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Evaluating Posttranscriptional Regulation of Cytokine Genes

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Abstract

A wide variety of cytokines are necessary for cell–cell communication in multicellular organisms, and cytokine dysregulation has detrimental effects, leading to disease states. Thus, it is a necessity that the expression of cytokines is tightly controlled. Regulation of cytokine gene expression takes place at different levels, including transcriptional and posttranscriptional levels. Ultimately, the steady-state levels of cytokine transcripts are determined by the equilibrium of transcription and degradation of this mRNA. Degradation rates of cytokine mRNAs can be measured in cells by blocking transcription with actinomycin D, harvesting RNA after different time points, and evaluating mRNA levels over time by northern blot. *Cis*-acting elements that mediate the rapid decay of numerous cytokine transcripts, including AU-rich elements (AREs), are found in the 3′ untranslated region (UTR) of these transcripts. Putative regulatory *cis*-elements can be cloned into the 3′ UTR of a reporter transcript in order to assess their function in regulating mRNA decay. *Cis*-elements, such as AREs, regulate cytokine mRNA decay by binding to *trans*-acting proteins, such as tristetraprolin or HuR. These RNA-binding proteins can be visualized using electromobility shift assays or UV crosslinking assays based on their binding to radioactively labeled RNA sequences. RNA-binding proteins that regulate cytokine mRNA decay can be purified using an RNA affinity method, using their target RNA sequence as the bait. In this chapter, we review the methods for measuring cytokine mRNA decay and methods for characterizing the *cis*-acting elements and *trans*-acting factors that regulate cytokine mRNA decay.

Keywords

mRNA decay; Actinomycin D chase; Northern blot; RNA–protein interaction; EMSA; UV crosslinking; One-step affinity purification; AU-rich element; Tristetraprolin; HuR

1. Introduction

Cytokines regulate a variety of different events in the human body. They provide signals to cells, telling them when to divide, what proteins to produce, what other cytokines to secrete, and how they should differentiate. Because of the importance of cytokines in maintaining homeostasis and the dangers involved when cytokines become dysregulated, it is crucial that the expression of cytokines is tightly regulated at multiple levels, including transcriptional and posttranscriptional levels. Steady-state mRNA levels are determined by the balance between transcription and mRNA degradation. The biochemical mechanisms that regulate

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the degradation of cytokine transcripts is not well understood, although there is evidence that failure to degrade pro-inflammatory cytokine transcripts such as TNFα, INFγ, IL2, IL6, IL8, or IL10 transcripts leads to chronic inflammation (1–5). The degradation of cytokine transcripts is regulated through *cis*-elements in their 3′ untranslated regions (3′ UTRs) and *trans*-acting factors that bind to them. One important and well-known destabilizing element is the AU-rich element (ARE), which is found in the 3′ UTR of many unstable cytokine mRNAs (6–9). AREs function by binding to *trans*-acting proteins that regulate the stability of the transcript. Some ARE-binding proteins, such as tristetraprolin and butyrate response factor 1, are responsible for rapid degradation of ARE-containing transcripts (10–12), whereas other proteins, such as HuR, have the potential to stabilize the same message (9, 13–18). Whether an ARE-containing cytokine transcript undergoes degradation or stabilization is dependent upon the activation status of the cells. Activation of a cell, for example, will lead to an inactivation of TTP or BRF1 (3, 19) and the recruitment of HuR to the ARE, which will result in increased stability of the ARE-containing message (9, 13–18). In this way, a cell can respond rapidly to changes in the outside environment and produce the required cytokine.

This chapter introduces the techniques used to measure degradation of cytokine transcripts and to characterize the RNA-binding proteins involved in their regulation. We have used the techniques described below to characterize the role of mRNA decay in regulating the expression of cytokine genes and other early response genes after activation of primary human T cells (7, 8, 20, 21), and to identify and characterize the function of AREs and ARE-binding proteins in the regulation of cytokine mRNA decay (1, 10, 13, 14, 17). Although we have used the techniques to study posttranscriptional cytokine gene regulation in human T cells, the techniques can be broadly applied to a variety of cell types and experimental systems.

2. Materials

2.1. Measuring mRNA Decay Rates

2.1.1. Isolation of Primary Human T Cells—

1. 40 mL Buffy coat white blood cell packs of 10^9 cells or more (American Red Cross). Buffy coat cells must be used immediately.
2. 2 mL RosetteSep human T cell enrichment cocktail (StemCell Technologies, Inc.).
3. Dulbecco’s Phosphate-buffered saline (PBS, Gibco) supplemented with 2% (v/v) FBS. Store at 4°C.
4. Ficoll-Paque™ Plus (GE-Healthcare Amersham).
5. Whole blood erythrocyte lysing kit (R&D Systems).
6. RPMI medium 1640 (Gibco) supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin (Gibco), and 1% (v/v) L-glutamine (Gibco).

