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Salmaso Lab TRAP Adaptation

Forked from a private protocol

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We use this protocol and it's working

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Abstract

This protocol details the procedure of Salmaso Lab TRAP Adaptation.

Attachments

516-1071.docx

34KB

Guidelines

Note

The following protocol is for 3-day TRAP; 3-Day protocol allows maximal coating of magnetic beads with anti-eGFP.

The **maximum** number of IP that may be performed using a single 1.5 mL tube is limited to 1mL of reagent; this works out to a MAXIMUM of 6 IPs per tube, this protocol should be limited to using a **maximum** of *20 IPs per 3-Day protocol* (limits of the equipment).

The main goals of the protocol are as follows:

- Preparing the magnetic beads for incubation with your sample. This is achieved through washing the beads and then coating them in *Protein L* and then incubating the coated beads with anti-GFP
- Homogenizing your tissues and preparing them to be incubated with anti-GFP coated magnetic beads
- Extracting the mRNA that has been extracted from your tissues currently bound to the anti-GFP magnetic beads
- Use RNAse Zap wipes throughout (Ambion AM#9786)

Materials

Day 1: Coating the Magnetic Beads & Preparing Stock Solutions

Solutions:

- Invitrogen Dynabeads™ MyOne™ Streptavidin T1 **Thermo Fisher Scientific Catalog #**65601
- Pierce™ Recombinant Protein L, Biotinylated **Thermo Fisher Catalog #**29997
- Anti-GFP antibodies (made for TRAP; stored at -20 °C)

- Stock [M] 0.15 Molarity (M) & 0 [M] 35 Molarity (M) KCl Wash Buffer, Homogenization buffer (for Day 2)

1. 1M MgCl2 **Ambion Catalog #**AM9530G
2. 2M KCl **Invitrogen - Thermo Fisher Catalog #**AM9640G
3. Surfact-Amps NP-40 **Thermo Fisher Scientific Catalog #**28324
4. 1M DTT **(added immediately before use**)
5. CHX-MeOH (**added immediately before use**)

- PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #**AM9625
- Protease-free BSA (make sure it is IgG free; BioShop#9048-46-8)

Materials & Equipment:

- 1.5 ml micro-centrifuge tube **Ambion Catalog #**AM12450
- Magnetic stand with tube carriage
- Slow-rotating rotisserie
- Access to a walk-in cold room **or** a cold fridge with electrical outlets

Stock Solution Preparation

- Prepare the stock solutions of Protein L, [M] 0.15 Molarity (M) KCl Wash Buffer, [M] 0.35 Molarity (M) KCl Wash Buffer, & Homogenization Buffer using sterile, biochemical grade water to save time during subsequent days.

- Reconstitute **Biotynlyated *Protein L*** to [M] 1 µg/µL with sterile, TRAP-grade *1x PBS*. Be sure to wash the sides of the bottle with the *1x PBS* and swirl gently to dissolve the *Protein L.*

To prepare a stock *100 mL* of ***0.15M KCl Wash Buffer:***

- Gather a 100mL biochemical grade sterile water container. Remove approximately 25mL into a CLEAN beaker, set aside for later use.
- To the biochemical water container add:
  a. 1 mL of HEPES-KOH buffer
  b. 500 µL of $MgCl_2$
  c. 7.5 mL of KCl
  d. 10 mL of 10% NP-40 (one glass vial; use caution when opening)
- Using the water set aside, fill container back to 100 mL and mix manually.

Note

This is not the final solution; immediately before use you will need to add activated CHX & DTT.

To prepare a stock *100mL* of ***0.35M KCl Wash Buffer***

- Gather a 100mL biochemical grade sterile water container. Remove approximately 30 mL into a CLEAN beaker, set aside for later use.
- To the biochemical water container add:
  a. 1 mL of HEPES-KOH buffer
  b. 500 µL of $MgCl_2$
  c. 17.25 mL of KCl
  d. 10 mL of 10% NP-40 (one glass vial; use caution when opening)
- Using the water set aside, fill container back to 100 mL and mix manually.

Note

This is not the final solution; immediately before use you will need to add activated CHX & DTT.

