

Chapter 2

iCODA: RNAi-Based Inducible Knock-In System in *Trypanosoma brucei*

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Abstract

In vivo mutational analysis is often required to characterize enzymes that function as subunits of the U-insertion/deletion RNA editing core complex (RECC) in mitochondria of *Trypanosoma brucei*. The mutations may skew phenotypic manifestation of a dominant negative overexpression if complex association is disrupted. Conditional knockouts and knock-ins of essential mitochondrial genes are time consuming and restricted to the bloodstream form parasites, thus limiting biochemical analysis. We have combined CODA (computationally optimized DNA assembly) technology with RNA interference to develop an iCODA inducible knock-in system for expeditious phenotype assessment and affinity purification of the RECC bearing a mutant subunit. For functional knock-in, the gene region targeted by RNAi is replaced with a synthetic sequence bearing at least one silent mutation per 12 contiguous base pairs. Upon co-expression of the double-stranded RNA targeting the endogenous transcript and modified mRNA in a stable cell line, the endogenous mRNA is destroyed and the cell survives on the RNAi-resistant transcript encoding the same polypeptide. In this chapter, we describe the generation of procyclic (insect) transgenic cell lines, RNAi rescue, complex purification, and validation methods for RNA editing TUTase 2 (RET2). These methods should be readily applicable for any gene in *T. brucei*.

Key words: *Trypanosoma*, Mitochondria, RNA editing, RNAi, TUTase

1. Introduction

Proteomic studies of trypanosomal mitochondrial RNA editing complexes, including the RECC (1–3) and the guide RNA binding complex (4), have been expedited by efficient constitutive and inducible overexpression systems. Introduction of a C-terminal affinity TAP tag (5), composed of protein A, calmodulin binding peptide, and a tobacco etch virus (TEV) protease cleavage site, enabled isolation and mass spectrometry analysis of these and other complexes involved in mitochondrial RNA

processing (2, 6). Because *Trypanosoma brucei* is a diploid asexually reproducing organism, inducible knockouts of essential genes require sequential generation of three clonal cell lines and as many selective markers (7); a fourth marker is required for a knock-in construction. For reasons that are not clear, successful knockouts and knock-ins of essential mitochondrial genes (8, 9) have been achieved only in blood stream (BF) parasites which are deficient in oxidative phosphorylation and grow to a low cell density ($\sim 10^6$ cells/mL). These technical restrictions made RNA interference (10) a method of choice for gene silencing in BF and procyclic (PF) parasites, which have an actively respiring mitochondrion and can be cultured at higher cell density ($\sim 10^7$ cells/mL).

Transgenic cell lines have been generated for both BF and PF *T. brucei* to allow tetracycline-regulated (11) ectopic expression of double-stranded (ds) RNA driven by either T7 RNA polymerase or RNA polymerase I promoters (12). Typically, the RNAi cassette is designed toward protein-coding regions, although achieving sufficient knockdown may require empirical selection among several gene fragments (13) or inclusion of untranslated regions (UTRs) (14). Targeting unique UTRs for RNAi also has been used as a knock-in method. In this strategy, RNAi targets exclusively 5'- or 3'-UTR triggering degradation of the endogenous mRNA while the gene of interest flanked by heterologous UTRs is expressed (15). Limitations of this approach include a requirement for precise UTR mapping to avoid knockdown of closely spaced adjacent mRNAs and UTRs that are too short for effective RNAi knockdown.

Although biochemical and structural analyses of recombinant editing enzymes were instrumental in defining catalytic residues and domain organization (16, 17), functional studies would greatly benefit from an *in vivo* mutagenesis system which also allows *in vitro* analysis of purified editing complexes. We have developed an iCODA *in vivo* complementation approach for PF parasites based on tetracycline-inducible co-expression of a dsRNA and a synthetic gene which encodes the same polypeptide as the one targeted by RNAi. A fragment corresponding to the RNAi-targeted region is assembled from overlapping DNA oligonucleotides using CODA technology (18) with at least one silent mutation per 12 bp. These mutations are designed based on a genome-wide analysis of codon bias and codon context to minimize effects on translation. Transcription of the synthetic gene produces an RNAi-resistant mRNA as the only source for the protein of interest. Consequentially, the cell survives unless a mutation disrupting catalysis or complex association is introduced into the synthetic gene. Introduction of the TAP tag allows for protein complex purification and downstream biochemical analysis (Fig. 1).

