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Investigating RNA editing factors from trypanosome mitochondria

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Abstract

Mitochondrial U-insertion/deletion mRNA editing is carried out by two principal multiprotein assemblies, enzymatic RNA editing core (RECC) and RNA editing substrate binding (RESC) complexes, and a plethora of auxiliary factors. An integral part of mitochondrial gene expression, editing receives inputs from primary mRNA and gRNA precursor processing pathways, and generates substrates for mRNA polyadenylation and translation. Although nearly all RECC-embedded enzymes have been implicated in specific editing reactions, the majority of proteins that populate the RESC are also essential for generating edited mRNAs. However, lack of recognizable motifs in RESC subunits limits the prowess of bioinformatics in guiding biochemical experiments and elucidating their specific biological functions. In this chapter, we describe a generic workflow for investigating mitochondrial mRNA editing in *Trypanosoma brucei* and focus on several methods that proved instrumental in assigning definitive functions to editing factors lacking known signature sequences.

Keywords

Trypanosoma; Mitochondria; Protein complexes; RNA editing; Guide RNA; TUTase; RNA ligase

1. Introduction

RNA editing alters DNA-encoded sequences and is distinct from splicing, 5' capping and 3' additions. Editing events are predominantly post-transcriptional and include nucleotide insertions and deletions, and base substitutions and modifications. The uridine insertion/deletion mRNA editing in mitochondria of kinetoplastid protists corrects frameshifts, introduces translation punctuation signals, and often adds and deletes hundreds of uridines to create the protein-coding sequence (reviewed in [1,2]). The sequential enzymatic reactions of mRNA cleavage, uridine addition to, or removal from, the cleaved mRNA 5' fragment,

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and re-ligation of two RNA pieces are carried out by well-defined catalytic subunits of the RNA editing core complex (RECC). This ~1.2 MDa (~20S) complex exists in three isoforms each of which contains 12 common and 2–3 distinct components [3]. Protein sequence analysis has been instrumental in identification of catalytic, zinc finger, OB-fold and others motifs thus steering biochemical characterization of enzymatic, structural and RNA binding RECC subunits [4,5]. Indeed, in many cases a knockdown of an individual enzyme inhibits a single reaction in the editing cascade thereby providing unambiguous functional assignment [6–9]. Discovery of the ~20-polypeptide RNA editing substrate binding complex (RESC), also referred to as the MRB1 complex [10–13], introduced a challenge of delineating the specific roles of multiple factors lacking known motifs or similarities to proteins outside of *Kinetoplastea*. The RESC complex is composed of at least three modules responsible for gRNA binding and stabilization (GRBC), interaction with RECC (RNA editing mediator complex, REMC) and interaction with the polyadenylation complex (polyadenylation mediator complex, PAMC) [12]. Ironically, only a single RESC subunit, an RNA binding proteins RGG2, contains a pronounced RRM RNA binding motif [14] while direct gRNA binding and stabilization have been attributed to GRBC1 and GRBC2, which lack discernible RNA binding motifs [10,12]. To that end, RNAi knockdowns of 15 RESC subunits inhibited production of edited mRNAs without affecting gRNA stability and, in most cases, structural integrity of the RESC complex (reviewed in [1]). It seems plausible that future studies will identify more RESC subunits or proteins otherwise essential for the editing process *in vivo*. The workflow outlined in Fig. 1 is designed for placing such candidate proteins into the context of known mRNA editing, polyadenylation and translation complexes, and understanding their potential biological functions. These methods rely on inducible protein and double-stranded RNA expression in transgenic Lister 427 procyclic (insect, PF) (29-13) and Lister 427 bloodstream (BF) “single marker” strains of *Trypanosoma brucei* that maintain nucleus-targeted *tet*-repressor and T7 RNA polymerase [15]. Hence, the main objectives include phenotypic analysis of genetic knockdowns and their impacts on specific RNA classes, and characterization of relevant protein complexes by mass spectrometry and biochemical fractionation. Available tools permitting, identification of *in vivo* RNA binding sites may provide some of the most important insights. The entire pipeline is typically completed within one year. Most of the equipment and reagents can be obtained from ThermoFisher Scientific unless otherwise stated.

2. Protein expression and purification from *T. brucei*

Inducible expression of C-terminally TAP-tagged proteins [16] under control of the ribosomal RNA promoter (pLEW100v5-TAP, developed by George Cross and modified in authors' laboratory) can be performed in PF and BF, or under PARP promoter (pLew79-MHTAP, [17]) in PF parasites. The former vector system is more universal, but provides lower expression level in PF; selecting a BF cell line with tightly-regulated expression typically requires screening of many clones. High expression levels in the latter system may lead to isolation of non-stoichiometric complexes, but the benefits of the overall efficiency and reliability often compensate the risks. Tagging an endogenous allele is also a viable option albeit constitutive expression levels can be low [18]. General protocols for parasite transfection, and selection and validation of stable inducible clonal cell lines are described in

[19]. The TAP tag is composed of Protein A and calmodulin binding peptide separated by the TEV protease cleavage site; it is compatible with protocols for isolating stable mitochondrial complexes and detecting transient co-complex interactions and bound RNAs. To that end, RNA-mediated interactions often withstand tandem affinity purification; therefore, isolation of stable complexes is preferentially conducted from RNase- and mock-treated mitochondrial lysates.

2.1. Purification of stable protein complexes

Enrichment of mitochondrial fraction by hypotonic lysis and centrifugation is recommended prior to tandem affinity purification. All procedures are done at 4 °C without interruptions unless specified. Collect aliquots at all steps to calculate purification efficiency by quantitative immunoblotting with anti-CBP antibody (GenScript). All incubations with beads are performed on Nutator platform.

1. Mitochondrial fraction enrichment. Inoculate 800 ml of SDM-79 media with 10% FBS and required antibiotics with stable parasite culture at final concentration of 10^6 cells/ml and grow at 27 °C in a roller bottle at 8 RPM to $10\text{--}15 \times 10^6$ cells/ml (50–60 h).
2. Collect cells by centrifugation at 3000g for 10 min.
3. Resuspend cell pellet in 50 ml of phosphate buffered saline (PBS) plus 6 mM sucrose, repeat centrifugation for 10 min and ensure complete removal of PBS buffer.
4. Calculate the required volume of DTE buffer (5 mM Tris-HCl, 1 mM EDTA, pH 8) to achieve final concentration of 1.2×10^9 cells/ml. Use half of the calculated DTE volume to initially resuspend cells, and then gradually add the rest with constant gentle pipetting until homogeneity is achieved. Proceed to Step 6 immediately.
5. During centrifugation at Step 3, prepare 50 ml conical tube (rated at g-force of 15,000 or higher) with pre-calculated volumes of 60% sucrose (12 ml of sucrose per 100 ml of DTE volume used for cell lysis), 150 µl of 1 M MgCl_2 , and 0.2 ml of DNase I solution (5000 U/mL, Sigma, D5025).
6. Transfer the lysate from Step 4 into 10 ml syringe fitted with 26-gauge needle and push intensely into the sucrose cushion prepared at Step 5. Mix gently, bring the volume to 50 ml with STE buffer (20 mM Tris-HCl, pH 7.6, 250 mM sucrose, 1 mM EDTA) and incubate on ice for 15 min.
7. Collect the pellet by centrifugation at 15,000g for 15 min. Resuspend in 50 ml of STE, repeat centrifugation; expect to recover ~1 g (wet weight) of mitochondrial fraction. Flash-freeze in liquid N₂, or proceed with affinity purification (preferred).
8. Extract preparation. Resuspend the pellet from Step 7 in 3 ml of Lysis Buffer (LB, 50 mM Tris-HCl, pH 7.6, 120 mM KCl, 1% NP40, 5 mM