2.1.2. T Cell Stimulation and Measurement of mRNA Decay After Addition of Actinomycin D—

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1. Tissue culture dish with 20 mm grid 150 × 25 (Falcon) for T cell stimulation.
2. Anti-hCD3ε Antibody (R&D Systems) and anti-hCD28 Antibody (R&D Systems) resuspended in 500 μL sterile water. Aliquot 50 μL and freeze at −20°C. Make coating solution fresh and use immediately; add 5 μg anti-hCD3ε antibody and 5 μg anti-hCD28-antibody into 10 mL PBS per plate to be coated. Antibody solutions will be stable for several months at −20°C.
3. Actinomycin D-mannitol (Sigma) is resuspended in 1 mL sterile PBS. Actinomycin D should not be used after 1 month in solution. Store at −20°C.
4. 70% (v/v) Ethanol.
5. 2-Mercaptoethanol.
6. Qiashredder (Qiagen).
7. RNeasy mini kit (Qiagen).

2.1.3. β-Globin Reporter Based Assays to Measure mRNA Decay Half-Lives—

1. HeLa Tet-Off cells (Clontech).
2. OptiMEM (Gibco).
3. Lipofectamine 2000 Reagent (Invitrogen).
4. BBB-plasmid (9).
5. pTracer™-EF C vector (Invitrogen).
6. TrypLE™ Express (Gibco).
7. Minimum essential medium alpha (MEMα, Gibco) supplemented with 10% (v/v) Tet system approved FBS (Clontech), 1% (v/v) penicillin/streptomycin (Gibco), and 1% (v/v) L-glutamine (Gibco).
8. Dissolve doxycycline (Clontech) to a concentration of 300 μg/μL in DMSO. This will result in a 1,000× stock solution. Store at −20°C.
9. 2-Mercaptoethanol.
10. Qiashredder (Qiagen).
11. RNeasy mini kit (Qiagen).

2.1.4. Northern Blotting—

1. NorthernMax®-Gly Sample Loading Dye (Ambion).
2. NorthernMax®-Gly 10× Gel Prep/Running Buffer (Ambion).
3. BrightStar®-Plus Positively Charged Nylon Membrane (Ambion).
4. NorthernMax® One-Hour Transfer Buffer (Ambion).
5. ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion).

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6. NorthernMax® Low Stringency Wash Buffer (Ambion).
7. NorthernMax® High Stringency Wash Buffer (Ambion).
8. 3 mm Chr Chromatography paper (Whatman).

2.1.5. Probe Preparation for Northern Blotting—

1. Taq DNA-Polymerase set (Qiagen).
2. The low dATP dNTP Mix contains 10 mM dGTP, 10 mM dTTP, 10 mM dCTP, and 2 mM dATP final concentrations in RNase-free water (Roche).
3. Primers for β-globin probe: β-globin+ (5′-GTC TAC CCA TGG ACC CAG AGG-3′), β-globin– (5′-AGG ATC CAC GTG CAG C-3′).
4. Primers for GFP probe: GFP+ (5′-CCA TGG CTA GCA AAG GAG-3′), GFP– (5′-CCA TGT GTA ATC CCA GCA GCA G-3′).
5. α32P-dATP (MP Biochemicals).
6. Microspin™ G-25 Columns (GE-Healthcare).

2.2. Characterization of RNA–Protein Interactions

2.2.1. Preparation of Cytoplasmic Extracts—

1. NP40 Lysis buffer: 10 mM HEPES/KOH, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 5% (v/v) glycerol, 0.2% (v/v) NP40. NP40 Lysis buffer can be stored at 4°C without DTT and inhibitors. Before cell lysis add 1 mM DTT, 2 ng/mL leupeptin, 2 ng/mL pepstatin, 8 ng/mL aprotinin, 0.1 mg/mL phenylmethylsulfonyl fluoride (PMSF).
2. Biorad protein assay reagent (Biorad).

2.2.2. Electrophoretic Mobility Shift Assay—

1. RBB buffer: 25 mM HEPES/KOH, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM DTT, can be stored at 4°C or aliquoted and frozen at −20°C.
2. Heparan sulfate (Sigma) is reconstituted in 100 μL RNase-free water to obtain a 50-mg/mL solution and should be aliquoted and stored at −20°C.
3. 5× TB-buffer: 54 g Tris–base and 27.5 g boric acid in 1 L of water. 1× TB-buffer is used for gel preparation and running buffer. Store at room temperature.
4. 30% (w/v) acrylamide/bis solution, 19:1 (Bio-Rad).
5. 6× DNA loading dye: 30% (v/v) glycerol and 0.25% (*w/v*) bromophenol blue in RNase-free water. Store at room temperature.

2.2.3. End-Labeling of Synthetic RNA Oligomer Probes—

1. Custom-synthesized RNA (Dharmacon) is dissolved as a 100 μM solution and is stored at −20°C.
2. T4 Polynucleotide Kinase (Invitrogen) which includes 5× forward reaction buffer.

