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-protein 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 200 pmol. The volume of the original bead suspension carrying 200 pmol of probes is variable.

Consult the MagIC Beads datasheet provided with your order to calculate the volume of the bead suspension carrying 200 pmol of capture probes.

Negative Control beads are recommended to be used with the kit and their use is described in the protocol. They have to, however, 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 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.

Other required materials (not provided):

- Magnetic rack.
- Temperature controlled mixer
- Centrifuge
- Reagents for the cross-linking method of choice (formaldehyde and glycine are 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 of the captured molecules
- 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.

Thermo blocks:

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

Harvesting adherent cells

1. Grow cells in tissue culture flasks or dishes to confluency (typically, a total of 200 million cells is needed per experiment).
2. Aspirate the media from the plates, rinse cells once with room temperature PBS and trypsinize.
3. Stop trypsinization by adding the complete media to the plates.
4. Resuspend the cells into a single cell suspension and count their concentration (either with an automated cell counter or hemocytometer).
5. Transfer the cells to 50 ml tubes (up to 50 million cells per tube).
6. Pellet the cells at **800 g** for **4 min** at **room temperature** and aspirate the media.
7. Resuspend the cells in ~0.8 ml PBS per million cells (~40 ml per 50 million cells).
8. Pellet the cells at **800 g** for **4 min** at **room temperature** and carefully aspirate as much PBS as possible.
9. Be ready to immediately move to the "***Cross-linking cells***" section of this protocol.

Harvesting suspension cells

1. Transfer cells with media to 50 ml conical tubes (typically, a total of 200 million cells is needed per experiment).
2. Pellet the cells at **200 g** for **5 min** at **room temperature** and aspirate the media. Centrifuge the cells again for **15-30 sec** and remove the remaining supernatant with a pipette tip.
3. Resuspend the pellets in 25 ml of ice-cold PBS.
4. Count cell concentration (either with an automated cell counter or hemocytometer).

5. If cell concentration is higher than 1 million per ~0.8 ml PBS adjust the concentration accordingly and make sure to keep up to 50 million cells per 50 ml tube (40 ml of the adjusted cell suspension per tube).
6. Pellet the cells at **200 g** for **5 min** at **room temperature** and aspirate the PBS. Centrifuge the cells again for **1 min** and remove the remaining supernatant with a pipette tip. Keep tubes on ice until.
7. Be ready to immediately move to the "***Cross-linking cells***" section of this protocol.

Cross-linking cells

Cells have to be chemically cross-linked to make the capture of RNA-protein complexes possible. Typically formaldehyde or glutaraldehyde are preferred in this type of experiment (formaldehyde is further preferred over glutaraldehyde as glutaraldehyde cross-links are irreversible and their presence may interfere with the downstream mass-spectrometry detection). 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% formaldehyde for cultured cells is described.

1. Prepare 1% solution of formaldehyde in PBS (0.9 ml of the solution per every million cells is required) freshly before use.
2. Prepare 1.25 M solution of glycine in PBS (1 ml of the solution will be needed per every 10 ml of the formaldehyde solution).
3. Take cellular pellets freshly harvested into 50 ml tubes (free of any excess liquid) and resuspend the cells in a small volume of 1% formaldehyde until no chunks are visible.
4. Add the remaining formaldehyde solution to the tube to the final required amount of 0.9 ml per million cells. Mix by inverting and cross-link for **30 min** on a rotator or in a shaker.
5. Add 1 ml of 1.25 M glycine solution per every 10 ml of formaldehyde solution used for the cross-linking and continue incubating for another **5 min**.
6. Pellet the cells at **2000 g** for **5 min** at **4 °C**.
7. Remove the supernatant from the cells and resuspend them in 20 ml of cold PBS, and pellet them again at **2000 g** for **5 min** at **4°C**.
8. Remove the supernatant and resuspend the pellet in 1ml of cold PBS per 20 mln cells.
9. Transfer the cells to 2 ml Eppendorf tubes, pellet them at **2000 g** for **3 min** at **4 °C** and **remove as much PBS as possible** with a pipette.
10. 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 or purified nuclei free of the collection buffer (recommended ~200 mln cells per 200 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.

Important: The standard input of 200 mln cells is on average sufficient for the successful downstream mass-spectrometry detection of proteins interacting with the target RNA. Some targets, however, may require a different amount of cells for a successful experiment.