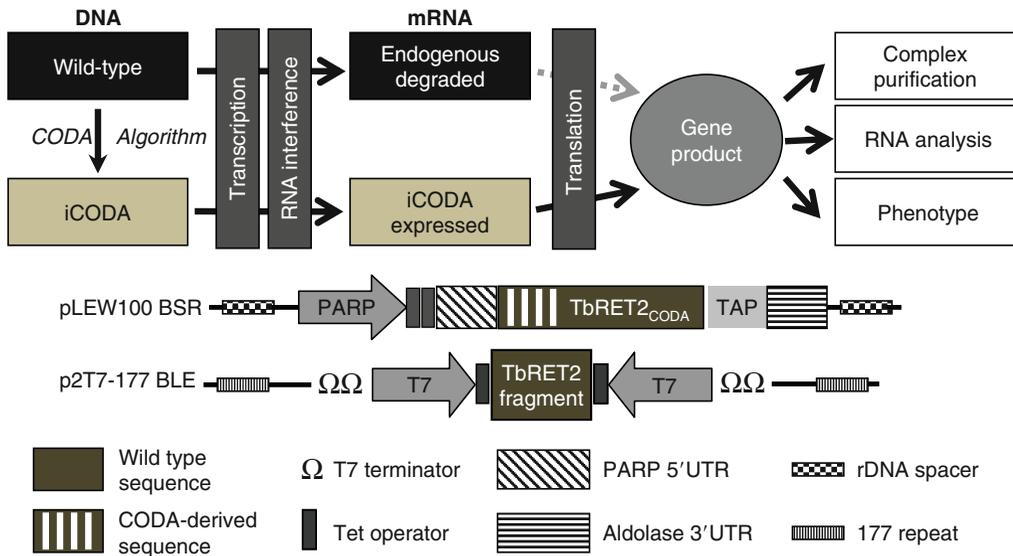


Fig. 1. iCODA knockdown/knock-in strategy. Silent mutations (at least one per 12 bp, and often more) were introduced into the RET2 gene to minimize potential effects on translation (18) and to prevent transcript targeting by the RNAi machinery. The expression of both RET2-iCODA protein and RET2 RNAi cassette is controlled by tet-operators positioned downstream of a procyclic acidic repetitive protein promoter (PARP), which is recognized by RNA polymerase I and T7 RNA polymerase promoter, respectively.

The iCODA technology has been applied to studies of RNA editing terminal uridylyl transferase 2 (RET2), an integral RECC component responsible for the U-insertion mRNA editing activity (19, 20). RET2 is the only nonredundant enzyme within the RECC and is essential for parasite viability in both PF (19) and BF (17). In addition, the iCODA technology has been useful in validating RNAi knockdown specificity for partial 3'-UTR targeting in the case of MEAT1 TUTase (14). Here, we present methods for design of an RNAi resistant gene, generation and validation of iCODA/RNAi cell lines, and complex purification.

2. Materials

2.1. Genetic Constructs

1. p2T7-177-BLE – RNAi construct with opposing T7 RNA polymerase promoters and phleomycin resistance (21).
2. pLEW100-TAP-BSR – Protein expression construct with blasticidin resistance (available from author's laboratory).

2.2. Cell Culture and Selection of Clonal Cell Lines

1. 29-13 strain of procyclic *T. brucei* (12).
2. SDM-79 (semi-defined) medium: dissolve 7 g S-MEM (Invitrogen), 2 g Medium 199 (Invitrogen), 1 g d-glucose, 8 g HEPES, 5 g MOPS, 2 g NaHCO₃, 100 mg sodium pyruvate,

200 mg L-alanine, 100 mg L-arginine, 300 mg L-glutamine, 70 mg L-methionine, 80 mg L-phenylalanine, 600 mg L-proline, 60 mg L-serine, 160 mg L-tyrosine, 160 mg L-tyrosine, 350 mg L-threonine, 200 mg L-tyrosine, 10 mg adenosine, 10 mg guanosine, 50 mg glucosamine-HCl, 4 mg folic acid, 2 mg *p*-aminobenzoic acid, 200 µg biotin, 8 mL MEM amino acids (50×) (Invitrogen), 6 mL MEM nonessential amino acids (100×) (Invitrogen) in 850 mL of water. Adjust pH to 7.3 with 5 M NaOH and bring volume to 900 mL. Sterilize by filtration and store at +4°C up to 2 weeks.