To prepare a stock *100mL* of ***Homogenization Buffer***

Note

You will need 1 mL of buffer PER sample (n); it is best to make more than required for the entire experiment to avoid having the remake more before a run.

- Gather a 100mL biochemical grade water container. Remove approximately 10 mL into a CLEAN beaker, set aside for later use.
- To the biochemical water container add:
    a. 1 mL of HEPES-KOH
    b. 500 µL of $MgCL_2$
    c. 7.5 mL of KCl
- Using the water set aside, fill container back to 100 mL and mix manually.

Note

This is not the final solution; immediately before use you will need to add activated CHX, DTT, Superasin, Protease Inhibitor Tablet.

Day 2: Tissue Homogenization & Bead Incubation

Materials Needed:

- Previously prepared stock solutions:
    1. Homogenization buffer
        a. Protease inhibitor tablets, SUPERase, CHX, DTT

2. [M] 0.15 Molarity (M) KCl Wash Buffer
   a. CHX, DTT
- 10% NP-40 (in a glass vial)
- DHPC **Avanti Polar Lipids, Inc. Catalog #**850306
- Large tubes (5mL), flat bottom preferably
- New set of 1.5mL low-bind tubes
- PCR tubes

Day 3: mRNA Extraction & Quantification

Materials Needed:

- Absolutely RNA Nanoprep Kit 50 preps **Agilent Technologies Catalog #**400753
  1.Lysis Buffer
  2. βME
  3. DNAse
  4.Salt Washes
- [M] 0.35 Molarity (M) KCl Wash Buffer
- 80% Sulfolane (warmed on the dry bath)

Before start

Day 3: mRNA Extraction & Quantification:

Before beginning; turn on the incubator to 37 °C ; and the dry bath to Level 3-5 on the “low” setting. Place the sulfolane bottle on top of the dry bath to warm and liquify.

Day 1: Magnetic Bead Preparation

1

Note

The below information is for 6 IPs, with single values in parentheses.
It is important to note that each run should encompass at least 1 whole N (one IP from each group) from your experiment.

Gather the *magnetic beads*; pipet the solution up and down to mix the beads.

Note

The magnetic beads settle on the bottom of the bottle and need to be mixed gently to ensure proper distribution and concentration.

2

Extract 360 µL of the *magnetic beads* into a 1.5mL low-bind tube [60 µL **per IP**]. Place the tube into the tube carriage and place onto the magnet. Wait for the beads to settle on the walls of the tube.

3

Wash beads **3** times: Discard the excess liquid from the tube, wash with 1 mL *of 1x PBS*. Remove the tube carriage and shake to suspend magnetic beads in 1x PBS. Replace the magnet, discard the excess 1x PBS and repeat 2 more times.

4

Collect the beads on the magnet and remove all excess liquid. Incubate the beads with 144 µL of ***Protein L*** ([M] 1 µg/µL) [24 µL **per IP**]. Fill the tube with 1x PBS for a **total** volume of 1 mL , in this case 496 µL of 1x PBS.

(360 µL beads + 144 µL Protein L + 496 µL 1 x PBS = 1000 µL , 1 mL)

5

Place the tube in the rotating rotisserie and spin slowly for ~ 01:00:00 **at** Room temperature .

1h

Note

Incubating beads with Protein L allows the beads to become coated in protein that is required to bind anti-eGFP.

6 While beads rotate, prepare 10 mL of 3% BSA in 1x PBS (weight/volume).

6.1 Measure out 300 mg of solid BSA and add to a 15mL conical tube.

6.2 Top up conical tube to 10 mL with 1x PBS, mix vigorously until BSA is dissolved.

7 Also prepare 10 mL of **FINAL** [M] 0.15 Molarity (M) *KCl Wash Buffer* in a 15mL conical tube by adding:

7.1 5 µL [M] 1 Molarity (M) DTT.

Safety information

Very toxic, dispose of in hazardous wastes; pungent smell.

7.2 10 µL activated 1000x CHX (100% MeOH, see dissection protocol).

8 After beads have been incubated with Protein L, collect the beads using the magnet and discard excess liquid. Wash the beads with the 3% BSA solution **5 times.**

Note

Washing with high volume of BSA collects the excess Protein L that has not been bound well to the magnetic beads.