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. The lysis of chemically cross-linked cultured cells can only be achieved with the aid of mechanical disruption of the cellular membranes. For mammalian cells, it is recommended to use a dounce homogenizer for this purpose (described in subsection **a**). For cells from organisms characterized by cell sizes too small to be disrupted by the douncer, it is recommended to use the appropriate procedure for the enzymatic disruption of the cells before the addition of the MagIC Lysis Buffer FA or to perform sonication directly in the MagIC Lysis Buffer FA (described in subsection **b**).

a. Transfer the cells into the cylinder. Initially, using a B-type pestle perform gentle homogenization. Apply a few gentle strokes until the cells look to be lysed and the lysates seem homogenous in appearance. Then apply 10-20 vigorous strokes, rotating the pestle when applying movements. Transfer the lysate to a fresh tube.

The advantage of the dounce homogenization compared to sonication is that it is not expected to fragment the RNA in the lysate at all, while at the same time, it is able to lyse the cells completely and if performed vigorously can also provide fragmentation of the chromatin to provide appropriate reduction of the viscosity of the lysate.

b. Sonicate the cells until all of them 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 be prohibitive for the downstream enrichment with MagIC Beads.
The sonication procedure required to obtain proper cell lysis is variable as it is heavily dependent on the model of the sonicator used as well as the type of biological material, the cross-linking protocol, and the number of cells used. The condition of sonication should 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 the desired amount of targeting (typically equivalent to 200 pmol of probes on the beads per capture reaction) and control beads (the same volume of the control beads suspension as taken for the targeting beads) 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 **5 sec 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 **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 **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 1 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 **5 sec 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 **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 **5 min** (the required concentration time might be considerably longer if tubes larger than standard Eppendorf tubes are used).
7. Remove the lysate from the beads. Optionally, transfer the entire volume of the lysate to a fresh Eppendorf tube for later use.

Important: The lysate might be transferred to a fresh tube and kept at room temperature if it is to be re-used. The lysate might be used for up to 2 subsequent rounds of capture with the same portion of the beads to maximize the pull-down efficiency. Performing 3 rounds of capture on the same sample ensure the maximum possible target yields. For details see the next section of the protocol.

8. Add 1 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 **5 sec on/30 sec** 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 1 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 **5 sec on/30 sec** 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 1 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 **5 sec on/30 sec** 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. Concentrate the beads on a magnet for at least **3 min** and discard the water.
19. Add 100 ul of nuclease-free 10 mM Tris pH 7.5 to all targeting and negative control beads, 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.
20. Take 1% of the volume of each eluate to a fresh Eppendorf tube for the analysis of the pull-down efficiency. Keep the remaining eluates at -80°C.

21. The beads after the elution may be discarded, processed for storage for later use (see the last section of this protocol), or immediately re-used with the same sample for maximizing the target yield from the sample (the next section of this protocol).

Subsequent rounds of enrichment (optional)

In most cases, a single round of enrichment captures >90% of the target molecules from a sample. For some targets, however, sub-optimal yields have been reported. When using custom-target MagIC Beads for a new target for the first time it is recommended to perform 3 rounds of the capture on the same sample with the same portion of the beads, analyze the pull-down efficiency from each round, and determine the optimal number of enrichment rounds in a given setup.

1. Directly after the heat elution (step 15 from the previous section of the protocol) add to the beads the entire volume of the sample previously set aside (step 7 from the previous section of this protocol), remove the tube from a magnet, and resuspend the beads in the lysate well by gentle pipetting.
2. Follow steps 3-18 from the previous section of the protocol for up to a total of 3 rounds of the target enrichment from a single sample.

The efficiency and specificity of each enrichment round should be analyzed separately, but if subsequent enrichment rounds are determined to be of good quality the eluates should be combined into one sample for the downstream detection of the target interactors with mass-spectrometry.

Analysis of the efficiency of the enrichment

Before performing protein mass-spectrometry on the enriched samples it is recommended to assay the specificity and efficiency of the capture by performing RT-qPCR analysis on the 1% of input and 1% of eluate samples with primer pairs specific to the target transcript and one or more highly expressed non-target transcripts. For that, extraction of RNA from the 1% control samples is necessary.

3. 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.
4. To the 1% of bead eluates samples 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.
5. 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!-----

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

17. Proceed to the preparation of the sequencing library with a chosen kit according to the manufacturer's instructions using the RNase eluted sample.

Mass spectrometry detection of captured proteins

Various strategies for preparing the samples for mass spectrometry can be employed after the MagIC Beads-based enrichment. The best results, however, were consistently observed when eluted samples were mixed with Tris pH 7.5 to a final concentration of 100 mM and SDS to a final concentration of 2%, incubated at 94°C for 15 min (to reverse the protein cross-links) and loaded onto FASP columns (if a high sample volume is reached a single column can be loaded more than once), and processed further with washing and on the column trypsin digestion following the standard protocol for the columns.

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.

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.