturer's instructions using the RNase eluted sample.

Reusability of the beads:

After each round of target capture, the beads are expected to lose a few % of their binding capacity. They are, however, reusable.

To ensure no carryover of biomolecules between experiments the beads need to be incubated in 10x volume of the original bead suspension of 10 mM Tris-HCl pH 7.5 for 2 min at 92°C after the elution of enriched molecules and before placing the beads into the storage buffer.

The potential carryover of RNase I is not a concern, because RNase I is fully inactivated by the MagIC Lysis Buffer FA.

For the short-term storage of used beads a buffer with the following components should be prepared: 0.05 % Tween®20, 10 mM Tris-HCl (pH 7.5 @ 25°C), 150 mM LiCl, 0.5 mM EDTA

For long-term storage, the storage buffer should be supplemented with NaN3 to a final concentration of 0.02%.

We do not recommend re-using the beads more than 2 times.