9 After the final wash, resuspend the beads with 175 µL of [M] 0.15 Molarity (M) *KCl* and 47 µL of anti-GFP solution. Rotate Overnight (~ 24:00:00) in a walk-in cold room, or fridge (4 °C). 2d

Day 2: Tissue Homogenization and Bead Incubation

10

Note

You will need a large **bin of ice to keep samples** cold between uses, for the beginning steps leave beads rotating in the cold room until samples are ready to be homogenized. Day 2 incubates the magnetic beads with sample to extract mRNA.

Note

Try to keep samples On ice whenever possible; do not let them sit at Room temperature for too long. Be sure to clean all surfaces being used with RNAse wipes. Day 2 requires utmost care for being clean and sterile.

Prepare 1mL/sample of **homogenization buffer** from stock solution. Measure into a 15mL conical tube (try to make at least 10 mL at a time; up to 12 mL at a time maximum).

10.1 Add 1 protease inhibitor tablet ***per*** 10 mL (can use 1 tablet up to 12 mL) (Fisher #A32955).

10.2 1 µL CHX ***per*** 1 mL of homogenization buffer.

10.3 100units SUPERase (RNAse inhibitor) ***per*** sample (Ambion #AM2694).

10.4 5 µL DTT ***per*** 10 mL of homogenization buffer ([M] 0.5 Mass Percent).

10.5 Mix the solution well, vigorously and until the protease inhibitor has dissolved completely (this may take a few minutes). Set aside On ice, keep cold.

11 Retrieve samples from freezer. Move sample to a new, labelled 5mL tube On ice. 5m
Add 1 mL of homogenization buffer to each sample. Let sit On ice (to thaw) for ~ 00:05:00.

> Note
>
> If you are doing fresh dissections, you do not need to let samples thaw On ice.

12 In a ***FUME HOOD***, set up homogenization blade or pestle. Gather enough dH_2O in a beaker to be able submerge the end of the blade or pestle. Turn on blade to a slow setting and submerge into homogenization buffer, moving the sample up and down until completely homogenized. Sample will be yellow and completely liquid. Clean blade with RNAse wipe + dH_2O between each sample.

13 Transfer homogenized tissues back into a new, labelled 1.5mL low-bind tube. Spin at 20000 x g for 00:10:00 at 4 °C. Collect supernatant, do not disturb the pellet, into a new, labelled 1.5mL low-bind tube. 10m

14 Add 100 µL **10% NP-40** & 115 µL **DHPC** (use a 1ml syringe because it is very thick/viscous) to each sample, mix with inversion and let sit On ice for 00:05:00. 5m

15 Spin the samples at 20000 x g for 00:15:00 at 4 °C. 15m

15.1 During the 15-minute cycle, begin to prepare the beads to be incubated with the sample. Prepare ~ 10 mL - 15 mL of [M] 0.15 Molarity (M) KCl Wash Buffer.

16 Suspend the beads on the magnet, discard excess liquid and wash ***3 times*** with 1 mL of [M] 0.15 Molarity (M) KCl wash buffer.

Note

Remember that these wastes contain CHX, DTT and need to be discarded in chemical waste.

17 After the 3rd wash, resuspend the beads using 1080 µL 0.15 Molarity (M) **KCl Wash Buffer +** 120 µL **DHPC** [180 µL 0.15 Molarity (M) **KCl,** 20 µL **DHPC per sample**].

Note

Following 15-minute cycle; collect supernatant.

18 Collect post-mitochondrial (S20), take care to avoid disturbing the pellet, into a new 1.5 tube. Collect 50 µL of the S20 post-mitochondrial supernatant into a new PCR tube, label and freeze at -80 °C .

Note

This S20 will act as “input” for validation of the model.

19 Add 200 µL of the bead solution to each sample, place on the rotisserie and let rotate Overnight at 4 °C . 10m

Day 3: mRNA Extraction and Quantification 42m

20

Note

It is vital to take care and use RNAse wipes liberally to keep surfaces and materials clean. Day 3 requires utmost care to keep samples cold to prevent RNA degradation. First you will need to strip the beads; then you will be able to extract the mRNA.