3. 29-13 Medium: SDM-79 medium supplemented with 50 µg/mL of Geneticin (G418), 50 µg/mL of Hygromycin, 10 µg/mL of hemin (EMD Chemicals), and 10% heat inactivated fetal bovine serum (FBS, see Note 1).
4. Limiting dilution (LD) medium: same as 29-13 medium with appropriate additional drug(s) and 20% FBS.
5. Humidified incubator set at 27°C with 5% CO₂.

2.3. Electroporation

1. Not I restriction endonuclease.
2. Cytomix: 120 mM KCl, 0.15 mM CaCl₂, 10 mM potassium phosphate, 25 mM HEPES, 2 mM EDTA, 5 mM MgCl₂, pH 7.6.
3. Phosphate sucrose buffer: 277 mM sucrose, 1 mM MgCl₂, 7 mM potassium phosphate, pH 7.4.
4. EM buffer: 3:1 mixture of Cytomix and phosphate sucrose buffer.
5. Bio-Rad Gene Pulser.
6. 0.4-cm electroporation cuvettes.

2.4. Tandem Affinity Purification

1. Extraction buffer (EB): 50 mM Tris-HCl pH 7.6, 150 mM KCl, 2 mM EDTA. Stock of Tris-HCl is prepared as 1 M solution with pH 7.6 at 20°C.
2. IgG binding buffer (IBB): 25 mM Tris-HCl pH 7.6, 150 mM KCl, 1 mM EDTA, 0.1% NP-40.
3. TEV cleavage buffer (TCB): IBB supplemented with 1 mM DTT (see Note 2).
4. Calmodulin binding buffer (CBB): 25 mM Tris-HCl pH 7.6, 150 mM KCl, 0.1% NP-40, 10 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl₂.
5. Calmodulin elution buffer 1 (CEB1) for activity assays: 25 mM Tris-HCl pH 7.6, 150 mM KCl, 3 mM EGTA, 1 mM EDTA, 0.1% NP40, 10% glycerol.
6. Calmodulin elution buffer 2 (CEB2) for mass spectrometry analysis of complexes: 25 mM Tris-HCl pH 7.6, 50 mM KCl, 3 mM EGTA, 1 mM EDTA, 0.1% NP40.

7. Disposable polystyrene columns (Pierce, product # 29920).
8. TEV protease (Invitrogen).
9. Sypro Ruby gel stain (Invitrogen).
10. Complete protease inhibitor (Roche). Dissolve one tablet in 1 mL of water.

3. Methods

3.1. Genetic Constructs Design

The choice of ~400 bp-long DNA fragment for the RNAi cassette is based on the RNAit algorithm (<http://trypanofan.path.cam.ac.uk/software/RNAit.html>) that uses BLAST searches to minimize off-targeting (22). The p2T7-177 genetic construct (partially diagrammed in Fig. 1) is a standard vehicle for inducible expression of dsRNA fragments in trypanosomes (21). The dual opposing T7 promoter architecture of this vector allows a one-step cloning of the target sequence or multiple gene knock-downs by cloning several target sequences. Integration into the mini-chromosome 177-bp repeat region provides for low base-line transcription.

The pLEW100 vector (23), partially diagrammed in Fig. 1, is widely used for stable expression in *T. brucei* via integration into the rRNA spacer. Procytic acidic repetitive protein (PARP) promoter-driven expression regulated by *tet*-operators allows for inducible expression in PF parasites. Alternatively, if work in BF parasites is planned, the pLEW100v5-BSD vector (<http://tryps.rockefeller.edu>) may be a better alternative. In this construct, expression is driven by the rRNA promoter that is active in both PF and BF *T. brucei*. While overexpression is often performed in the pLEW79-based mhTAP vector (24), pLEW100 variants provide a balance of efficient integration and tightly regulated expression which is better suited for iCODA.

