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Multiplex analysis of RNA interference defects in *Trypanosoma brucei*

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Genome sequencing is now complete for a number of trypanosomatids [1] (see www.genedb.org) but functional analysis techniques that allow an increase in throughput are urgently needed if we are to fully exploit this data. RNA interference (RNAi) is a biological response to double-stranded RNA (dsRNA) that knocks down expression from complementary genes. RNAi has revolutionised research on gene function, been exploited as a high-throughput experimental tool [2] and is currently the method of choice for loss-of-function experiments in *Trypanosoma brucei.* We have established a simple quantitative RNAi barcode methodology that generates a read-out of relative cell number from a mixed *T. brucei* culture engineered for inducible RNAi. The RNAi target sequence, usually between 400 and 600 bp, is flanked by head-to-head inducible promoters and stably integrated into the genomic DNA of each cell type. Each target then serves as the template for dsRNA synthesis and as the barcode that can be amplified and labelled through multiplex competitive PCR with a single primer. The mixed barcode probe is then hybridised to specific sequences to produce the read-out. Proof-of-principle experiments demonstrate that the read-out can provide a quantitative report of relative drug resistance, relative RNAi-induced growth defects and RNAi-induced auxotrophy. Increased throughput, a range of alternative assay systems and read-out using microarray should all be compatible with the methodology.

A construct with head-to-head tetracycline inducible promoters is commonly used for RNAi experiments in *T. brucei* [3]. We routinely select specific gene targets using the RNAit software [4] (see http://trypanofan.path.cam.ac.uk/software/RNAit.html) that are then amplified by PCR, cloned in the p2T7^{TAblue} RNAi vector (Fig. 1A), which has been optimised for direct cloning of PCR products [5], and introduced into *T. brucei.* Throughput can be limited by laborious phenotype analysis and subtle defects can be difficult to confirm however. We have explored the use of RNAi barcode analysis to report relative proportions of bloodstream-form cells within mixed cultures.

First, a primer specific for the tetracycline operator was used in a competitive PCR reaction with three RNAi constructs with different sized targets as template. The PCR products, separated on an agarose gel, indicated efficient and specific amplification of all three inserts (data not shown). *T. brucei* clones with each of the three constructs integrated into the genome were then mixed in different proportions. Genomic DNA was extracted from the cultures and used as PCR template. One of the cell lines represented from 0.5 to 33% of the population and this ‘standard curve’ could be accurately reported by the relative amount of

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PCR product as quantified by phosphorimager analysis of Southern blots hybridised with a mixed target probe (Fig. 1B and 1C). Thus, RNAi targets can be used as molecular barcodes to assess relative cell number.

Using conventional 'growth-curve' analysis (data not shown), the three RNAi targets used above had distinct effects on cell growth when RNAi was induced. The largest target directed against telomerase (TERT) produces no apparent growth defect, the middle-sized target directed against a putative methyltransferase (MT) produces an intermediate growth defect and the smallest target directed against histone H3 produced a severe growth defect. To test whether the method outlined above can be used to report relative RNAi-induced growth defects, mixed cultures were grown for two or five days in the presence or absence of tetracycline prior to DNA extraction. Taken together, the PCR products and phosphorimager analysis of the Southern blot shown in Figure 1D clearly report the relative growth defects observed previously. H3 cells appear to be reduced to ~2% of non-induced after only two days (Fig. 1D, lower panel) and MT cells are clearly reduced relative to TERT cells after five days (Fig. 1D, upper panel). Thus, RNAi-induced differences in growth can be quantified in mixed cultures using multiplex barcode PCR. Importantly, phosphorimager analysis indicates no relative change in cell number after five days in the absence of induction showing that non-induced cells grow at similar rates and that growth differences brought about through RNAi are exquisitely tetracycline-dependent.

To explore the sensitivity of the technique we mixed small numbers of H3 cells with another RNAi cell line that displayed no apparent growth defect when RNAi is induced (MRE). Mixed cultures containing 1%, 0.1% and 0.01% H3 cells were induced for RNAi for two days. Multiplex PCR reveals MRE DNA (Fig. 1E, upper panel) while H3 signal is detected only on the Southern blot (Fig. 1E, lower panel). H3 cells at 0.1% of the culture were detectable and shown to be depleted following RNAi suggesting that a pool with a complexity of at least 1,000 could be effectively analysed using multiplex barcode PCR.