Prepare 20 mL of **FINAL** [M] 0.35 Molarity (M) *KCl* in a 50mL conical tube by adding:

20.1 10 µL [M] 1 Molarity (M) DTT.

Safety information

Very toxic, dispose of in hazardous wastes; pungent smell.

20.2 20 µL activated 1000x CHX (in 100% MeOH, see dissection protocol).

21 Retrieve the rotating samples from the rotisserie and collect in the magnet caddie, wait until beads have settled on the magnet. Extract the liquid into a labelled 1.5mL tube, this is the ***"unbound"*** fragment used for validation.

Note

The beads here contain the mRNA that you are interested in, be careful to collect all the beads before collecting the liquid portion.

22 Wash the beads with 200 µL *of* [M] 0.35 Molarity (M) *KCl* Wash Buffer ***4 times.***

23 During the 4th wash, prepare the lysis solution *in the fume hood* by mixing 600 µL ***lysis buffer*** with 4.2 µL ***βME*** in a 1.5mL tube.

Note

Both can be found in the extraction kit.

(100 µL **Lysis buffer,** 0.7 µL **βME *per* sample**)

24 Resuspend each of the sample beads with 100 µL of the prepared lysis solution, mix well and let incubate at Room temperature for 00:10:00 . Take this time to label out a new set of tubes, as well as the filters from the RNA extraction kit. 10m

Note

Be careful when handling the filter cup not to disturb the filter, wash any surface that the tubes or your hands will touch with RNAse wipes.

25 Place the samples back on the magnet and collect the liquid (which now has the mRNA) into a new, labelled, low-bind 1.5mL tube; **begin with RNA extraction from the solution.**

26 Add 100 µL of the warmed 80% sulfolane to each of the samples and mix by inversion.

Note

The sulfolane should be warm, not hot.

27 Make the DNAse soltution by combining 15 µL **reconstituted DNAse** with 75 µL **DNAse Digestion Buffer.** Set aside for now.

(2.5 µL DNAse, 12.5 µL DNAse Digestion Buffer *per* sample)

28 Put the whole sample into a labelled filter cup with a collection tube underneath, cap and spin at 12000 x g for 00:01:00 at 4 °C .

1m

Note

The filter cup now contains your RNA, keep track of the filters.

29 Discard the liquid in the collection tube (**filtrate**), add 300 µL ***Low-Salt Wash*** to the filter cup, spin at 12000 rpm for 00:01:00 at 4 °C . Discard the filtrate and spin at 12000 x g for 00:02:00 to dry the filter.

3m

30 Add 14.5 µL ***DNAse + Digestion Buffer*** *directly* onto the filter, taking care not to touch the filter of each sample, incubate at 37 °C for 00:15:00 .

15m

Note

During this time, gather 40 µL Elution Buffer (from kit) per sample in a 1.5mL tube, and heat in the warm bath until needed, collect and label PCR tubes to collect RNA for the run, use a cold caddy to keep the tubes cold.

31 Add 300 µL ***High-Salt Wash*** to each sample, spin at 12000 x g for 00:01:00 at 4 °C. Discard filtrate. 1m

32 Add 300 µL ***Low-Salt Wash*** to each sample, spin at 12000 x g for 00:01:00 at 4 °C. Discard filtrate. 1m

33 Add 300 µL ***Low-Salt Wash*** to each sample, spin at 12000 x g for 00:03:00 at 4 °C. Discard filtrate. 3m

33.1 Spin at 12000 x g for 00:01:00 at 4 °C. Discard filtrate and collection tube. Replace with a new collection tube. 1m

34 Add 20 µL heated **Elution Buffer** directly to the filter of each sample, incubate at Room temperature for 00:02:00. 2m

34.1 Spin samples at 12000 x g for 00:05:00 at 4 °C. 5m

35 Collect the resulting filtrate into a cold PCR tube, label as the “Primary Elution” (1°E).

36 Repeat steps 34 & 35, labelling the second set of PCR tubes as “Secondary Elution” (2°E).

37 Bring 1°E & 2°E to the NanoDrop; record the RNA concentrations for each sample and the 260nm/280nm values.

38 Freeze the samples at -20 °C for short term use (~2 weeks) or -80 °C for a longer period (good for up to 2 years, depending on storage conditions).