The synthetic gene replacement construct is designed to (a) be identical in amino acid sequence to the wild-type gene except for deliberately introduced mutations; (b) differ from the wild-type gene DNA sequence by at least 1 bp in any contiguous 12 bp; (c) avoid off-target cross-hybridization, which is known to be a source of mutagenesis failure (25); (d) respect known or hypothesized codon usage (26) and codon pair (27–29) preferences; (e) avoid any undesired DNA sequences, e.g., restriction sites used in cloning; and (f) be easily assembled from purchased synthetic oligonucleotides by polymerase extension.

These goals are all accomplished by making silent (synonymous) codon substitutions in the designed synthetic gene replacement construct. For this purpose, we used the computationally optimized DNA assembly technology (CODA, (18)) to design the gene assembly and mfold (30) to identify undesired RNA

secondary structures. However, any modern gene design software that accepts sequence constraints and removes undesired RNA secondary structures may be used instead of CODA. Conceptually, the CODA system works by beginning with (a) a desired amino acid sequence, (b) a candidate DNA sequence, and (c) a set of constraints to satisfy. It then iteratively introduces silent codon substitutions into the candidate DNA sequence until all constraints are satisfied. Finally, it outputs (a) a list of DNA oligonucleotide sequences to purchase and (b) a set of instructions for combining the purchased oligonucleotides by polymerase extension. The actual CODA implementation involves more technical details; see Larsen et al. for further explanation (18).

1. Define the desired synthetic replacement sequence. To minimize the DNA synthesis and assembly burden, an approximately 400 bp gene fragment is recommended for iCODA.
2. Prepare an initial candidate DNA sequence by replacing every codon in the sequence from step 1 by the most prevalent (most highly used) codon for that amino acid in the host organism (26), unless the wild-type DNA sequence also uses the most prevalent codon there, in which case choose the second most prevalent codon. The result of this step is a DNA sequence that is identical in encoded amino acid sequence to step 1 and differs from the wild-type DNA sequence at every codon (except for methionine and tryptophan, which each have only one codon).
3. Prepare a list of desired DNA sequence constraints, which includes any required DNA subsequences while prohibiting (a) any four consecutive codons from the wild-type gene; (b) restriction sites used in cloning from occurring anywhere in the coding region of the gene; (c) low-usage codons or undesired codon pairs; and (d) any other DNA sequences to exclude. Prohibiting any four consecutive codons from the wild-type gene guarantees that the desired synthetic gene replacement construct has no more, and often fewer, than 11 contiguous base pairs that are identical to the wild-type gene DNA sequence.
4. Compare the current candidate DNA sequence to the list of sequence constraints from step 3. If any constraint violations are found, introduce silent (synonymous) codon substitutions into the candidate DNA sequence to eliminate them while avoiding low-usage codons (26) and go to step 4.
5. Analyze the current candidate DNA sequence for undesired RNA secondary structures, such as hairpins, cross-hybridizations, etc. (18, 25). If any undesired RNA secondary structures are found, introduce silent (synonymous) codon substitutions

into the candidate DNA sequence to eliminate them while avoiding low-usage codons (26), and go to step 4.

6. Divide the DNA sequence of step 5 into DNA oligonucleotide sequences that can be purchased commercially and assemble them by polymerase extension as described in Larsen et al. (18).

3.2. Transfection of Genetic Constructs into 29-13 *T. brucei* by Electroporation

The following protocol may be used for transfection with a single plasmid or co-transfection with two plasmids (see Note 3). All materials and solutions that will come in contact with cells should be kept on ice. Sterile technique must be employed when handling cells.