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3. γ^{32} P-ATP (MP Biochemicals).
4. Phenol/chloroform/isoamyl alcohol (Invitrogen).
5. Prepare a 3-M sodium acetate solution in RNase-free water. Store at room temperature.
6. Microspin™ G-25 Columns (GE-Healthcare).

2.2.4. UV Crosslinking Assays—

1. Heparan sulfate (Sigma) is reconstituted in 100 μL RNase-free water to obtain a 50-mg/mL solution and should be aliquoted and stored at −20°C.
2. RNase T1 (Ambion).
3. RBB buffer: 25 mM HEPES/KOH pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM DTT, can be stored at 4°C or aliquoted and frozen at −20°C.
4. 5× Sample buffer: 25 mM Tris–HCl pH 6.8, 500 mM dithiothreitol (DTT), 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol. Aliquot and store at −20°C.

2.2.5. Probes for UV Crosslinking Assays—

1. Promega kit (Promega).
2. rNTP-mix: combine 10 mM rATP, 10 mM rCTP, and 10 mM rGTP final concentration in RNase-free water. Store at −20°C.
3. α^{32}P-UTP (MP Biochemicals).
4. RNA stop solution mix: 10 mL Tris/EDTA (10 mM/1 mM), pH 8.0 with 10 μL 0.5 M EDTA, pH 8.0, and 100 μL 10% (w/v) SDS. Store at room temperature.
5. Phenol/chloroform/isoamyl alcohol (Invitrogen).
6. Prepare a 3-M sodium acetate solution in RNase-free water. Store at room temperature.

2.2.6. Isolation of RNA-Binding Proteins by One-Step Affinity Chromatography —

1. Custom 5′ biotinylated RNA and unbiotinylated competitor RNA (synthesized by Dharmacon).
2. Dissolve heparan (Sigma) as 100 mg/mL solution in RNase-free water. Aliquot and store at −20°C.
3. M-280 Streptavidin beads (Dynal Biotech).
4. RBB buffer: 25 mM HEPES/KOH, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 5% (v/v) glycerol, 1 mM DTT, can be stored at 4°C or aliquoted and frozen at −20°C.

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3. Methods

This methods section is divided into two parts. The first part describes methods to measure decay rates of cytokine mRNAs, and the second part details methods to characterize RNA-binding proteins that bind to specific decay elements, such as the ARE.

The methods described below all involve RNA in one way or another. Since RNA is prone to degradation by RNases, which can be found in dust and sweat, it is crucial that gloves are worn at all times and that the workspace is clean. To clean the workbench, we use RNA-ZAP (Ambion). Another source for RNase contamination is water used to make solutions and buffers; therefore, RNase-free water should be used for preparing every buffer and reaction. RNase-free water can be prepared relatively inexpensively using DEPC, which will destroy enzymes (reaction with histidines), and its preparation is described (see Note 1). DEPC needs to be inactivated by thoroughly autoclaving this solution. Failure to inactivate DEPC will result in nonreproducible results.

3.1. Measuring mRNA Decay Rates

The half-lives of different transcripts expressed in T cells vary greatly (8). Also, the activation status of T cells can greatly influence the rate of mRNA decay (8). This section focuses on the isolation of T cells, on how to stimulate them and how to prepare total RNA in order to measure transcript half-lives of the endogenous cytokine mRNAs. We describe methods for measuring cytokine mRNA decay rates by northern blot following transcriptional arrest with the RNA polymerase II inhibitor, actinomycin D.

After it is ascertained that a certain transcript is regulated at the level of mRNA decay, the *cis*-elements that are responsible for this regulation can be identified using a reporter based assay. The most widely used reporter in the field of mRNA degradation is the β-globin based reporter plasmid, BBB, which produces the β-globin transcript under control of a tetracycline-regulated promoter (9). The putative degradation sequence can be cloned into the BBB plasmid via a unique *Bgl*II site in the 3′ UTR. This plasmid can then be transfected into HeLa cells expressing a tet-repressor, and β-globin mRNA decay can be measured by northern blot after turning off transcription by the addition of doxycycline. The advantage of measuring mRNA decay in this system compared to the actinomycin D system is that doxycycline has minimal effects on the cells, whereas actinomycin D is extremely toxic. Another advantage is, that the function of each putative decay element can be singled out and studied individually. The disadvantages of this system are that reporter transcripts are studied, rather than the endogenous native transcripts, and the experiments cannot be performed in primary cells.

3.1.1. Isolation of Primary Human T Cells—

1. Separate 40 mL of buffy coat into two 50 mL tubes equally (see Note 2).

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[1] In all experiments RNase-free water is required. To prepare RNase-free water add 0.9 mL of diethylpyrocarbonate (DEPC) to 1 L of water. Stirr overnight and autoclave for 1 h to inactivate the DEPC.