- MgCl₂, 5% glycerol), add 1/5 of Complete protease inhibitor tablet (Roche), transfer to 15 ml tube and incubate on ice for 15 min.
9. Add extraction buffer without detergent to bring the extract volume to 11 ml, sonicate 3 × 10 s with microtip at 9 W.
 10. Centrifuge at 200,000*g* for 15 min in SW41 rotor (Beckman). Immediately transfer supernatant into 10 ml syringe fitted with low-protein binding 0.45 μ filter and push out 5 ml into 15 ml conical tube with 0.1 mg of RNase A and 2000 U of RNase T1, and 5 ml into control tube. Incubate on ice for 10 min.
 11. First chromatographic step. During Step 10, transfer 0.3 ml of IgG Sepharose slurry (GE Life Sciences) into 15 ml conical tube and wash 2× with 10 ml of IgG Binding Buffer (IgG-BB, 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 5% glycerol, 0.1% NP40) using centrifugation at 500*g* for 30 s.
 12. Transfer the extract into tube with IgG resin and incubate for 30 min.
 13. Transfer the suspension into 2-ml disposable column with bottom filter and re-load twice from the incubation tube to collect the entire resin in the column.
 14. Wash with 5 full column volumes (CV) of IgG-BB and allow some overflow to rinse the rim of the column.
 15. Wash with 2 CV of IgG-BB plus 1 mM DTT, close the outlet. Add 150 U of AcTEV protease and 1/200 of Complete inhibitor tablet in 1.5 ml of IgG-BB plus 1 mM DTT to the drained resin, close upper end of the column with Parafilm, and incubate for 16 h at 4 °C.
 16. Second chromatographic step. Pre-treat 2 ml disposable columns and 15 ml conical tubes with 2% solution of Tween 20, wash extensively in water. Use low protein binding 1.5 ml Eppendorf tubes to collect fractions.
 17. Transfer 0.4 ml of calmodulin resin (Agilent) into 15 ml plastic tube and wash 2× with 15 ml of calmodulin binding buffer (CBB, 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 mM CaCl₂, 1 mM MgAc, 0.1% NP40, 10 mM β-mercaptoethanol, 1 mM imidazole, 5% glycerol).
 18. Drain the IgG column into 15 ml plastic tube with pre-washed calmodulin resin.
 19. Rinse the IgG column with 4 × 1 ml of CBB, collect rinses into the tube with calmodulin resin, push out the leftover with a thumb. Target volume is ~6 ml.
 20. Add 6 μl of 1 M CaCl₂ and incubate for 1 h on Nutator.
 21. Transfer suspension into 2-ml disposable column and re-load 3 times to collect the resin.

22. Wash the column with 3 full column volumes of CBB.
23. Close the column with bottom plug, add 0.6 ml of Calmodulin Elution Buffer (CEB, 20 mM Tris-HCl, pH 7.6, 100 mM KCl, 2 mM EDTA, 3 mM EGTA, 0.1% NP-40, 10 mM β -mercaptoethanol, 1 mM imidazole, 5% glycerol) gently mix, and incubate for 5 min at room temperature. Collect the fraction and elute three additional 0.5 ml fractions likewise. Freeze fractions in liquid N₂ and store at -80°C . For enzymatic activity assays, add glycerol to final concentration of 50% and store at -20°C . Examples of RECC and RESC purifications via TAP-tagged RNA editing TUTase 2 (RET2) and gRNA binding complex subunit 1 (GRBC1), respectively are shown in Fig. 2A.

2.2. Purification of unstable ribonucleoprotein complexes

In contrast to the lengthy and stringent protocol described in Section 2.1., critical steps of the rapid affinity pulldown (RAP) are performed in less than 30 min. This method takes advantage of cryogenic cell disruption and fast binding kinetics of TAP-tagged proteins to nonporous IgG-coated magnetic beads. The technique is also beneficial for purification of large RNPs, e.g., the ribosome, which can be size-excluded from polysaccharide-based resins. Finally, RAP provides a viable alternative to immunoaffinity purification of RNA-protein UV-crosslinking adducts (CLIP protocol) whenever the antibody is not available (Section 4). Solutions used in purification are the same as in Section 2.1 unless specified; there should be no interruptions between steps. If necessary, purification can be performed in the presence of RNase cocktail.

2.2.1. Preparation of IgG-coated magnetic beads

1. Resuspend the entire bottle of Rabbit IgG (100 mg, Sigma I5006-100MG) in 7 ml of water and dialyze against 2 L of 1 $\times$ PBS overnight at 4°C . Recovered solution should contain ~ 14 mg/ml of IgG.
2. Transfer the entire contents of Dynabeads[®] M-270 Epoxy vial (300 mg) with 20 ml of 0.1 M sodium phosphate buffer (pH 7.4) into 50 ml conical tube. Use 6–7 ml of buffer at a time to rinse the vial. Vortex for 30 s. Incubate on Nutator for 10 min at room temperature. Split equally between four 15 ml conical tubes and collect the beads on a magnetic stand.
3. Centrifuge 3.5 ml of IgG solution at 21,000 g for 10 min, and prepare coupling mixture in the following order: add 9.85 ml of 0.1 M sodium phosphate buffer to the IgG solution and mix; add 6.65 ml of 3 M ammonium sulfate and mix. Let the suspension sit on the bench for 5 min. Filter through 0.22 μm low protein binding filter, expect to recover ~ 20 ml.
4. Add 5 ml of coupling mixture to beads in each 15 ml tube. Close and seal caps with Parafilm and place on Nutator for 20 h at 30°C . Expect to see cloudy solution at the end.

5. Collect the beads on magnetic stand and discard the supernatant. Rinse beads with 10 ml of PBS three times by brief vortexing, collecting beads on magnetic stand and decanting the supernatant.
6. Add 3 mL of 100 mM Glycine-HCl (pH 2.5), vortex, collect the beads and decant as soon as possible.
7. Resuspend beads in each tube in 3 ml of 20 mM Tris-HCl, pH 8.8 and collect in a single 15 ml conical tube. Rinse the remaining two tubes to collect all beads.
8. Wash beads three times with 5 ml of 100 mM triethylamine (make fresh, adjust to pH 8.0 with concentrated HCl) for 5 min. This and subsequent washes are done at room temperature.
9. Wash with 3 times with 10 ml of PBS for 5 min.
10. Wash once with PBS plus 0.5% Triton X-100 for 5 min, then 3 times with 10 ml of PBS for 5 min.
11. Resuspend all beads in 6 ml of 1× PBS plus 0.02% sodium azide (50 mg/ml). Store at 4 °C for up to three months.

2.2.2. Rapid affinity pulldown (RAP)

1. Collect and freeze cell pellet in liquid N₂. Break up the frozen pellet with a spatula into pieces smaller than 5 mm, and transfer into pre-cooled 25 ml Cryomill jar (Retsch) with 3 stainless steel 10-mm balls. Perform a single cycle with 3 min pre-cooling and 6 min grinding at maximum frequency.
2. Transfer powder with cold spatula into N₂-cold 15 ml tube, keep in N₂. Move to room temperature just prior to lysis.
3. Pre-warm 3 ml of Lysis Buffer plus 40 U of Turbo DNase and 1/5 of Complete protease inhibitor tablet to 30 °C and add to the cell powder with gentle mixing on ice; do not exceed 3–5 min at this step.
4. Add Lysis Buffer sans detergent to bring the final volume to ~11 ml and sonicate with microtip at 9 W for 10 s to break up clumps.
5. Centrifuge in SW41 at 200,000*g* for 10 min at 4 °C.
6. During step 5, pellet 20 mg of IgG-coated beads in 15 ml tube on magnet stand. Wash with IgG-BB three times for 1 min.
7. Filter the supernatant through 0.45 µ low-protein binding filter into the conical tube with beads and incubate on Nutator for 10 min at 4 °C.
8. Pellet beads on magnetic stand, decant supernatant and quickly rinse beads 2× with 10 ml IgG-BB with mild vortexing.
9. Wash beads 3× with 10 ml of IgG-BB for 5 min at 4 °C and transfer into 2 ml tube.