3.2.1. Preparation of Plasmids and Cell Lines

1. From a mid-logarithmic culture maintained at $\sim 5 \times 10^6$ cells/mL in 29-13 medium, seed $1-2 \times 10^6$ cells/mL to achieve a cell density of $4-5 \times 10^6$ cells/mL on the following day. Assume that the division time is ~ 12 h and 1.4×10^7 cells per transfection are required. Incubate 25–30 mL of suspension culture at 27°C with mild agitation (~ 100 rpm) in a 75 cm² cell culture flask in vertical position.
2. Digest 10–12 μ g of plasmid DNA purified with QIAprep kit (Qiagen) with 5 units of Not I restriction endonuclease in a 200- μ L reaction volume at 37°C overnight.
3. Precipitate the DNA with 500 μ L of ethanol for 20 min in dry ice. Do not add extra salt or extract with organic solvents. Wash the pellet with 70% ethanol, air-dry in the laminar hood, and resuspend in 100 μ L of sterile water (50 μ L for co-transfection).

3.2.2. Electroporation

1. When the cell count reaches $\sim 5 \times 10^6$ /mL, transfer cells into a conical tube.
2. Using a fixed-angled rotor, centrifuge the cells $2,000 \times g$ at 4°C for 5 min. Aspirate the supernatant completely.
3. Fill the tube to 50% volume with EM buffer and gently resuspend cells with pipetting. Repeat centrifugation and aspiration.
4. Resuspend cells in EM buffer at 3×10^7 /mL.
5. Transfer the DNA solution into a chilled 4-mm electroporation cuvette. Use sterile water for the control transfection.
6. Add 450 μ L of cell solution to each cuvette with 1-mL pipette tip. Gently pipette twice.
7. Set electroporator to 1,500 V and 25 F. Electroporate cells with two pulses with a 10-s interval. Expected time constant is 0.8–1 ms. Chill on ice for 2 min (see Note 4).

- Using a sterile transfer pipette, transfer cells to a 25-cm² culture flask with 10-mL 29-13 medium prewarmed to 27°C and incubate with mild agitation at 27°C for 24 h.

3.2.3. Add Drug at 24-h Posttransfection

For single-plasmid transfection, add 10 μL of 2.5 mg/mL phleomycin (2.5 μg/mL final) or 10 μL of 10 mg/mL blasticidin (10 μg/mL final). For co-transfection, add both drugs. Incubate cells in 25 cm² culture flask with mild agitation at 27°C for 24 h.

3.2.4. Limiting Dilution

- Add 100 μL of LD medium into wells 2–12 (all rows) of a 96-well flat-bottom plate.
- Add 100 μL of LD medium into wells B1, D1, F1, and H1.
- Add 300 μL of culture into wells A1, C1, E1, and G1.
- Transfer 100 μL of culture from A1 to B1, C1 to D1, E1 to F1, and G1 to H1.
- Using a multichannel pipetman, perform 1:1 serial dilutions (100 μL) from lane 1 through lane 12.
- Starting from lane 12, add 100 μL of LD medium to each well to achieve 200 μL of total volume.
- Incubate the 96-well plate at 27°C in humidified incubator with 5% CO₂.
- Monitor growth under an inverted microscope. Expect to see cell growth in 10–14 days. If no live cells are present after 2 weeks, repeat the transfection.
- When the well with highest dilution becomes confluent and the next dilution remains clear, transfer the entire 200-μL culture into 3 mL of LD medium in a 25-cm² flask and incubate upright without agitation for 24 h (see Note 5).
- Start agitation (100 rpm) and continue until cell density reaches 5–10 × 10⁶ cells/mL.
- Add 3 mL of 29-13 medium with drugs and continue incubation for another 24 h.
- Stabilize culture by diluting to 10⁶ cells/mL two to fourfold every 24 h until division time is consistent.

