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-RNA complexes from chemically cross-linked material.

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

Beads:
The provided 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 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 or the label on the bead container to calculate the volume of the bead suspension carrying 20 pmol of capture probes.

Negative Control Beads can be used with the kit and their use is described in the protocol. Their use, however, is optional and they have to be purchased separately.
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 for formaldehyde/glutaraldehyde cross-linked material as in the MagIC Beads system the probe binding dependent experimental background is minimal or non-existent and the vast majority or all of the background signal originates from the binding of the cross-linked molecules to the surface of the beads.

Buffers:
All buffers should be kept at 4°C for long-term storage. The components of the buffers, however, precipitate in low temperatures and need to be re-dissolved before each use.

MagIC Lysis Buffer FA – optimized for cell lysis, solubilization of cross-linked molecules, and RNase inhibition.

MagIC Lysate Dilution Buffer FA – provides optimal conditions 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.

Other required materials (not provided):
- Cross-linked biological material
- Magnetic rack.
- Temperature controlled mixer
- Centrifuge
- 1M Dithiothreitol (DTT)
- Protease inhibitors of choice
- Fresh MiliQ water
- Nuclease-free 10 mM Tris pH 7.5

Protocol:
Before you start

Buffer preparation:

MagIC Lysis Buffer FA – Remove the bottle from 4°C and equilibrate to room temperature. Occasionally gently shake the bottle until all precipitated buffer components dissolve and the buffer is clear. Transfer to a fresh tube 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 (up to 45 µl per ml of the buffer, **do not exceed the 1x concentration of the protease inhibitors**), mix well, and keep at room temperature until needed.

MagIC Lysate Dilution Buffer FA – Remove the bottle from 4°C and equilibrate to room temperature. Occasionally gently shake the bottle until all precipitated buffer components dissolve and the buffer is clear. Take additional care when looking for any undissolved buffer components. Re-dissolving the buffer components may be accelerated by incubating the bottle at 30-40°C if necessary. Keep at room temperature until needed.

MagIC Wash Buffer I FA - Remove the bottle from 4°C and equilibrate to room temperature. Occasionally gently shake the bottle until all precipitated buffer components dissolve and the buffer is clear. Re-dissolving the buffer components may be accelerated by incubating the bottle at 30-40°C if necessary. Keep at room temperature until needed. Keep at room temperature until needed.

MagIC Wash Buffer II FA - Remove the bottle from 4°C and equilibrate to room temperature. Occasionally gently shake the bottle until all precipitated buffer components dissolve and the buffer is clear. Take additional care when looking for any undissolved buffer components. Re-dissolving the buffer components may be accelerated by incubating the bottle at 30-40°C if necessary. Keep at room temperature until needed.

Thermo blocks:

Prepare thermo blocks, which will be used at 40°C for hybridization, 40°C and 45°C for the washes, and 92°C for the elution of captured molecules.

Preparation of the lysates for the enrichment

1. Take desired amount of cells cross-linked with formaldehyde, free of the collection buffer (typically, ~40 mln cells per 20 pmol of capture probes) 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 with 3 cell pellet volumes of room temperature MagIC Lysis Buffer FA containing DTT and protease inhibitors by pipetting the cells with the buffer up and down until the pellet is evenly dissolved in the buffer.
3. The lysis of chemically cross-linked cultured cells can only be achieved with enzymatic or mechanical methods. For mammalian cells, it is recommended to always use sonication. For other organisms, whenever applicable, it is recommended to use the appropriate procedure for the enzymatic disruption of the cells before adding the MagIC Lysis Buffer FA or to perform sonication directly in the MagIC Lysis Buffer FA.
4. Sonicate the cells in MagIC Lysis Buffer FA with a device of choice until all of the cells have lysed and the viscosity of the lysate is reduced. **Avoid excessive sonication**.

It is critical to not over-fragment the RNA in the samples as this may negatively influence the efficiency of the enrichment with MagIC Beads.
The exact sonication procedure for the optimal cell lysis may have to be empirically defined and optimized as it is heavily dependent on the specific type of the sonicator used, the nature of the biological material, the

cross-linking protocol, and the number of cells used. The condition for optimal sonication outcome may have to be established in advance for each specific setup before performing the MagIC Beads enrichment procedure.

5. Centrifuge the lysates at **22°C**, **18 000 - 20 000 g** for **15 min** to pellet the insoluble cellular components.
6. Transfer the supernatant to a fresh tube, while avoiding the disruption of the pellet.
7. Centrifuge the lysates again at **22°C**, **18 000 - 20 000 g** for **15 min** to pellet any remaining insoluble cellular components.
8. Transfer the supernatant to a fresh tube, while avoiding the disruption of the pellet.
9. Add to the cleared supernatant room temperature MagIC Lysate Dilution Buffer FA at a volume equal to that of MagIC Lysis Buffer FA used for cell lysis.
10. Mix lysates well by pipetting or vortexing, try to avoid excessive foaming of the sample. The MagIC Lysate Dilution Buffer FA is viscous and thorough mixing of the sample after adding it is critical.
11. 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 (optional) beads on the bench and allow the content to equilibrate to room temperature.
2. After the bead suspension has warmed up resuspend the particles thoroughly.
3. Transfer the desired amount of targeting and control (optional) beads to a fresh tube.

