p2T7^{TAblue}

For inducible RNA interference experiments in *T. brucei* (See Alibu *et al*., 2005).

RNAi is now the method of choice for loss-of-function studies in *T. brucei*. Inducible (conditional) expression allows a change in phenotype to be monitored in a single cell-line and also permits the ablation of proteins essential for growth.

- The plasmid was derived from p2T7TiB/GFP (LaCount *et al*., 2002).
- Primers: We use a software tool (RNAit – Redmond *et al*., 2003) for the selection of RNAi targets that provides primer information and minimises off-target effects (see Durand-Dubief *et al*., 2003).
- Should integrate into any *T. brucei* genome (ribosomal spacer) following digestion with *Not*I.

Upon integration into *T. brucei*, the construct lies between *RRNA* spacer sequences. RNAi targets are flanked by opposing *T7* promoters with downstream operators. The operators bind Tet-repressor in the absence of tetracycline so dsRNA is only synthesised when tetracycline (1 μg/ml) is added to the growth medium. *T7* terminators prevent transcription from extending into *RRNA*.

Key features

- Complete sequence available.
- Optimised for direct cloning of PCR products .
- *Hpa*I and *Bam*HI/*Xho*I sites allow alternative cloning strategies where desired and facilitate diagnostic digests.
- *LacZ* allows blue/white colour screening (vector/insert separation not required).
- All inserts can be sequenced using a 'universal' primer (see map).
- Hygromycin (*HYG*) for stable selection (*BLA* and *BLE* versions also available).
- Inducible expression using tetracycline (or analogues).
- Inducible cassette is independent of selectable marker.
- Modular nature allows components to be exchanged.
- Compatible with bloodstream (Single Marker Bloodstream form recommended: Wirtz *et al*., 1999 – see Fig. 7) or insect-stage (1313-1333 recommended: Alibu *et al*., 2005) *T. brucei* that express T7 RNAP and Tet-repressor.

New technology development

- Some leaky expression is seen from these vectors. Inducible T7 RNAP expression appears to solve this problem in insect stage cells (Alibu *et al*., 2005). We are establishing a similar system in bloodstream-form cells.
- These vectors transform bloodstream-form cells at a low efficiency (~10^{-7}) and can integrate in the genome at any one of several ribosomal spacer loci. Subsequent position effects may generate variable results. We are optimising a system to increase efficiency and eliminate position effects.

Other questions / comments, contact David Horn (david.horn@lshtm.ac.uk).

Protocols for the Generation of RNAi constructs

PCR – recommendations

- Use RNAit – (Redmond et al., 2003) for the selection of RNAi primers and targets.
  - o http://trypanofan.path.cam.ac.uk/software/RNAit.html
- Use MinElute PCR purification kit (Qiagen #28106). Elute in 30 μl of EB.
  [PCR products can be stored at -20°C].

Vector Preparation – for TA-cloning

*Eam*1105I sites that flank the LacZ "stuffer" are engineered to leave 3´ T overhanging ends.
Use a polymerase that generates 3'-A overhanging ends for TA-cloning (e.g. *Taq*).

1. For ~20 ligations, prepare the following in a 1.5 ml eppendorff: [Scale if necessary].
   - 4 μl p2T7^{TAblue} vector (stock = 1μg/μl)
   - 5 μl 10x reaction buffer (Fermentas)
   - 39 μl dH_2O
   - 2 μl *Eam*1105I (Fermentas)
2. Incubate at 37°C for 1-2 hrs (**NB:** *longer incubations may lead to damaged DNA ends and higher background*).
3. Check 5μl of the digested DNA on a 1% agarose gel (1xTBE / 0.1μg/ml ethidium bromide) with an uncut control.
4. Clean-up remaining digested vector using a MinElute Reaction Cleanup kit (Qiagen #28204).
   Elute in 20 μl of EB.
   [it is not necessary, or recommended to purify the vector backbone away from the LacZ stuffer].

Ligation

1. Add the following to 10μl of PCR product:
   - 1 μl *Eam*1105I digested p2T7^{TAblue}
   - 1.3 μl 10x ligase buffer (NEB)
   - 0.5 μl T4 ligase (NEB: #M0202S).
2. Incubate o/n at 4^{o}C.

E. coli Transformation - recommendations

Use one Shot DH5α cells (Invitrogen #12297-016) or prepare your own electrocompetent cells – we follow the protocol in *Current Protocols in Molecular Biology*.

1. Plate *E. coli* onto LB containing ampicillin, X-gal and IPTG and grow overnight.
2. Screen DNA from white colonies using *Hpa*I digestion and confirm by sequencing (see primer sequence on map).