3.3. Validation of iCODA/RNAi Cell Lines

Successful concurrent knockdown of endogenous protein and knock-in of exogenous protein can be verified at both the transcript and protein level. If antibodies are available, the depletion of endogenous protein and expression of longer TAP-tagged polypeptide can be verified by Western blotting (Fig. 2a). Parasite cells are collected by centrifugation at different induction time points, washed with phosphate buffered saline (PBS) buffer, and boiled in SDS loading buffer for 3 min. Cell extract is separated by SDS gel (3–5 × 10⁶ cells/well) and transferred by standard

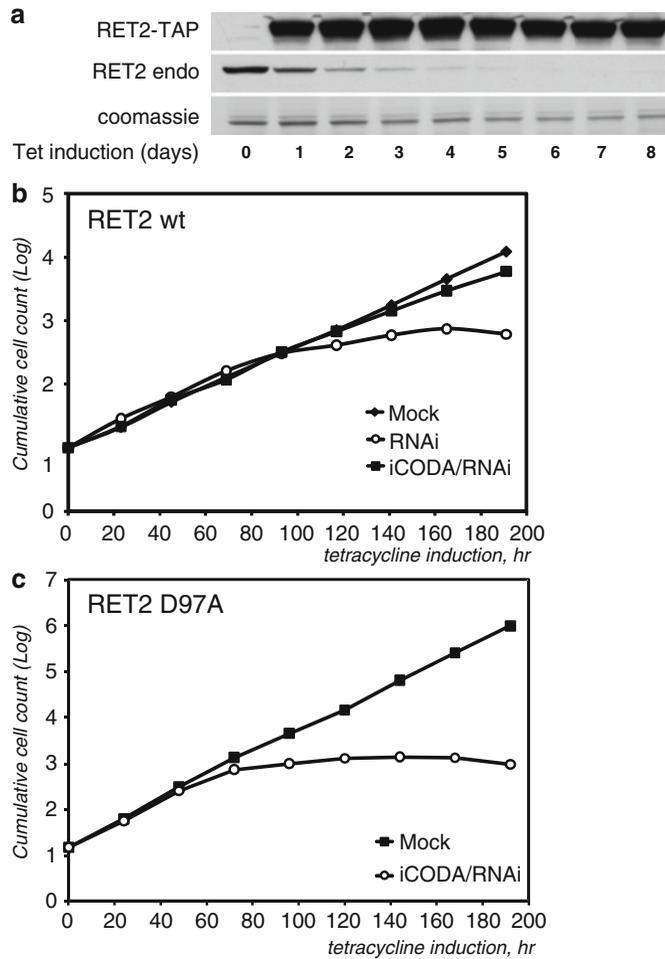


Fig. 2. Functional complementation of RET2 RNAi by co-expression of the RNAi-resistant transcript. (a) Western blotting analysis of RET2-iCODA/RNAi cells. (b) Growth kinetics of RET2-RNAi and RET2-iCODA/RNAi cell lines. (c) Growth kinetics of RET2-D97A-iCODA/RNAi cell line.

techniques. If antibodies are not accessible, Western blotting with commercially available PAP reagent (Sigma) which recognizes the Protein A moiety of the TAP tag and quantitative RT-PCR (qRT-PCR) can be used to confirm expression of TAP-tagged proteins and knockdown of the endogenous transcript, respectively. For total RNA isolation, 20–50 mL cultures are sufficient. Since transcripts derived from the iCODA-optimized sequence will be present in iCODA/RNAi cell lines, both qRT-PCR primers must selectively hybridize with the RNAi-targeted region of the wild-type transcript, but not with the iCODA-derived sequence. The knockdown of the endogenous transcript after ~48 h of RNAi should be normalized to mock-induced cells (see Note 6).

The RET2-RNAi-mediated growth phenotype is successfully rescued via co-expression of the iCODA-modified transcript (Fig. 2b). To confirm that the functional complementation requires RET2's enzymatic activity, a single amino acid mutation (D97A) has been introduced into the active site. As seen from Fig. 2c, the expression of the inactive RET2 does not counteract the RNAi growth inhibition phenotype.

3.4. Tandem Affinity Purification of Complexes from iCODA/RNAi Whole Cell Lysates

The following protocol is used to purify complexes from either iCODA/RNAi cells or from cells expressing the TAP-tagged protein. All purification steps are performed at 4°C.