10. Wash beads 3× with 1 ml of IgG-BB for 5 min at 4 °C.
11. Elute bound proteins with 100 U of AcTEV protease in 0.3 ml of IgG-BB plus 1 mM DTT and equivalent of 1/500 of the Complete inhibitor tablet with gentle mixing at 4 °C for 12–16 h. Use of the Eppendorf ThermoMixer at 800 rpm is recommended.

2.3. Isolation and analysis of RNA from affinity purified complexes

While bound RNA is largely removed or degraded during tandem affinity purification, the rapid pulldown described in Section 2.2.2 typically yields RNA samples compatible with downstream analysis by Northern blotting, RNA-Seq and other analytical techniques [12].

1. Resuspend beads recovered at Step 10 in Section 2.2.2 in 0.25 ml of 0.5% SDS, 100 mM NaOAc, pH 5.5, 1 mM EDTA, 5 mM DTT, 25 µg/ml of glycogen and incubate at 65 °C for 5 min at 1000 RPM in Eppendorf ThermoMixer. Collect the beads, transfer the supernatant to a fresh tube. If comparing different complexes, save 5% aliquot for immunoblotting to normalize RNAs to the amount of bait protein.
2. Repeat bead extraction, combine both supernatants and extract with 0.5 ml of phenol/chloroform by shaking at 2000 rpm on Eppendorf ThermoMixer for 5 min at room temperature.
3. Extract with 0.5 of chloroform and concentrate ~3-fold with anhydrous *sec*-butanol. Precipitate RNA by adding 4 V of ethanol.

2.3.1. Guide RNA analysis—The presence of bound guide RNAs can be expeditiously assessed by radio labeling with vaccinia virus guanylyltransferase in the presence of [α -³²P]GTP. The majority of small mitochondrial RNAs, including guide RNAs, maintain 5' triphosphate characteristic of primary transcripts [12]. Specific sequences can be detected by Northern blotting while a more comprehensive analysis requires RNA-Seq. Commercial RNA-Seq library preparation kits can be applied directly after converting the 5' triphosphate into monophosphate by RNA 5' polyphosphatase (Epicentre) and gel purification.

1. GTP labeling. Re-suspended RNA from Section 2.3. in 10 µl of water, incubate at 85 °C for 2 min and place on ice for 2 min. Add 10 µL of 2× reaction buffer (120 mM Tris-HCl pH 8.0, 12 mM MgCl₂, 10 mM DTT, 2 units/µL RNaseOut, 20 µCi [α -³²P] GTP (3000 Ci/mmol, Perkin Elmer)) and 1 µg of guanylyltransferase. Incubate at 37 °C for 30 min. Stop the reaction with equal volume of 0.1% SDS, 10 mM EDTA, 0.6 M NaAc, pH 5.5. Extract reaction mixture with phenol/chloroform (pH 8.0) and precipitate with 4 volumes of ethanol. Dissolve reaction products in 60% formamide, 1 mM EDTA, 0.025% of Xylene Cyanol, and Bromophenol Blue. Analyze on high-resolution 12% polyacrylamide/8 M urea gel (40 cm × 20 cm × 0.45 mm). Run the gel at 40 W until Xylene Cyanol migrates 2/3 of the gel. Vaccinia virus guanylyltransferase can be purified in-house from *Escherichia coli* carrying pET-HisD1/D12 plasmid as

described [20]. Typical labeling patterns of total cellular RNA, and RNA extracted from purified mitochondrial fraction and from rapid affinity pulldown of GRBC1 are shown in Fig. 2B.

2. RNA isolation for RNA-Seq. Re-suspend RNA from Section 2.3 in 17 μ l of water and proceed with pyrophosphate removal as recommended by manufacturer using 20 units of the RNA 5' Polyphosphatase (Epicentre). Extract reaction mixture with phenol/chloroform (pH 8.0) and precipitate with 4 volumes of ethanol. Dissolve products in 60% formamide, 1 mM EDTA, 0.025% of Xylene Cyanol, and Bromophenol Blue. Separate RNA on 12% polyacrylamide/8 M urea gel (10 cm $\times$ 10 cm $\times$ 1.5 mm) along with Low Range ssRNA Ladder (NEB). Using the 50-nt band as marker, excise the gel area corresponding to 40–70 nt RNA fragments (~1 cm). Crash the gel into a fine powder and elute into five volumes of 0.1 M sodium acetate, 10% phenol, 1 mM EDTA, 0.1% SDS, pH 5.5 for 1 h at 20 °C in Thermomixer. Remove gel by centrifugation, add glycogen to 20 μ g/ml and extract RNA solution with phenol/chloroform. Concentrate aqueous phase ~3-fold with anhydrous *sec*-butanol and precipitate RNA with four volumes of ethanol. Re-purify RNA with RNA Cleanup & Concentrator-5 from Zymo Research.
3. Proceed with library preparation using TruSeq Small RNA Library Preparation Kit from Illumina. Extract final PCR reaction with phenol/chloroform and purify by PAGE. An example of RNA-Seq library separated on 7% polyacrylamide gel is shown in Fig. 2C. Stain the gel with SYBR Green I Nucleic Acid Gel Stain and excise under blue light (Safe Imager 2.0). Elute DNA into 5 volumes of 0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS overnight. Extract with phenol/chloroform, concentrate 3-fold with *sec*-butanol, precipitate with ethanol and re-purify with DNA Clean & ConcentratorTM-5 (Zymo Research). Proceed with 75 or 100 nt single-end sequencing on Illumina HiSeq2500 or similar instrument using small RNA sequencing primer (5' CGA CAGGTTTCAGAGTTCTACAGTCCGACGATC3'.) The objective is to read through the 3' adaptor in order to capture the entire 3' end of small RNA.

2.3.2. Data Processing for RNA-Seq dataset—To obtain an accurate picture of small RNA transcriptome by RNA-Seq, a two-step clean-up pre-processing procedure is needed. First, because the 40–60 nt size range of guide RNAs is shorter than the sequencing read length, an adaptor trimming step is necessary. Second, filtering out reads that align to nuclear genome is needed to remove contamination by nuclear-encoded transcripts. In addition, the heterogeneity of 5' start sites, and 3' ends generated by 3'–5' exonucleolytic processing and subsequent uridylation needs to be accounted for [21]. For these reasons, the common K-mer based methods are not suited for the assembly of a small RNA reference. Instead, read clustering approach is recommended to group the sequencing reads to generate

the small RNA reference dataset. The final step is to predict the gRNAs among small RNA transcripts based on alignment to fully edited maxicircle transcripts.