[2] When using more than one buffy coat for an experiment be sure to never mix T cells from different donors. This will lead to activation of these cells and will compromise the experiment.

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2. Add 1 mL of RosetteSep Human T Cell Enrichment Cocktail (StemCell Technologies Inc) to each tube.
3. Mix by gently shaking the tubes.
4. Incubate the reaction for 20 min at room temperature to allow the antibodies to agglutinate everything except CD4-positive cells.
5. Bring each sample up to 35 mL with PBS supplemented with 2% (v/v) FBS (PBS/FBS) after the incubation is finished.
6. Prepare two new tubes containing 15 mL Ficoll-Paque™ Plus.
7. Layer the blood carefully over the Ficoll. Avoid mixing of the layers at all times (see Note 3).
8. Spin the samples at 1,700 × *g* for 20 min with the brake turned off.
9. Remove the top layer (serum) carefully to avoid disturbing the white, cloudy interphase which contains CD4 positive T cells.
10. Transfer the cells in the interphase to two new 50 mL tubes.
11. Add PBS/FBS solution up to 50 mL and centrifuge at 230 × *g* for 8 min.
12. Remove the supernatant carefully and as completely as possible without disturbing the cell pellet.
13. Remaining red blood cells are removed with the whole blood erythrocyte lysing kit (R&D Systems). Resuspend both pellets in 5 mL 1× lysis buffer and pool them in a 50 mL tube and incubate for 10 min at room temperature to allow erythrocyte lysis.
14. Add 40 mL of 1× wash buffer and spin tube at 230 × *g* for 8 min.
15. Remove the supernatant; the pellet should appear white now.
16. Resuspend cells in 50 mL RPMI medium 1640 supplemented (Gibco, 10% FBS, 1% penicillin/streptomycin (Gibco), 1% L-glutamine (Gibco)).
17. Count cells using a hemocytometer (use a dilution of 1:100 cell suspension to PBS).
18. Cells are incubated overnight in an incubator at 37°C, 5% (v/v) CO_2.

3.1.2. T Cell Stimulation and Measurement of mRNA Decay after Addition of Actinomycin D—

1. Coat four 15 cm petri dishes overnight at 4°C with coating solution (10 mL PBS containing 5 μg anti-hCD3ε antibody and 5 μg anti-hCD28 antibody) and four 15 cm dishes with PBS only. Note: Use one plate for each time point of the actinomycin D chase experiment (see Note 4).

[3] Be very careful when overlaying the Ficoll-paque with blood. Mixing of the phases results in dramatically reduced T cell yields. Also, keep the brake of the centrifuge of. Reducing the speed of the rotor too fast will result in mixing the phases and an increased loss of T cells.

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2. Remove coating solution or PBS.
3. Wash plates with 10 mL PBS. Remove PBS.
4. Equilibrate plates with 5 mL RPMI 1640 medium with supplements.
5. Remove medium.
6. Add 3×10^7 cells in 15 mL RPMI-medium with supplements to each plate.
7. Incubate cells for 3 h at 37°C and 5% (v/v) CO_2.
8. Add actinomycin D at a final concentration of 5 μg/mL to each plate (see Note 5).
9. Incubate the plates at 37°C and 5% (v/v) CO_2 for 0, 1, 2, or 3 h.
10. Remove cells by scraping. Note: Do not remove medium before scraping because the medium may contain floating cells.
11. Transfer cells and medium to a 50 mL tube.
12. Collect cells by spinning at $340 \times g$ for 5 min.
13. Remove medium as completely as possible without disturbing the pellet.
14. Extract total RNA with the RNeasy Mini kit following manufacturer's recommendations (see Note 6).
15. Elute total RNA in 50 μL RNase-free water.
16. Estimate RNA concentration using a spectrophotometer. The RNA concentration should be between 0.5 and 3 μg/μL (see Note 7).
17. Analyze the stability of the cytokine mRNA by northern blot; probes for endogenous RNA can be prepared (see Note 8).

3.1.3. β-Globin Reporter Based Assays to Measure mRNA Decay Half-Lives—

1. HeLa Tet-Off cells are propagated in HeLa Tet-Off medium (see Note 9).
2. Seed HeLa Tet-Off cells into 15 cm dishes with HeLa Tet-Off medium; the cells should be about 90% confluent the next day.

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[4]To stimulate T cells with anti-hCD3ε antibody and anti-hCD28 antibody use no other plates than the ones indicated in Subheading 2.1.1, item 2. We tested different plates and these were the best in immobilizing the antibodies on their surface.

[5]Swirl the plates well to equally distribute the Actinomycin D. Do not add Actinomycin D to the 0 h timepoint.

[6]Be sure to dry the membranes of the RNeasy column extensively. Contamination of ethanol in the RNA samples will make the sample leak out of the pockets when northern blot is performed. Residual ethanol also has negative effect on reverse transcription reactions.