1. Build up the 150 mL culture with cell density of $\sim 10^7$ cells/mL (29-13 medium plus appropriate antibiotics). Avoid dilutions larger than five to tenfold as this can kill cells. Seed 2×850 mL cultures (1×10^6 cells/mL) in roller bottles (110 \times 475 mm, Bellco) and add tetracycline to 1 μ g/mL. Incubate in the roller apparatus until cell density reaches 12–15 $\times 10^6$ cells/mL, typically 48–72 h.
2. Harvest cells in a fixed-angle rotor at 5,000 $\times g$ for 10 min.
3. Resuspend pellet in 100 mL cold PBS pH 7.6 and repeat centrifugations.
4. Resuspend pellet in 50 mL of PBS, transfer into a conical centrifuge tube, collect cells at 3,000 $\times g$ for 10 min, and proceed with complex purification. Pellet can be frozen in liquid nitrogen and stored at -80°C for later use.
5. Resuspend cells in 6-mL EB (final volume). Add NP-40 to 0.4% and 300 μ L of Complete protease inhibitor (Roche).
6. Sonicate the extract three times at 9 W for 10 s.
7. Spin the extract at 100,000 $\times g$ for 15 min at 4°C in a TLA 100 rotor and promptly transfer supernatant to a clean 15-mL conical tube.
8. Re-extract the pellet in 6 mL of EB (no NP-40) with sonication and centrifugation. Pool the two extracts – target volume is ~ 12 mL.
9. Prepare the IgG Sepharose resin. Transfer 0.3 mL of slurry (GE Healthcare) to a 15-mL tube and add 15-mL IBB. Centrifuge 300 $\times g$ for 2 min (no brakes). Aspirate supernatant.
10. Filter the extract from step 8 through a syringe-driven low-protein binding membrane into the resin-containing tube from step 9. Incubate the extract with resin for 1 h with gentle agitation.
11. Transfer the material into a disposable column (Pierce). Reload three times to maximize recovery of resin. Collect flow-through.

12. Wash the column with 5–6 full volumes of IBB. Gently resuspend the resin with each wash and rinse the rims of the column well.
13. Wash IgG column with two column volumes of TCB.
14. Mix 150 units TEV protease, 20 μ L Complete protease inhibitor, and 1.5 mL TCB. Close the column outlet, add TEV solution, seal both ends of the column with parafilm, and incubate overnight with gentle agitation.
15. Transfer 0.3 mL calmodulin resin into a 15-mL conical tube pretreated with 2% Tween 20 (see Note 7). Fill the tube with CBB, centrifuge at $300\times g$ for 2 min and then aspirate the supernatant. Repeat wash once.
16. Drain the IgG column (~1.5 mL) directly onto the prewashed calmodulin resin.
17. Wash the IgG column with 3-mL CBB and collect the wash into the tube containing the calmodulin resin. Expect to recover ~4.5 mL total.
18. Add 6 μ L 1 M CaCl_2 to the suspension and incubate the tube with gentle agitation for 1 h.
19. Transfer the suspension to a disposable column pretreated with 2% Tween 20. Reload flow through 3–5 times. Collect the flow-through.
20. Wash the column with 5–6 full column volumes with CBB with resin.
21. Close the bottom of the column. Apply 0.5 mL CEB to the resin and incubate for 10 min at room temperature with periodic gentle mixing to resuspend the resin. Collect the elution in a low-protein binding microcentrifuge tube. Repeat thrice (see Note 8).
22. Flash-freeze fractions in liquid nitrogen and store at -80°C .
23. To visualize the complexes, load ~10 μ L of eluted fractions onto an SDS-PAGE gel and stain the gel with Sypro Ruby.

Protein profiles of RECCs purified from RET2-mhTAP, RET2-iCODA-TAP, and RET2-iCODA/RNAi cells are indistinguishable (Fig. 3). Collect aliquots at all purification steps starting from total cell lysate. Quantitative Western blotting with anti-CBP antibody (GenScript) can be used to estimate purification yield for the bait protein, which is expected to be 25–30%. For mass spectrometry analysis, final fraction is precipitated by adding trichloric acid to 20% and deoxycholate to 0.1%, washed with acetone twice and digested with trypsin by standard protocols.