2.3.2.1. Adaptor trimming: Use adaptor trimming tools of your choice to remove the sequencing adaptor. An example of using trimgalore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) for single-end adaptor trimming: `trim_galore -a Your_adaptor_sequence -q 20 --fastqc Your_input_fastq`

2.3.2.2. Filtering out reads that align to nuclear genome

1. Use short read aligner, such as BWA (<http://bio-bwa.sourceforge.net/>), to align the trimmed fastq file to the nuclear genome reference (<http://tritrypdb.org/tritrypdb/>). When performing alignment for the first time, create index for your nuclear reference genome using the following command: `bwa index -a is Your_nuclear_genome_fastaFile`

2. Next step is to align trimmed fastq reads to the nuclear reference genome using the following commands (single-end read example):

```
bwa aln Your_nuclear_genome_fastaFile Your_trimmed.fastq > aln_sa.sai
bwa samse Your_nuclear_gnnome_fastaFile aln_sa.sai Your_trimmed.fq
> aln-se.sam
```

3. Last step is to extract the unmapped sequences and generate cleaned fastq files; command line example:

```
samtools view -f4 aln-se.sam > unmapped.sam (generate unmapped
alignment output)

samtools view -bS unmapped.sam -o unmapped.bam (convert SAM to
BAM file)

samtools bam2fq unmapped.bam > cleaned.fastq (Convert unmapped
BAM to fastq format)
```

2.3.2.3. Reducing diversity by read clustering: Read clustering can be performed by cd-hit: http://weizhong-lab.ucsd.edu/cd-hit/wiki/doku.php?id=cd-hit_user_guide. Before applying cd-hit to perform sequence clustering, the identical sequences need to be eliminated by FASTX tools (http://hannonlab.cshl.edu/fastx_toolkit/) using the following command:

```
fastx_collapser -v -i cleaned.fq -o cleaned.fa -Q 33
```

After this step, the similarity percentage cutoff can be set. For example, at 96% a single mismatch is allowed for 25 nt.

```
cd-hit -i cleaned.fa -o cleaned.cd-hit.96.fasta -c 0.96
```

2.3.2.4. Predict gRNA by alignment to edited mRNA reference: The sequences of edited mitochondrial mRNAs can be downloaded at <http://splicer.unibe.ch/kiss/>. Guide RNAs can be predicted by aligning the reads from previous step to edited mRNA sequences using tools

developed in Koslowsky et al. [22]. There are two scripts: one is designed to find perfect alignment without mismatch in the matching region (fasta-align-pro-noTtail), the other one allows mismatches (fasta-align-LCS). In addition, an alignment with each edited transcript needs to be performed separately.

The command line example for gRNA prediction without mismatch for mRNA encoding subunit 6 of the mitochondrial ATP synthase (Tba6):

```
fasta-align-pro-noTtail cleaned.cd-hit.96.fasta Tba6ed.fasta 18 0.2 ./Tba6ed.gRNA
> ./Tba6ed.ali
```

The command line example for gRNA prediction with N mismatches (N normally ≤ 2), with mismatch number set at 1 for Tba6:

```
fasta-align-LCS ./cleaned_filtered.fa Tba6ed.fasta 18 0.5 Tba6ed. m1.gRNA 1 2000
1 >Tba6ed.m1.ali
```

2.3.3. mRNA and rRNA analyses—General methods described for mRNA and rRNA analysis by Northern blotting and quantitative RT-PCR for total RNA (Section 3) are fully applicable for samples isolated from affinity-purified complexes. However, relative abundance of a given transcript in different preparations cannot be calculated based on a reference RNA, such as tubulin mRNA. Instead, the Ct values can be normalized by the bait protein eluted from beads (Section 2.3). Alternatively, an enrichment fold can be calculated for the transcript of interest vs. tubulin mRNA in affinity purified fraction and in total cellular RNA. Examples of Northern blotting analysis of mRNA, rRNA and gRNA in samples extracted from rapid affinity-purified RESC and RECC complexes are shown in Fig. 2D.

2.4. Sample preparation for mass spectrometry (LC-MS/MS)

Affinity purified samples often contain less than 10 $\mu\text{g/ml}$ of protein and require careful handling to minimize contact with plastic and keratin contamination. The total amount of protein can be estimated by staining the SDS gel with Syro Ruby vs. protein standard with known concentration. Depending on sample complexity, 0.1–1 μg of protein is typically sufficient for two replicate LC-MS/MS runs. Always use the highest purity reagents available and low protein binding untreated tubes. All centrifugations are performed at 4 °C.

1. Add trichloroacetic acid (TCA) and sodium deoxycholate to final concentrations of 20% and 0.1%, respectively. Incubate on ice for 30 min, centrifuge for 20 min at 21,000g, remove supernatant without disturbing the pellet. Centrifuge for 1 min, remove residual liquid.
2. Wash the pellet with 1 ml of ice-cold acetone 3 times. Gently invert the tube to lift the pellet, do not vortex. Spin for 10 min after each wash. The pellet is significantly reduced during washes and may become invisible and easy to lose. Air dry on the bench for 2–3 min.
3. Add 25 μl of LysC Buffer (8 M Urea, 100 mM Tris-HCl, pH 8.5, 1 mM DTT) with LysC (Promega) to achieve 1 to 50 mass ratio of protease to total protein. Resuspend vigorously, incubate at 37 °C for 4 h.

4. Add 75 µl of 50 mM NH₄HCO₃ (pH 7.8) with Trypsin Gold (Promega) to achieve 1 to 100 mass ratio of protease to total protein, continue incubation overnight.
5. Dilute the sample with 0.1 ml of 0.5% of trifluoroacetic acid (TFA) and proceed with purification as described by the manufacturer of Pierce C18 Spin Columns.

3. RNAi knockdown, cell growth phenotype, mitochondrial RNA and complex analyses

Inducible RNA interference remains the technique of choice for a rapid assessment of gene function [23]. The gene silencing is achieved by *tet*-repressor controlled expression of ~500-nt double-stranded RNA either in the form of an extended hairpin, or from opposing T7 phage RNA polymerase promoters (reviewed in [24]). A single transfection is required to introduce expression construct into transcriptionally-silent rRNA or 177-bp repeat loci [25] of the Lister 427 procyclic (insect, PF) (29-13) or Lister 427 bloodstream (BF) “single marker” strains of *Trypanosoma brucei* [15]. The limitations of RNAi include incomplete knockdown and “leakiness” of RNAi expression caused by tetracycline contamination of the fetal bovine serum (FBS) used in media preparation, or by cellular RNA polymerase read-through transcription. Conversely, off-targeting does not appear to be a significant problem with probes verified by RNAi software (<http://trypanofan.bioc.cam.ac.uk/software/RNAi.html>). The mRNA ablation typically occurs within few hours of RNAi induction while the rate of protein decline varies dramatically depending on its turnover rates. Alternatively, true null mutants for non-essential genes can be readily obtained by published methods while repression on an essential gene requires conditional knock-in [26]. Although more laborious to generate, gene knockouts are more reliable especially in bloodstream parasites. In any event, analyzing the outcomes of RNAi knockdown or conditional knock-in over several time points is preferred in order to observe kinetic responses to a gradual protein decline rather than the end-point result. Quantitative RT-PCR is recommended for rapid assessment of gross RNA editing defects. However, Northern blotting analysis is often indispensable for analyzing accumulation of editing intermediates, and pre-editing and post-editing mRNA polyadenylation events. The protocols are described for procyclic parasites, but can be readily adapted for the bloodstream form assuming approximately twice shorter division time and 10-fold lower cell density in axenic culture.

3.1. RNAi-induced cell growth phenotype

1. Dilute stabilized cells to 1×10^6 cells/ml in 20 ml of SDM-79 media containing 10% of FBS and appropriate antibiotics. Use tetracycline-free serum.
2. Divide cells into two 25-ml T-flasks and add tetracycline to one flask to 1 mg/L. Grow in upright position with mild agitation at 27 °C for 24 h and determine cell count using Beckman Coulter Counter.

3. Dilute cultures back to 1×10^6 cells/ml and count every 24 h. Prepare enough media to complete the entire experiment; tetracycline is added fresh daily. Do not dilute cultures if less than one division occurred in 24 h. Continue for seven days and beware of phenotypes appearing later than 96 h, these may be unreproducible. Knockdown of an essential editing factor typically inhibits cell division at 24–72 h albeit the severity varies widely. In some instances, cells resume normal growth after division arrest due to loss of RNAi at later time points.