[7]T cells do not have much RNA, do not expect high yields.

[8]To test the stability of specific cytokine mRNAs, specific probes have to be generated for each transcript. For this reverse transcribe mRNA into cDNA following the instructions of the Superscript II reverse transcriptase protocol (Invitrogen). Generate specific primers for a 300–500 nucleotide long portion of the desired cytokine. Run a standard PCR to amplify that fragment, which needs to be gel purified subsequently. The purified fragment can now be used to generate a radiolabeled probe as indicated in Subheading 3.1.4.

[9]When using the β-globin reporter based decay assay in HeLa Tet-Off cells be sure to use Tet system approved FBS at all times. Other types of FBS may contain tetracycline as a contaminant which will lead to irreproducible results. If the cells are cultured with nonapproved FBS you can recover them by growing them on a plate for a week with frequent changes of Tet system approved FBS.

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3. Prepare a Lipofectamine master mix by adding 4 mL of OptiMEM and 100 μL of Lipofectamine 2000 per plate to be transfected. Use 5% more of each reagent to allow for pipetting error. Incubate the Lipofectamine master mix for 5 min at room temperature.
4. Meanwhile, prepare the DNA master mixes: mix 15 μg of BBB decay reporter plasmid and 8 μg pTracer plasmid in 4 mL OptiMEM. Vortex.
5. Mix 4 mL of the Lipofectamine master mix slowly using stirring motions into 4 mL of the DNA master mix and incubate at room temperature for 20 min.
6. Remove the medium from the HeLa Tet-Off cells and replace with 8 mL of OptiMEM.
7. Add the transfection mixes from step 4 to each plate.
8. Incubate the plates with the transfection mixes for 4–5 h at 37°C, 5% (v/v) CO_2 (see Note 10).
9. Remove the transfection mix from the cells and add 18 mL of HeLa Tet-Off medium to each plate. Let the cells recover overnight.
10. The next day, remove the medium completely, wash cells with PBS and remove cells from the plate with 2 mL TrypLE express.
11. Split the cells 1:4 on p10 plates in a total of 8 mL medium the next day. And incubate again over night at 37°C, 5% (v/v) CO_2.
12. The next day, add doxycycline to a final concentration of 300 ng/mL (8 μL) to each plate.
13. Incubate the plates at 37°C and 5% (v/v) CO_2 for 0, 1, 2, or 3 h.
14. Remove medium as completely as possible.
15. Extract total RNA with the RNeasy Mini kit following manufacturer's recommendations.
16. Elute total RNA in 50 μL RNase-free water.
17. Estimate RNA concentration using a spectrophotometer. The RNA concentration should be between 1 and 3 μg/μL.

3.1.4. Northern Blotting—

1. Dilute 10 μg of total RNA into 10 μL of RNase-free water.
2. Mix 10 μL of NorthernMax® Gly Sample Loading Dye with the sample.
3. Spin sample for 30 s to collect all liquid at the bottom.
4. Denature the samples at 50°C for 30–60 min in a heat block.

[10]Do not exceed 5 h of transfection due to cytotoxicity of the Lipofectamine 2000 reagent.

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5. Meanwhile, melt 1 g of agarose in 90 mL of RNase-free water; be sure that the agarose melted completely.
6. After the agarose has cooled down to about 60°C add 10 mL of 10× NorthernMax® Gly 10× Gel Prep/Running Buffer and swirl.
7. Pour the gel solution into a level tray to a thickness of about 6 mm and let the gel solidify for about 30 min; the comb should be about 1 cm from the end.
8. Prerun the gel at 85 V for about 1 min in 1× NorthernMax®Gly Gel Prep/Running Buffer prior to loading the samples.
9. Spin samples briefly to collect all liquid at the bottom prior to loading.
10. Run the gel at 5 V/cm of distance between anode and cathode until the blue dye front reaches the bottom of the gel.
11. Take a picture of the gel under UV-light (see Note 11).
12. Remove excessive gel by cutting the sides and top below the slots.
13. Assemble blotting apparatus: place the gel upside down on a mirror.
14. Prewet the membrane in NorthernMax® One-Hour Transfer Buffer and place on top of the gel and roll out trapped air bubbles.
15. Construct a filter paper stack and revert the gel (membrane down) onto the stack.
16. Prewet a long filter paper and put it one end on top of the gel and the other end into a chamber containing NorthernMax® One-Hour Transfer Buffer; remove air-bubbles.
17. Blot gel for 15–20 min per mm gel thickness.
18. Remove the membrane from the blotting apparatus and rinse with 1× NorthernMax®Gly Gel Prep/Running Buffer to remove salt.
19. UV crosslink RNA to the membrane at 500 mJ/cm^2 in a UV stratalinker.
20. Wrap the membrane in plastic foil.
21. View the membrane under UV light and mark the prominent 18S and 28S rRNA bands.
22. The membrane can be frozen at this point at −20°C.
23. Preheat the ULTRAhyb® Ultrasensitive Hybridization Buffer at 42°C.
24. Place blot without the plastic wrap facing inward into a glass hybridization tube and add 10 mL of preheated ULTRAhyb buffer.
25. Prehybridize blot for at least 30 min prior to adding the probe.