The amount of control beads used should be equal to the mass of targeting beads used in the experiment.

4. Keep the beads in the storage buffer at room temperature, until needed.

If not using the Negative Control Beads skip the following section of the protocol.

Preparation of the experimental background control bead samples (optional)

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. Centrifuge the tube briefly to collect all the liquid at the bottom of the tube.
4. Place the tube into a thermo block preheated to **40°C** and incubate with **750-1400 RPM** with **5 sec on/30 sec off** cycles. **The optimal RPM value will vary depending on the size of the tube and the volume of the sample. It is critical to provide shaking conditions, which prevent the particles from accumulating at the bottom of the tube and at the same time do not lead to splattering of the lysate on the cap of the tube. The optimal RPM value may be determined at this stage.**
5. Incubate the sample for **10 min** at **40°C** with **5 sec 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 **4 min**.
7. Transfer the entire volume of the lysate directly to the targeting beads free of the storage buffer (step 2 of the next section of the protocol).
8. Add 0.4 ml of MagIC Wash Buffer I FA to the control beads. Leave them on the bench at room temperature. Later, perform all wash steps on the control beads simultaneously with washes on the targeting beads (next section of the protocol, steps from 7 on).

Enrichment of the target RNA-RNA complexes

1. Concentrate the prepared targeting beads on a magnet and remove the storage buffer.
2. Add the processed lysate to the beads (if using Negative Control Beads transfer the lysate from step 7 of the previous section of the protocol instead) 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 shake at **750-1400 RPM** until the particles spread evenly across the lysate (confirmed visually). **The optimal RPM value will vary depending on the size of the tube and the volume of the sample. It is critical to provide shaking conditions, which prevent the particles from accumulating at the bottom of the tube and at the same time do not lead to splattering of the lysate on the cap of the tube. The optimal RPM value may be determined at this stage.**
4. Incubate the sample for **30 min** at **40°C** with **5 sec 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 **4 min** (the required concentration time might be considerably longer if tubes larger than standard Eppendorf tubes are used).
6. Remove the lysate from the beads.
7. Add 0.4 ml of MagIC Wash Buffer I FA per 20 pmol of capture probes used to the beads, resuspend them by gentle pipetting, and wash by incubating for 10 min at **40°C** with interval mixing with **5 sec on/30 sec** off cycles at **750-1400 RPM**.
8. After the wash, concentrate the beads on a magnet for at least **4 min** and discard the buffer.
9. Steps 7-8 should be repeated for a total of **2 washes**.
10. Add 0.4 ml of MagIC Wash Buffer II FA to the beads, resuspend them by gentle pipetting, and wash by incubating for 10 min at **45°C** with interval mixing with **5 sec on/30 sec** off cycles at **750-900 RPM**.
11. After the wash, concentrate the beads on a magnet for at least **2 min** and discard the buffer.
12. Add 0.4 ml of MagIC Wash Buffer II FA to all bead samples, resuspend them by gentle pipetting, and transfer the entire sample volume to a **fresh tube**.
13. Wash the beads by incubating the tube for 10 min at **45°C** with interval mixing with **5 sec on/30 sec** off cycles at **750-900 RPM**.
14. After the wash, concentrate the beads on a magnet for at least **2 min** and discard the buffer.
15. Resuspend the beads in 1 ml of room temperature nuclease-free 10 mM Tris pH 7.5 per 20 pmol of capture probes by gentle pipetting and concentrate them on a magnetic rack for at least **3 min**.
16. Discard all the liquid.
17. Resuspend the beads in the desired volume of Elution Buffer (nuclease-free Tris-HCl, pH 7.5). The recommended volume of Elution Buffer is 40-50 µl per 20 pmol of capture probes.
18. Heat the sample for **2 min at 92°C** in a thermo block with constant shaking at 600 RPM.
19. Concentrate the beads on a magnet for at least **2 min** and transfer the liquid containing eluted RNA-RNA complexes to a fresh tube and keep it on ice.
20. The beads after the elution may be discarded or processed for storage for later use (see the last section of this protocol).

Isolation of the nucleic acids and analysis of the pull-down efficiency

1. To the 1% of the 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 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/ml 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 **~30 sec** 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.
13. Proceed to cDNA synthesis with random primers on the nucleic acids isolated from the 1% of input sample and 1% of bead eluate 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 main samples for downstream analysis.

Analysis of RNA co-captured with target transcript

The bead eluates remaining after the analysis of the pull-down efficiency can be subjected to the RNA sequencing library prep method of choice and the RNA interactors can be analyzed by sequencing. Alternatively, cDNA synthesis can be performed on the remaining eluates to assess the presence of candidate interactors with RT-qPCR.

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 nucleic acids and proteins 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.

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.