3.2. RNA analysis

3.2.1. RNA isolation

1. Dilute stable culture in 200 ml of SDM-79 media to 2×10^6 cells/ml in a 225-cm² flask. Transfer 100 ml into fresh 225 cm² flask (uninduced control), and add tetracycline to 1 mg/L to the remaining volume. Incubate with mild agitation for 24 h, target is $8\text{--}10 \times 10^6$ /cells/ml. At each time point, the remaining cells are diluted back to 100 ml according to analytical growth curve, e.g., if cells only divide twice between 72 and 96 h, dilute to 4×10^6 cells/ml.
2. Collect 40 ml of culture every 24 h for five consecutive days by centrifugation at 3000g for 10 min. Wash cells by re-suspending in 40 ml of cold PBS and spinning down at 3000g for 10 min. Re-suspend the pellet in 2 ml of cold PBS, transfer into 2 ml microfuge tube and centrifuge at 3000g for 5 min. Remove as much liquid as possible by aspiration. Freeze the pellet in liquid N₂ and store at -80°C .
3. Re-suspend frozen cell pellets ($1\text{--}4 \times 10^8$ cells) in 0.8 ml of cold Solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M β -mercaptoethanol). Add 0.1 mL of 2 M sodium acetate (pH 4.0) and 0.9 mL of water-saturated phenol, mix gently. Add 0.3 ml of chloroform/isoamyl alcohol (49:1) and incubate for 10 min on Nutator at 4°C .
4. Separate phases by centrifugation at 21,000g for 10 min, and transfer the supernatant into a Phase Lock Gel Heavy 2-ml tube (5Prime) and extract vigorously by vortexing for 1 min with 0.8 ml of chloroform/isoamyl alcohol. Transfer supernatant into a fresh tube and precipitate RNA with 1 ml of isopropanol at -20°C for 1 h.
5. Collect RNA by centrifugation at 21,000g for 15 min and wash with 80% ethanol. Dissolve RNA in 0.2 ml of water and re-precipitate with sodium acetate/ethanol by standard technique. Expected yield is 0.2–0.5 mg of RNA.
6. For Northern blotting, treat 50 μg of RNA with 5 U of RNase-free DNase I in 0.1 ml of manufacturer-supplied buffer for 30 min at 30°C , extract with

equal volume of phenol (pH 5), and phenol-chloroform, and precipitate with ethanol. Expected A260/280 ratio is 2.0–2.2.

7. For quantitative RT-PCR, perform digestion with Turbo DNase for 30 min at 37 °C and purify RNA with RNeasy MiniElute Kit (Qiagen) as recommended by the manufacturer.

3.2.2. Northern blotting—Polyacrylamide/8M urea gel electrophoresis is recommended for high-resolution separation of guide RNAs or short mitochondrial mRNAs, such as ribosomal protein RPS12-coding transcript (~325 nt); denaturing agarose/formaldehyde gels are useful for longer mRNAs and rRNAs. Validated oligonucleotide probes for detection of guide RNAs and gRNA-like molecules are listed in Table 1. Examples of mRNA and gRNA analyses in RNAi knockdown cell lines are shown in Fig. 2E and F, respectively.

3.2.2.1. Polyacrylamide/8 M urea gel electrophoresis

1. Prepare 20-cm long, 20 cm-wide and 1.5-mm thick acrylamide/bis-acrylamide (19:1 ratio)/8 M urea gel with 8-mm wells in an apparatus that allows temperature stabilization; use 5% gel to separate short mRNAs (RPS12, MURF5) and 10% gel for gRNAs.
2. Mix 8 µg of RNA for mRNA analysis or 10 µg for small RNA analysis dissolved in water with 15 µl of 95% formamide, 10 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. Immediately before loading, heat at 65 °C for 2 min and place on ice.
3. Perform pre-electrophoresis at 20 W for 30 min.
4. Run the gel at 20 W until the upper dye migrates 2/3 of the plate (10% gel), or exits the gel (5% gel).
5. Transfer RNA onto BrightStar-Plus membrane by electroblotting in a cooled tank (GE Life Sciences) in 0.5× TBE buffer at 100 V for two hours (5% gel) or for one hour (10% gel).
6. Immediately expose the membrane to UV light at 120 mJ/cm² using CX-2000 UV Crosslinker (UVP).
7. Perform hybridizations to detect gRNAs and tRNA (loading control) with 5'-radiolabeled oligonucleotide probe (Table 1 and Ref. [27]) at 42 °C in ULTRAhyb-Oligo buffer as recommended by the manufacturer. Membranes with small RNAs can be stripped by incubating at 75 °C for 30 min and membranes with mRNAs by boiling in microwave oven for 30 min in 0.1% SDS. Membrane can be re-hybridized or stored indefinitely in a sealed plastic bag at –20 °C.
8. Messenger RNAs and rRNAs are detected by hybridization with single-stranded DNA probe. Templates for preparation of the ssDNA probe are generated by gel-isolating PCR product from qRT-PCR reaction. DNA recovered from a single 25 µl qRT-PCR reaction is typically sufficient for

20 single-strand probe preparations by asymmetric two-step PCR as follows: 10 pmol of 5'-radiolabeled anti-sense oligonucleotide in a standard PCR reaction with Taq polymerase (95 °C, 2 min; 95 °C, 15 s, 50 °C, 30 s; 45 cycles). Purify the probe on G25 Sephadex spin column (GE Life Sciences). The specific activity of the probe can be increased several-fold by reducing dATP concentration to 10 µM and adding 12.5 µl of [α -³²P] dATP (6000 µCi/ml) to PCR reaction.

3.2.2.2. Agarose formaldehyde urea gel electrophoresis

1. Prepare 1.7% gel by melting 5.1 g of agarose in 216 ml of RNase-free water, cool to 65 °C add 30 ml of 10 × MOPS Running Buffer. Add 54 ml of 12.3 M formaldehyde under chemical hood and pour the 20 × 30 cm gel that is approximately 7-mm thick.
2. Prepare samples by mixing 10 µg of RNA dissolved in 5 µl of water with 25 µl of Ambion Northern blotting loading solution. Before loading, heat the sample at 65 °C for 2 min and place on ice.
3. Run the gel immediately after polymerization at 120 V for 4.5 h. Transfer RNA onto BrightStar-Plus membrane in 10 × SSC using vacuum blotting system at 50 mbar for at least 4 h or overnight. Proceed with UV-crosslinking and hybridization as described above.

3.2.3. Quantitative RT-PCR—It is desirable to have the reactions with reference transcripts on each plate and to calculate primer pairs efficiencies based on the slope of the standard curve. It will not change the trend, only the absolute values. This is essential when comparing the abundance of different targets in the same cDNA. Nuclear encoded TERT and β -tubulin transcripts are used routinely as normalization standards. Because of the U-rich nature of edited transcripts, annealing/extension step is performed at a lower temperature for some primer pairs (Table 2). Beacon Designer (Premier Biosoft) software is recommended for primer design for SYBR Green chemistry-based assays. Different amounts of cDNA preparation are used for some targets in order to achieve closer Ct values. The dilution factors are then accounted for in calculating relative abundance.

1. Synthesize cDNA from 2 µg of Turbo DNase-treated, column purified total RNA in 0.1 ml reaction with TagMan Reverse Transcription Reagents (N808-0234, Applied Biosystems) as recommended by the manufacturer. If more than one reaction is planned, prepare a master-mix for RT buffer plus hexamers, and a separate master-mix for dNTP/Mg²⁺ and RT.
2. Pre-mix primer pairs at 1.5 µM final concentration of each in water.
3. Dilute cDNA according to Table 2, if necessary.
4. In a 1.5 ml tube, mix 8 µl of cDNA, or calculated amount of cDNA and water to achieve 8 µl, and add 18 µl of primer mix.
5. Add 26 µl of SYBR Green mix (Power SYBR Green Master Mix), total volume 52 µl to allow for pipetting error.