[11]When performing northern blot, the gel as well as the membrane should show the two rRNA bands. If these bands are absent it hints either at the loss of a sample (probably due to ethanol contamination), or the degradation of the RNA sample.

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26. Add the radioactive probe (preparation see Subheading 3.1.4) to the blot and hybridize the membrane at 42°C for 3–4 h.
27. Discard the radioactive hybridization solution into the liquid radioactive waste.
28. Wash blot twice with 15 mL NorthernMax® Low Stringency Wash Buffer for 10 min at 42°C. (If precipitate has formed in the buffer prewarm at 37°C). Discard all washes into radioactive waste container.
29. Perform a high stringency wash with 15 mL NorthernMax® High Stringency Wash Buffer at 50°C for 15 min. Discard wash into radioactive waste container.
30. Wrap blot into clear plastic wrap and expose to a phosphorimager plate for at least 4 h.
31. Visualize Northern blot by scanning the plate in a phosphorimager.

3.1.5. Probe Preparation for Northern Blotting—Here, we describe the preparation of the GFP and β-globin probes. Other probes for detection of endogenous mRNAs can be prepared (see Note 8).

1. Mix 20–40 ng of β-globin or GFP PCR template with 5 μL 10× PCR-buffer, 1 μL 10 mM low dATP dNTP-mix, 25 pmol of primer β-globin+ or GFP+, 25 pmol of primer β-globin− or GFP−, and 5 μL α^{32}P-dATP in a final volume of 50 μL water in a PCR-tube.
2. Add 0.3 μL of Taq-Polymerase and transfer the tubes into a PCR machine.
3. Amplify the probe with the following cycles: 94°C 5 min, (94°C 30 s, 48°C 30 s, 72°C 1 min) for 37 cycles, 72°C 10 min, 4°C indefinite (see Note 12).
4. The probe is purified afterward over a Microspin™ G-25 column following the manufacturer's instruction.
5. Measure specific activity of the probe in scintillation counter. (Add 1 μL of probe to 5 mL of scintillation fluid, mix and count).
6. Denature probe at 95°C for 5 min and immediately place on ice.
7. Add 1×10^6 cpm per mL hybridization buffer per probe into the hybridization solution of the northern blot.

3.2. Characterization of RNA–Protein Interactions

As mRNA half-lives of cytokine transcripts vary with the activation status of a T cell, so does the composition of the proteins bound to mRNA decay elements. After stimulation of primary human T cells, the cells can be lysed to generated cytoplasmic extracts. We perform gentle lysis in a NP40-buffer which is compatible with electrophoretic mobility shift assay (EMSA), UV crosslinking assays, and the one-step affinity purification described below.

[12]The cycling of different PCR machines will be different which may result in a poor radiolabeled probe. Adjust the annealing temperatures in a nonradioactive PCR to solve that problem.

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EMSA is an easy way to see protein–RNA interactions in cytoplasmic lysates and is a good tool to identify a RNA-binding protein associated with a *cis*-element of interest. If candidate RNA-binding proteins are known, this assay can be used to confirm binding by simply adding a specific antibody against the protein of interest to the reaction to determine if the antibody supershifts the RNA-binding complex. If the proteins that associate with the cytokine mRNA are not yet known, UV-crosslinking experiments are able to give some information about the sizes of bound proteins and thus might lead to the identification by comparing to the sizes of known candidate RNA-binding proteins. Another method to identify proteins bound to the RNA decay elements is RNA affinity purification followed by mass spectrometry. We describe a one-step affinity purification in which biotinylated bait RNA is incubated with the T cell lysate to allow association of the RNA-binding protein to the RNA. The protein–RNA complex is captured by streptavidin beads, washed several times and eluted. The proteins in this complex can be analyzed by mass spectrometry.

3.2.1. Preparation of Cytoplasmic Extracts—

1. T cells are purified and stimulated as described in Subheading 3.1.1.
2. Transfer the cells into 50 mL conical tubes and collect by centrifugation at 230 × *g* for 8 min.
3. Pool cells of unstimulated or stimulated cells into one 50 mL tube each (Never mix T cells from different buffy coats).
4. Bring the volume in each tube up to 50 mL with PBS.
5. Spin cells at 230 × *g* for 8 min and remove the supernatant.
6. Repeat steps 6 and 7 once more. Remove the supernatant as completely as possible without disturbing the pellet.
7. Estimate the pellet volume.
8. Resuspend cell pellets in 3 volumes of NP40 lysis buffer by pipetting up and down a few times and transfer suspension to a 1.5 mL precooled centrifugation tube.
9. Incubate on ice for 10 min.
10. Homogenize lysates with 50 strokes in a precooled dounce homogenizer.
11. Remove nuclei and unbroken cells by centrifugation at 850 × *g* for 5 min at 4°C.
12. Transfer supernatant to a new precooled 1.5 mL centrifugation tube. The lysates of the different buffy coats can now be pooled.
13. Measure protein concentration at OD_{595} using the Biorad protein assay, following manufacturer's recommendations.
14. Snap freeze the protein samples in liquid nitrogen and store at –80°C.