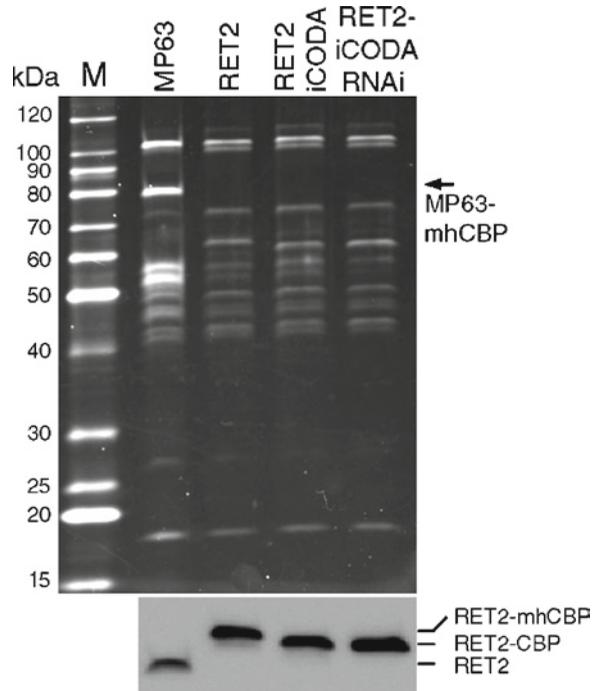


Fig. 3. Protein profiles of complexes purified from RET2 overexpression (mhTAP), RET2-iCODA-TAP, and RET2-iCODA/RNAi cell lines. (*Top panel*) TAP-purified complexes were separated on an 8–16% gradient SDS-PAGE gel and stained with Sypro Ruby staining. Cell lines are listed above the gel. mhCBP – 6His plus calmodulin binding peptide which remain on the tagged protein upon TEV protease release from the IgG resin. (*Bottom panel*) TAP-purified complexes were analyzed by Western blotting with anti-RET2 antibodies. Affinity purification of structural RECC subunit (MP63) was used to demonstrate gel mobility of the endogenous RET2.

4. Notes

1. Many commercial batches of FBS are contaminated with tetracycline. Some manufacturers (BD, Invitrogen) claim to have a tetracycline-free serum which does not always guarantee the leak-proof transcriptional control of RNAi expression. Apparently, mild to severe growth inhibition phenotypes can be induced in *T. brucei* RNAi cells by tetracycline present at levels undetectable with current testing methods. It is advisable to collect several serum samples and test for growth kinetics of an RNAi cell line in which an essential gene is targeted (available from the author's laboratory). Typically, serum supporting the fastest growth would be most suited for cloning procedures and maintenance of uninduced cells.

2. When preparing TCB, DTT should be added immediately before applying to the IgG beads.
3. Co-transfection of both TAP and RNAi constructs requires less time and is a default protocol. If obtaining clones resistant to all four drugs is unsuccessful, sequential transfection is recommended. First, transfect 29-13 cells with the protein expression construct (blasticidin resistance) and stabilize cells in a 10 mL of culture without clonal selection (~3 weeks). Second, transfect cells with the RNAi construct and proceed with clonal selection.
4. Incubation of the electroporated cells on ice longer than 5 min is not recommended. When performing multiple transfections, electroporations should be staggered to decrease the time between electroporation and inoculation into medium. If an electric arc occurs, expect to obtain fewer or no clones.
5. The medium color changes with cell growth from red at low density to yellow in the stationary culture. Cells can be ready for passage at 3–4 weeks posttransfection. Care should be taken to avoid oversaturated (yellow) cultures. Transfer at least 3–5 clones, but continue incubating the 96-well plate for a few more days to ensure lack of cell growth in wells with higher dilutions. If this occurs, discard previously collected clones and transfer new ones from the highest dilution wells.
6. For RNAi knockdown of essential genes, induced and mock-induced cell should be harvested at the same cell density. Typically, RNAi is induced at 10^6 cells/mL and the culture is maintained for 24–48 h; cell density should not exceed $5\text{--}6 \times 10^6$ cells/mL.
7. Pretreatment of plastic tubes and columns during the calmodulin binding step with 2% Tween 20 is recommended to improve purification yields. Pretreated plastic should be rinsed with large amounts of distilled water before use.
8. Fractions 1 and 2 should contain most of the eluted material. The first 50–100 μL of the Fraction 1 is the void volume and does not need to be collected.

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