6. Split 16.5 μ l into triplicate wells in 96 well plate (951022043, Eppendorf). Heat-seal the plate with a film (951023060, Eppendorf).
7. Perform PCR reactions in Eppendorf Realplex 2S cycler or similar, as follows: 95 °C, 10 min; 95 °C (15 s), 60 °C (1 min, measure point, check Table 2 for Ta), 45 cycles. Analyze melting profile with 0.2 °C resolution.
8. Calculate relative abundance or change in relative abundance vs. uninduced RNAi sample by using LinRegPCR free software [28] available at <http://www.gene-quantification.info>.

3.3. Complex integrity analysis

RNA editing complexes vary in stability and sub-unit interdependence. For example, the intrinsically stable RNA editing core complex (RECC) can be virtually eliminated by knockdown of a critical structural subunit [29,30], or may only lose proteins that directly interact with the repressed subunit [6,31], or may not be significantly affected by the loss of an individual component [32]. Conversely, a more dynamic RNA editing substrate binding complex (RESC) remains largely unaltered upon depletion of any single constituent [12]. A combination of glycerol gradient sedimentation and native gel separation provides a time-effective assessment of complex integrity upon RNAi knockdown of one or more subunits of interest. RECC is readily detected by self-adenylation of RNA editing ligases in the presence of [α - 32 P]ATP while other complexes can be visualized by Western blotting. An example of such analysis is shown in Fig. 2G.

1. Based on analytical RNAi growth curve (Section 3.1), build up an ~400 ml parasite culture to achieve a cell density of $\sim 10^7$ /ml after 72 h of RNAi induction. Isolate crude mitochondrial fraction as described in Section 2.1. Add one more wash step to collect mitochondrial fraction in 2 ml Eppendorf tube and determine wet weight of the pellet. Proceed to Step 2 immediately or freeze the pellet in liquid N₂.
2. Add 3 ml of Gradient Lysis Buffer (GLB, 50 mM Tris-HCl, pH 7.5, 120 mM KCl, 12 mM MgCl₂, 1 mM DTT, 1/10 of Complete Protease Inhibitor, 2 U of Turbo DNase and 1.2% NP40) to 1 g of mitochondrial pellet. Resuspend with pipette tip, incubate on ice for 10 min, sonicate with microtip for 5 s at 6 W.
3. Centrifuge the lysate for 15 min at 21,000g and recover the supernatant without disturbing loose membrane pellet.
4. While the extract is being centrifuged, prepare 10–30% glycerol gradient in 25 mM Tris-HCl, pH 7.5, 100 mM KCl and 10 mM MgCl₂ for SW41 rotor (Beckman) using Gradient Master (Biocomp Instruments). Use ice-cold 10% and 30% solutions, short caps and following settings: 2.5 min, 81.5° tilt, 17 rpm.
5. Take out 0.2 ml from the top of the prepared gradient and load 0.25 ml of mitochondrial extract. Centrifuge gradients for 5 h at 38,000 RPM in SW41 rotor (Beckman) with slow breaking.

6. Collect 4 mm fractions from the top with Gradient Station fractionator (Biocomp Instruments). Expect to collect 18 of 0.56 ml fractions.
7. To detect RNA editing core complex, incubate 10 µl aliquots from fractions 2–16 with 5 µCi of [α - 32 P]ATP for 10 min at 30 °C. Otherwise, add 1 µl of NativePAGE™ 5% G-250 Sample Additive and load onto 15-well NativePAGE Novex 3%–12% Bis-Tris gel.
8. Perform electrophoresis in 1× NativePAGE Running Buffer without additives at 4 °C in XCell SureLock Mini-Cell. Fill lower and upper chambers to the top and place on stirrer plate in the cold chamber for at least one hour prior to run. Run conditions: 150 V constant for 60 min, then increase voltage to 250 V constant until free Coomassie dye exists the gel (60–90 min).
9. Transfer the gel onto nitrocellulose membrane in 1× NuPAGE Transfer Buffer in XCell II™ Blot Module at 4 °C on a stirring plate at 25 V constant for 2 h.
10. Expose the membrane to phosphor storage plate or continue with immunoblotting by standard techniques.

4. Identification of RNA binding sites *in vivo*

Methods described in this section are based on high-throughput sequencing of RNA fragments isolated by crosslinking immunoprecipitation (HITS-CLIP) technique [33], which has been adapted for RNA editing factors. Immunoaffinity purification is a technique of choice because target protein is expressed at the normal level, but the successful outcome ultimately depends on the antibody quality. The use of monoclonal or antigen-purified polyclonal antibodies is recommended. Rapid affinity pulldown of an overexpressed TAP-tagged protein represents a viable alternative when antibody is not available. Once the antibody-coated Protein A/G magnetic beads and rabbit IgG-coated magnetic beads (Section 2.2.1) are prepared, the protocol is identical for both approaches. Samples are handled at 4 °C unless otherwise specified. All incubations are performed on Nutator platform; on-bead enzymatic treatment are done in ThermoMixer (Eppendorf).

1. Pellet 0.1 ml (1 mg) of Pierce Protein A/G Magnetic Beads on a magnetic stand and wash two times for 1 min with Tris-buffered saline (TBS) containing 0.05% Tween-20 at room temperature using Nutator rocking platform. Add 5–10 µg of specific antibody and incubate for 1 h. Wash the beads with 1 ml of 1× PBS three times for 5 min.
2. Cross-link bound antibody by adding 0.5 ml of 0.45 mM freshly-prepared DSS (disuccinimidyl suberate) solution in 1× PBS and incubating for 1 h. Pellet the beads and wash with TBS three times for 10 min. Store in 1× TBS at 4 °C for up to three months.
3. Grow *T. brucei* culture (0.8 L) until cells reach $\sim 10^7$ cells/ml. If TAP-tagged protein is expressed, ensure ~ 72 h of induction. All steps prior to

UV-crosslinking must be carried out expeditiously. Pellet cells at 3000 g for 10 min, resuspend in 50 ml of ice-cold PBS with 6 mM sucrose, and collect cells by centrifugation at 3000g for 5 min.

4. Re-suspend cells in 40 ml ice-cold PBS with 6 mM sucrose and distribute 5 ml per cover plate for 4× 100 mm pre-chilled Petri dishes. Keep the remaining 20 ml on ice. Set up Petri dishes on pre-cooled 5 cm-tall enzyme storage blocks in UVP Cross-linker and irradiate three times at 400 mJ/cm². Swirl the plates gently between UV-exposures. Transfer cells into 50 ml tube, use 30 ml of PBS to collect the remaining material from Petri dishes. Collect cells at 3000 g for 10 min and store at –80 °C. Care must be taken for identical processing of UV+ and UV– samples.
5. Resuspend the pellet in 3 ml of Extraction Buffer (EB, 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 1% NP-40, 1/10 of Complete Protease Inhibitor tablet, 40 U of Turbo DNase) and incubate on ice for 15 min. Dilute with 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM MgCl₂ buffer to 11 ml, transfer into 15 ml tube, sonicate with microtip 3 times for 20 s at 12 W with intermediate incubations on ice. Centrifuge the extract at 40,000 RPM for 20 min in SW41 rotor. Filter the supernatant through 0.22 µm low protein binding filter, add EDTA to 10 mM and split 3 × 3 ml into 15 ml conical tubes.
6. Add 2, 0.5 and 0.1 µl of RNaseA/T1 cocktail (RNase A, 500 U/mL; RNase T1, 20,000 U/ml, Ambion) per tube. Incubate at 25 °C for 15 min, and place on ice. The amount of RNase is critical and may need to be titrated.
7. Dispense 0.3 mg of antibody-coated Protein A/G magnetic beads or 1 mg of rabbit IgG-coated Dynabeads (Section 2.2) per tube and incubate on Nutator for 30 min at 4 °C. For low affinity antibodies, the incubation time may need to be extended to 1 h.
8. Rinse with 10 ml of WB (20 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2% NP40) and wash 2× with 10 ml of WB for 5 min.
9. Transfer beads into a 2 ml tube, wash 2× with 1 ml of WB with mild vortexing for 10 s.
10. Wash 2× with 1 ml of HS buffer (20 mM Tris pH 7.6, 500 mM NaCl, 1 mM EDTA, 0.2% NP40) for 10 min.
11. Wash 2× with 1 ml of CIP buffer (50 mM Tris pH 8.0) for 5 min, add 25 U of calf intestinal phosphatase in 50 µl of 1× CIP buffer and incubate at 37 °C for 15 min at 1000 RPM.
12. Wash 1× with 1 ml of HS buffer and 2× with 1 ml of PNK buffer (40 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 0.2% NP40) for 5 min. Wash 1× with 1 ml of 0.1 mg/ml of BSA in water.