3.2.2. Electrophoretic Mobility Shift Assay—

1. To prepare cytoplasmic extract from stimulated and unstimulated T cells follow the instructions of Subheading 3.2.1.

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2. Prepare probe following the instructions of Subheading 3.2.3.
3. Prepare a native 5% polyacrylamide gel in 1× TB buffer.
4. Mix on ice in a test tube 10 μg of cytoplasmic extract, 100 μg heparan sulfate, cold competitor probe at a final concentration of 10–100 molar excess over probe, and 20,000–100,000 cpm of specific probe. For supershift assays add 2 μL of specific or control antibody. Bring reaction to a total of 20 μL with buffer RBB. Probes and competitor RNA can be diluted in RBB buffer.
5. Incubate the reaction on ice for 30 min.
6. Add DNA loading dye and immediately load the entire sample on a 5% native polyacrylamide gel (see Note 13).
7. Run the gel in 1× TB buffer at 100 V until the blue dye is 1 cm from the end of the gel. Do not let the dye run out because the probe is running in the front.
8. After the gel is finished remove one of the glass plates and cover the gel with a Whatman filter paper. The gel will stick to it and can be easily removed from the glass plate. Cover the gel with plastic wrap. Do not wrap the plastic around the gel, otherwise it will not dry.
9. Dry the gel in the gel dryer at 80°C for 2 h.
10. Expose the dried gel to film for approximately 2–48 h depending on signal strength.

3.2.3. End-Labeling of Synthetic RNA Oligomer Probes—

1. Resuspend deprotected RNA Oligos in RNase-free water at a concentration of 100 μM. Dilute RNA oligos to a working concentration of 1 μM with RNase-free water.
2. Heat oligos to 95°C for 3 min and put immediately on ice to linearize the oligos. Linearized oligos can be stored at −20°C.
3. Mix 23 μL RNase-free water, 8 μL 5× forward reaction buffer, 4 μL 1 μM linearized RNA-oligomer, 4 μL γ^{32}P-ATP (4,500 Ci/mmol), and 1 μL of T4 PNK on ice.
4. Incubate the mixture at 37°C for 30 min.
5. Purify sample over a Microspin™ G-25 column into a 1.5 mL screw cap tube.
6. Add RNase-free water to a final volume of 200 μL.
7. Extract protein and free nucleotides by adding 200 μL of phenol/chloroform/ isoamyl alcohol (24:25:1, v/v/v) (see Note 14).
8. Vortex for 30 s.

[13]The DNA loading dye may interfere with protein–RNA interaction. Alternatively, the dye can be loaded into a free well or mixed with the probe-only control.
[14]When phenol extracting the probe radioactive aerosols may develop. Always perform phenol extractions in a chemical fume hood.

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9. Spin sample at maximum speed for 5 min in a microfuge.
10. Transfer aqueous phase to a new tube.
11. Precipitate RNA by adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol followed by an incubation of 2 h at −20°C.
12. Pellet the radiolabeled RNA by centrifugation at 14,000 × *g* for 20 min.
13. Remove the supernatant and dry the pellet in a vacufuge for 3 min.
14. Resuspend the probe in 20 μL of RNase-free water.
15. Measure specific activity of the probe in scintillation counter. (Add 1 μL of probe to 5 mL of scintillation fluid, mix and count).
16. Calculate the specific activity of the probe (see Note 15).

3.2.4. UV Crosslinking Assays—

1. To prepare cytoplasmic extract from stimulated and unstimulated T cells follow the instructions of Subheading 3.2.1.
2. Prepare probe following the instruction of Subheading 3.2.5. Dilute the probe to 50,000 cpm/μL with buffer RBB before adding to reaction.
3. Mix 100 μg heparan sulfate, 2 μL RNase T1, and 50,000 cpm of radiolabeled probe. Add RBB buffer to a final volume of exactly 24 μL including the amount of cytoplasmic extract.
4. Add 8–10 μL of cytoplasmic protein to each tube, mix the tubes, and then quick spin in a shielded microcentrifuge.
5. Incubate tubes at room temperature for 30 min behind a shield.
6. Place the tubes on ice with the caps open and make sure that water or ice does not get inside the tubes. Crosslink the protein to the RNA at 250 mJ/cm^2 in a UV-Stratalinker.
7. Remove the samples from the stratalinker and add 24 μL of 2× protein loading buffer.
8. Boil the samples for 5 min at 95°C on a heat block.
9. Spin down the contents of the tube in a microfuge.
10. Subject samples to PAGE on a 12% SDS-gel; do not forget to load a protein size marker. Do not let the dye run out of the gel.

