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 UV 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 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 or the label on the bead container to calculate the volume of the bead suspension carrying 200 pmol of capture probes.

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):

- Magnetic rack
- Temperature controlled mixer
- Centrifuge
- 1M Dithiothreitol (DTT)
- Protease inhibitors of choice
- Nuclease-free 10mM Tris-HCl 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.

Cross-linking cells

Cells have to be UV cross-linked to make the capture of RNA-protein complexes possible. Cross-linking with 254 nm wavelength can be used, but 4-Thiouridine (4SU) dependent cross-linking with 365 nm wavelength is heavily preferred due to its superior efficiency.

Below, the recommended 4SU-mediated cross-linking procedure for adherent cells cultured on plates is described.

1. Supplement cell culture media with a final concentration of 200 µM 4SU and grow cells for 8 h before cross-linking.
2. Remove the growth media from the cells and wash them once with ice-cold PBS.
3. Remove as much PBS as possible and cross-link the cells while keeping the plates on ice using 0.8 J/cm^2 of 365-nm UV light.
4. Immediately after the cross-linking scrape the cells from culture dishes with a few milliliters of ice-cold PBS and transfer them to tubes (cells can be counted at this stage).
5. Pellet the cells by centrifugation at 500g for 5 min at 4°C.
6. Discard as much PBS as possible from the pellet and use immediately for the experiments or flash free-freeze in liquid nitrogen and store at -80°C for later use.

Preparation of magnetic beads for the enrichment

1. Place the container with magnetic 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 beads to a fresh tube.
4. Keep the beads in the storage buffer at room temperature, until the cellular lysates are prepared.

Preparation of the lysates for the enrichment

1. Take desired amount of cells or purified nuclei free of the collection buffer (typically, ~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 lyse 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. Incubate the cells with the buffer at room temperature for ~10 min.
4. Reduce the viscosity of the lysate by either:
    a. Passing the entire lysate volume, using a syringe, through a 21G needle 5-20 times (until the viscosity reduction can be observed visually).
    b. Applying the entire lysate volume to a QIAshredder column according to the guidelines provided by the manufacturer – remember that a single column can be loaded multiple times in case the lysate volume exceeds the maximum loading capacity of the column.
5. Centrifuge the lysates at 4°C at 20 000 g for 10 min to pellet the insoluble cellular components.
6. Transfer the supernatant to a fresh tube, while avoiding the disruption of the pellet.
7. Add to the lysate room temperature MagIC Lysate Dilution Buffer FA at a volume equal to that of MagIC Lysis Buffer FA used for the lysis.
8. 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.

Enrichment of the target RNA-protein complexes

1. Concentrate the prepared 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 well by gentle pipetting.
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. Optionally, transfer the entire volume of the lysate to a fresh 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 ensures the maximum possible target yields. For details see the next section of the protocol.

7. Add 1 ml of MagIC Wash Buffer I FA per 200 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 1 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. Steps 11-12 should be repeated for a total of **2 washes**.

13. Resuspend the beads in 1 ml of room temperature Elution Buffer (10mM Tris-HCl, pH 7,5) per 200 pmol of capture probes by gentle pipetting and concentrate them on a magnetic rack for at least **3 min**.
14. Discard the buffer.
15. Resuspend the beads in the desired volume of Elution Buffer (nuclease-free Tris-HCl, pH 7.5). The recommended volume of Elution Buffer is 50-100 µl per 200 pmol of capture probes.
16. Heat the sample for **2 min at 92°C** in a thermo block with constant shaking at 600 RPM.
17. Concentrate the beads on a magnet for at least **2 min** and transfer the liquid containing eluted RNA-protein complexes to a fresh tube and keep it on ice.
18. The eluted sample is suitable for downstream processing and analysis with the methods of choice.
19. It is recommended to take 1% of the volume of the eluate for the analysis of the enrichment efficiency.
20. 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 17 from the previous section of the protocol) add to the beads the entire volume of the sample previously set aside (step 6 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 3-20 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 eluted 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% of the input sample is necessary. The sample eluted from the beads, however, should not be subjected to additional RNA extraction. As long as random hexamers are used as primers in the reverse transcription reaction RT-qPCR will be successful without further sample purification.

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. 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!-----

3. 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**.
4. Transfer the aqueous phases from the sample to fresh tubes without disrupting the interphase of water-chloroform.
5. Add 1ul of 5mg/ml Glycogen and 1/10 of 3M lithium chloride to the recovered samples.
6. Mix well, centrifuge briefly, and add 1 sample volume of Isopropanol to each tube.
7. Mix samples well and centrifuge at **20 000 g** for **30 min** at 4**°C**.

8. Discard the alcohol from the nucleic acids pellet and centrifuge them again for **~30 sec** at **20 000 g**.
9. Discard any remaining liquid from the pellets and air dry them for **5-10 min**.
10. Resuspend the nucleic acid pellets in desired volumes of fresh MiliQ water.
11. The samples may be used immediately for downstream processing or stored at -80°C for later use.
12. Proceed to cDNA synthesis with 1% of the eluted sample and the RNA extracted from the 1% of the input sample with random hexamers, 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.
13. Calculate the efficiency and specificity of the target capture to assess the quality of the enrichment before preparing the samples for protein mass spectrometry.

Remember: The bead eluate can be used directly for cDNA synthesis with random hexamers followed by qPCR without any need for additional processing. The presence of UV-mediated protein crosslinks on the target RNA is not prohibitive for cDNA synthesis as long as it is done with random hexamers.

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 processed with a Filter Aided Sample Preparation (FASP) system involving the trypsinization of the proteins bound to the column. FASP Protein Digestion Kits are available from various providers and following the specific guidelines for a chosen kit variant is recommended.

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.