13. Add 10 μCi of [γ - ^{32}P] ATP, 10 U of polynucleotide kinase in 50 μl of PNK Forward Buffer. Incubate at 37 °C for 10 min at 1000 RPM. Add ATP to 0.2 mM, continue incubation for 20 min.
14. Wash 2 $\times$ with 1 ml of WB and 1 $\times$ with PNK for 5 min.
15. Collect beads, add 40 μl of 1 $\times$ LDS-MOPS loading buffer with 50 mM DTT. Incubate in Thermomixer at 70 °C for 10 min at 1000 RPM. Collect beads on magnet, transfer supernatant into a clean tube, centrifuge for 5 min at 21,000g, transfer into a fresh tube, load into 2 wells of 10-well 4–12% NuPAGE gel and run at 120 V.
16. Transfer proteins to nitrocellulose membrane in 1 $\times$ MOPS buffer as recommended by manufacturer and expose to phosphor storage screen for 0.5–12 h depending on signal. Expect to see RNA-protein crosslinks as smear that becomes more compact with increased RNase concentration. There should be at least ten-fold difference between no-UV control and irradiated samples. The radiolabeled pattern that looks like a sharp protein band is indicative of RNA overdigestion.
17. Stain the membrane with Sypro Ruby for 2–3 min and de-stain in water. The band of interest can be often visualized under UV light. Cut the area just above the stained protein band plus 4–5 mm up. Re-expose the membrane, cut out bigger area above if necessary. Alternatively, place several dots with radioactive ink to merge the membrane and radioactive pattern. Examples of stained nitrocellulose membrane and corresponding RNA-protein UV-crosslinked patterns are shown in Fig. 2H.
18. It is recommended to pre-incubate Proteinase K solution for 15 min at 37 °C. Cut the membrane into strips and incubate in 200 μl of PK buffer (100 mM Tris-HCl, 50 mM NaCl, 10 mM EDTA) with 4 mg/ml of proteinase K at 37 °C for 20 min at 1000 RPM. Add 0.53 ml of phenol-chloroform (3:1, pH 5.2) and continue incubation for 20 min at 37 °C at 1000 RPM in ThermoMixer.
19. Separate by centrifugation at 21,000g for 5 min at room temperature and collect the upper aqueous phase, add 50 μl of 3 M Sodium Acetate (pH 5.2), 5 μg of Glycogen and 0.2 ml of chloroform. Vortex for 2 min and separate phases by centrifugation at 21,000g for 5 min.
20. Precipitate RNA from the aqueous phase with 1 ml of ethanol: isopropanol (1:1) mixture, wash the pellet with 80% ethanol and dissolve in 5 μl of RNase/DNase free water.
21. Proceed with small RNA library preparation using TruSeq Small RNA Library Preparation Kit (Illumina). Separate PCR products on 20-cm long, 1 mm-thick 8% PAGE in 1 $\times$ TBE gel and stain with SYBR Green I (ThermoFisher). Expect to see an uneven smear 2–3 mm above the major primer-dimer band. Cut out ~1–2 cm-long strip to include the smear and going up, and the same area from no-UV control line. The use of Safe

Imager is recommended to visualize DNA bands. An example of a typical CLIP-HITS library purification is shown in Fig. 2I. Elute, clean up and sequence DNA as described in Section 2.3.1.

22. The data preprocessing step for HITS-CLIP Seq is identical to small RNA-Seq adaptor trimming and the removal of nuclear contamination (2.3.2). To identify the binding targets, pre-processed CLIP-Seq fastq reads can be mapped to small RNA reference, maxicircle sequence and edited mRNAs. The alignment can be done similarly as described in the first two steps of section 2.3.2. The results can be visualized using IGV genome browser (<http://www.broadinstitute.org/igv/>).

5. Summary

Proteomic studies of mitochondrial mRNA editing, polyadenylation and translation complexes unraveled an extremely complex apparatus responsible for synthesis of few, but nonetheless essential, mitochondrially-encoded proteins. The prevalence of polypeptides lacking recognizable motifs among more than two hundred proteins that populate these complexes calls for a uniform approach to their systematic investigation. The described methods constitute an initial characterization workflow to place a candidate protein into the context of mitochondrial gene expression and to set the stage for a detailed mechanistic analysis.

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Fig. 1.
Analysis pipeline.

Fig. 2.

Representative experimental outcomes. **A.** Tandem affinity purification of RNA editing core and substrate binding complexes via C-terminally TAP-tagged RET2 terminal uridylyltransferase and GRBC1 subunit, respectively. Final fractions eluted from calmodulin column were separated on 8–16% SDS gel and stained with Sypro Ruby. Positions of bait proteins are shown by arrows. **B.** Labeling of small mitochondrial RNAs with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ in the presence of vaccinia virus guanylyltransferase. **C.** RNA-Seq library was constructed from gel-purified 40–70 nt RNA fragments derived from rapid affinity purified GRBC1

complex. PCR products were separated on 7% PAGE in 1× TBE buffer and DNA was eluted from the area indicated by brackets. Synthetic 18-mer RNA (25 fmol) was used as library preparation efficiency control. Primer-dimer band is shown by arrow. **D.** Distribution of pre-edited and fully-edited mRNAs and guide RNA among RNA editing core and gRNA binding complexes. Following rapid affinity pulldown, RNA was extracted from magnetic beads, separated on 5% polyacrylamide/8 M urea gel and probed for respective RNA species. Beads, IgG-coated magnetic beads were incubated with extract from the parental cell line. RNA amounts were normalized to TAP-tagged bait proteins detected by quantitative Western blotting with antibodies against calmodulin binding peptide. Cytosolic 5.8S rRNA served as contamination control. **E.** Guide RNA decline and accumulation of gRNA precursors upon RNAi knockdown of RNA editing TUTase 1, RET1 [34]. **F.** Northern blotting analysis of pre-edited and fully-edited RPS12 mRNAs. RNAi knockdown of GRBC1 triggers degradation of gRNAs, which inhibits mRNA editing [10] and also leads to accumulation of pre-edited mRNA. Total RNA was separated on 5% polyacrylamide/8 M urea gel and hybridized with single-stranded DNA probe. Cytosolic ribosomal RNA (5.8S) was used as loading control. **G.** Mitochondrial fraction from the insect stage of *T. brucei* was extracted with detergent and soluble contents were separated for 5 h at 178,000g in 10%–30% glycerol gradient. Each fraction was incubated with [α -³²P]ATP and further resolved on 3%–12% Bis-Tris native gel. Positions of native protein standards are indicated by arrows. Thyroglobulin (19S) and bacterial ribosomal subunits were used as apparent S-value standards. **H.** Isolation of *in vivo* RNA-protein crosslinks of RET1 TUTase. Immunoaffinity purified fractions were subjected to partial RNase digestion and RNA fragments bound to the protein were radiolabeled. Upon separation on SDS gel, RNA-protein crosslinks were transferred onto nitrocellulose membrane. Protein patterns were visualized by Sypro Ruby staining (left panels) and radioactive signals by exposure to phosphor storage screen. RNA was eluted from areas indicated by brackets and sequenced. UV, live parasites were subjected to UV-irradiation (+) or mock-treated (–). **I.** RNA-Seq library constructed from RNA fragments obtained in panel H. PCR products were separated on 8% PAGE in 1× TBE buffer and DNA was eluted from the area indicated by brackets.