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[15]Calculations of the specific activity of the radiolabeled probes for EMSA and UV-crosslinking: 4 pmol of synthetic RNA were added for radiolabeling and it is anticipated that 75% of RNA remains after purification and reconstitution. This results in a total of (4 pmol RNA/20 μL reconstitution volume) × 0.75 = 0.15 pmol/μL of purified RNA probe. After counting the probe the amount of radiolabeled per picomol of RNA can be determined. Count (cpm/μL)/0.15 pmol/μL RNA = cpm/pmol. For example, the specific activity of the probe is 200,000 cpm/pmol as measured in the scintillation counter and 50,000 cpm will be used in the experiment: 50,000 × 0.15 pmol/μL/200,000 cpm/μL = 0.0375 pmol/reaction.

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11. After the gel is finished open the glass plates and carefully cut of the lower part containing the blue dye.
12. Cover the gel with a Whatman filter paper. The gel will stick to it and can be easily removed from the glass plate. Cover the gel with plastic wrap. Do not wrap the plastic around the gel, otherwise it will not dry.
13. Dry the gel in the gel dryer at 80°C for 2 h.
14. Expose the dried gel to film for approximately 2–48 h depending on signal strength.

3.2.5. Probes for UV Crosslinking Assays—

1. Mix 6 μL 5× buffer, 3 μL 100 mM DTT, 6 μL rNTP-mix, 1.2 μL rUTP (100 mM), 2 pg template, 1 μL RNAsin, 10 μL α^{32}P-UTP, 1 μL T7-polymerase in a total of 30 μL RNase-free water in a PCR-tube.
2. Incubate reaction in a PCR machine for 1 h at 37°C.
3. Add 1 μL of DNase RQ1 to each sample and incubate for another 15 min at 37°C in a PCR machine.
4. Stop the reaction by adding 20 μL RNA stop solution.
5. Purify samples over a Microspin™ G-25 column following manufacturers' instructions.
6. Bring volume up to 200 μL with RNase-free water and transfer to a screw cap tube.
7. Extract samples with 200 μL of phenol/chloroform/isoamyl alcohol (see Note 14).
8. Vortex sample for 1 min.
9. Spin sample at max speed for 10 min in a microfuge.
10. Transfer the aqueous phase to a new screw cap tube.
11. Add 200 μL chloroform and repeat steps 8–10.
12. Add 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol.
13. Precipitate probes for at least 2 h at −20°C.
14. Spin samples at max speed for 20 min in a microfuge.
15. Remove the supernatant and discard in radioactive waste.
16. Dry the pellet in a speedvac for 5 min.
17. Resuspend the RNA-probe in 20 μL of RNase-free water.
18. Measure specific activity of the probe in scintillation counter. (Add 1 μL of probe to 5 mL of scintillation fluid, mix, and count).

3.2.6. Isolation of RNA-Binding Proteins by One-Step Affinity Chromatography—Note: All steps are performed at 4°C or on ice.

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1. Add 300 pmol of biotinylated RNA (bait), 750 pmol competitor RNA, and 1.5 mg heparan to 10 mg of T cell lysate (total volume about 1 mL) prepared as indicated under Subheading 3.2.1.
2. Incubate reaction at 4°C for 4 h tumbling on a wheel to assure protein–RNA interaction.
3. Meanwhile transfer 150 μL of M-280 streptavidin beads per pull down to a tube.
4. Wash the beads two times with 1 mL RNase-free water, once with 1 mL RBB buffer and finally add the 50 μL of RBB buffer per reaction to the beads.
5. Stir up beads by inverting the tube a couple of times and add 50 μL of M-280 streptavidin bead slurry to each tube.
6. Tumble reaction at 4°C for 1 h on a wheel to allow RNA-bead binding.
7. Collect beads on the side of the tube with a magnet.
8. Remove the supernatant.
9. Add 1 mL of cold RBB buffer to wash unbound protein off the beads.
10. Collect beads on the side of the tube with a magnet.
11. Remove the supernatant.
12. Repeat steps 7–9 twice more.
13. Resuspend the beads in 20 μL of RBB buffer supplemented with 0.05% (w/v) sodium dodecylsulfate (SDS).
14. Incubate sample at 65°C for 15 min to elute the protein from the beads.
15. Collect beads using a magnet.
16. Transfer eluate to a new tube.
17. Spin sample at 7,600 × *g* for 2 min to remove residual beads.
18. Transfer the supernatant to a new tube.
19. Freeze samples in liquid nitrogen.
20. Samples can be store at –80°C prior to mass spectrometric analysis.

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