Table 1

Validated DNA oligonucleotide hybridization probes for detection of nuclear-encoded control RNAs, mitochondrial ribosomal RNAs, guide RNAs and small RNAs transcribed as anti-sense to guide RNAs.

Name	Sequence, 5'-3'	Application
A851	GGAAGCCAAGTCATCCATCGCGACACGTTGTGGGAGCCGTGG	5.8S
A343	TGGTAAAGTTCCCCGTGTTGA	18S rRNA
A872	GGGGACCATTCGGACTGCAGCCG	tRNA ^{Cys}
A304	TGAACAATCAATCATGGTAATAAGTAGACGATG	12S rRNA
A504	ACGGCTGGCATCCATTC	9S rRNA
A798	TAATTAAATCTTCTCATTGTCACTGTCTTATACTACGATTGAGTTTGTAT	CO3[147] gRNA
A662	CATTCAATTACTCTAATTTAATTTTATTTTGTGC	Murf2 II gRNA
B111	TTATTTACTCACTTTATCTCACTACATAAATCCATGATTACCCAGTATA	RPS12[100] gRNA
B108	ATAATTATCATATCACTGTCAAAATCTGATTCGTTATCGGAGTTATAGTATAT	A6[14] gRNA
B100	ATACAACTCAATCGTAGTATAAGACAGTGACAATGAGAAGATTTAATTA	CO3[147] anti-gRNA
B101	GGAAATTATAGAAAGCACAAAAATAAAATTAAATTAGAGTAATTGAATG	Murf2 II anti-gRNA
B107	TATACTGGGTAATCATGGATTATGTAGTGAGATAAAGTGAGTAAATAA	RPS12[100] anti-gRNA
B105	ATATACTATAACTCCGATAACGAATCAGATTTTGACAGTGATATGATAATTAT	A6[14] anti-gRNA

Table 2

Primer pairs and annealing temperatures for qRT-PCR analysis of mitochondrial transcripts in *T.brucei*. E, edited; P, pre-edited. Recommended cDNA volumes are indicated for each RNA target. Dilution factors of highly abundant RNAs (β -tubulin, and mitochondrial rRNAs) are also provided. Anti-sense primers can be used to generate single-stranded DNA hybridization probes for Northern blotting.

Primer	Ta, °C	Sequence, 5'–3'	Polarity	cDNA, μ l	Target
A344	60	TTCCGCACCCTGAACTGA	Sense	1:100, 8	β -tubulin
A345	60	TGACGCCGGACACAACAG	Anti	1:100, 8	β -tubulin
B290	60	GAGCGTGTGACTTCCGAAGG	Sense	8	TERT
B291	60	AGGAACTGTCACGGAGTTTGC	Anti		TERT
A503	60	ATTAGATTGTTTTGTAAATGCTATTAGATG	Sense	1:100,1	9S rRNA
A504	60	ACGGCTGGCATCCATTTC	Anti		9S rRNA
A303	60	GGGCAAGTCCTACTCTCTTTACAAAG	Sense	1:100, 8	12S rRNA
A304	60	TGAACAATCAATCATGGTAATAAGTAGACGATG	Anti		12S rRNA
A293	60	GTTTACTACTTGCATGTCTCTTTCTTTG	Sense	2	MURF1
A294	60	AAAGCCAATACAAATACAAAGGTAACCTTAG	Anti		MURF1
A348	60	GATTTTAAATGTTTGGTTGTTTAAATTTAG	Sense	2	Murf2, E
A346	60	GATTTTAAAGATTGGCTTTGATTGA	Sense		Murf2, P
A347	60	AATATAAAATCTAGATCAAACCATCACA	Anti		Murf2, P/E
A295	60	GGACTGCTTCTTGATGGATTACGTTTACC	Sense	1	ND1
A296	60	AGATAATTCAGTAACAAGGCCAGCAACAAG	Anti		ND1
B292	55	CGTTGTGTGTTGTGGTTT	Sense	8	ND3, E
B293	55	ACAAATAATGGAATTTAACAATACA	Anti		ND3, E
A351	60	GAATGGGAGATGGGTTTTGG	Sense	1	ND3, P
A352	60	AACAAATCTCTTTACCCCTTCAG	Anti		ND3, P
A297	60	CAATCTGACCATTCCATGTGTGACTACC	Sense	5	ND4
A298	60	TGCTATAATACTAAACCCACACAATTACACTATC	Anti		ND4
A299	60	TTTCTATATGTTTGTAGTAGGATGTGCGTTC	Sense	2	ND5
A300	60	GCGTGATTAAATGCTGATACTGGGATAGG	Anti		ND5
A353	60	GCATCCCGCAGCACATG	Sense	2	ND7, E
A354	60	CTGTACCACGATGCAAATAACCTATAAT	Anti		ND7, E
A355	60	GCGGGCGGAGCATTATT	Sense	2	ND7, P
A356	60	GATCTACGGTCCCTCTTTCCT	Anti		ND7, P
B266	52	GTTGTATTGCTTGTTCGTT	Sense	8	ND8, E
B267	52	ACAATGAATGCGTAATGG	Anti		ND8, E
B183	60	AAGCCCATTTTGAGCAGGAG	Sense	1	ND8, P
B184	60	TTGGCAAAAATCTGTCTGGGC	Anti		ND8, P
B189	52	TTAGAATTACAACGGTGAA	Sense	8	ND9, E
B190	52	TGTTGAAGTGTATCCATT	Anti		ND9, E
B187	60	AACATCGAGGAGTTTGGGG	Sense	1	ND9, P
B188	60	TTAAGGTTGCCCTGTTGTCTG	Anti		ND9, P
A357	60	CGTATGTGATTTTGTATGGTTGTGTG	Sense	4	RPS12, E

Primer	Ta, °C	Sequence, 5'-3'	Polarity	cDNA, µl	Target
A358	60	ACACGTCGGTTACCGGAACT	Anti		RPS12, E
A360	60	CCCCCACCACAAATCTTT	Anti	2	RPS12, P
A359	60	CGACGGAGAGCTTCTTTTGAATA	Sense		RPS12, P
A301	60	TGCCTATACTATGGGTGGGTTTACAAAC	Sense	1	CO1
A302	60	ACTAAGCAACCAATCCTCCAATAAACATTC	Anti		CO1
A312	60	ATTACAGTGTAAACCATGTATTGACATT	Sense	2	CO2, P/E
A314	60	ATTTCATTACACCTACCAGGTATACAA	Anti		CO2, E
A313	60	TTCATTACACCTACCAGGTTCTCT	Anti		CO2, P
A206	60	GAAACCAGATGAGATTGTTTGCA	Sense	6	CO3, E
A205	60	TTCATTCCAATAAACCCCTTTC	Anti		CO3, E
B288	60	GGGAAACCAGATGAGATTG	Sense	2	CO3, P
B289	60	ACTACCTCTTCATTCCAATA	Anti		CO3, P
B260	55	TTGCCGCCATATTACAGT	Sense	8	A6, E
B261	55	TCTATAACTCCAATAACAAACCAAT	Anti		A6, E
B262	60	GAGAAGCAAGGAGGAGAA	Sense	2	A6, P
B263	60	GCAAAGGCAATTCCCAAT	Anti		A6, P
B274	60	ATATAAATATGTTTCGTTGTAGATT	Sense	8	Cyb, E
B275	60	CTAAACACACTCCACAAAT	Anti		Cyb, E
A207	60	ATATAAAAGCGGAGAAAAAGAAAG	Sense	2	Cyb, P
A208	60	CCCATATATTCTATATAAACACCTGACA	Anti		Cyb, P