In *T. brucei*, the expression of a neomycin phosphotransferase (NPT) reporter is repressed when the gene is close to a *de novo* telomere formed adjacent to an *rDNA* promoter (DH, unpublished), so we tested whether multiplex PCR could be used to report loss-of-silencing and subsequent increased G418-resistance. Growth in 250μg of G418 for four days brings about more than 100-fold enrichment of cells with an *NPT* reporter 5 kbp distal to the telomere relative to cells with a similar reporter only 2 kbp from the telomere where NPT expression is significantly repressed (Fig. 1F, right-hand lanes). These data provide proof-of-principle for a multiplex assay for loss-of-silencing as assessed using G418 selection.

Next, we examined the ability of multiplex PCR to report RNAi-induced auxotrophy. Since ornithine decarboxylase (ODC) catalyses conversion of L-ornithine to putrescine, the precursor of the essential polyamines, spermine and spermidine, ODC null mutants are putrescine auxotrophs [6]. An irreversible inhibitor of ODC, α-difluoromethylornithine (DFMO), is used to treat African trypanosomiasis, so the effect of depleting ODC activity in *T. brucei* is of particular interest. An ODC RNAi cell line was constructed and mixed with cells containing RNAi target controls. The severe growth defect in the histone H3 cells described above provides a control for efficient RNAi-induction. The other two controls were directed against non-essential genes not thought to be involved in putrescine biosynthesis (MLH and MRE). All the cell lines were mixed in equal proportions and genomic DNA samples were prepared following different treatment regimes (Fig. 2A). The non-induced mixture or 'library' was also frozen for future analysis. This time, the multiplex PCR products derived from these samples were isotopically labelled and used as probes for hybridisation to identical dot-blots (Fig. 2A). Multiple targets of similar size can be analysed in parallel using this approach.

All the controls show the expected results (Fig. 2). H3 cells are severely depleted following induction, MLH and MRE are not affected by induction or by putrescine and there is no 'background' hybridisation to negative control DNA (RAD). In contrast, RNAi against ODC produces a growth defect that is alleviated by putrescine. An independent ODC RNAi clone was tested using a conventional growth-curve assay and also found to have an inducible growth defect that was only partially alleviated by putrescine at 0.125 or 1mM (data not shown). Interestingly, the current results suggest that an ODC deficit causes reduced fitness independent of exogenous putrescine. This effect was not seen in ODC null cells [6] and the difference may be explained by non-specific RNAi [7], complex interactions between intermediate levels of ODC and other factors or metabolic compensation during generation of the null mutant. Non-specific RNAi is unlikely since the RNAit software used to select the target sequence is designed to optimise specificity [4]. Whichever the case, putrescine dependency is clearly a consequence of ODC knockout [6] and RNAi (Fig. 2).

RNAi barcode analysis allows a read-out of relative cell number to be extracted from a mixed culture. When growth data are derived in this way, each experimental sample is internally controlled for fluctuations in the growth medium, other supplements or density sensing effects [8]. Proof-of-principle is demonstrated in that read-out reports relative drug resistance, RNAi-induced growth defects and RNAi-induced auxotrophy. Since any phenotype that affects relative cell number can be reported using this approach, it should be possible to report a range of additional phenotypes. This could be particularly important for trypanosomatids since 50-75% of the genes have no function inferred through bioinformatics analysis and are currently annotated 'hypothetical' (see www.genedb.org). Phenotypes that should be relatively easily reported include auxotrophy and altered response to drugs, chemicals, temperature or oxidative stress. Many functional screens are limited to selection of cells with increased resistance. Since inducible RNAi allows the study of defects incompatible with sustained growth, comparing RNAi barcodes from a mixed culture exposed to selective pressure with those from an unselected population can simultaneously reveal cells with increased resistance and those that are sensitised to selection. It should also be possible to establish systems to detect developmental defects and cell sorting could be used to enrich or deplete cells displaying other altered phenotypes. Fluorescence-activated sorting could be used if a fluorescent marker, sensor or antigen is affected or methods could be devised to sort cells that lose mobility through depletion of flagellar components for example. Similar approaches have been proposed to explore human gene function [2].

It should also be possible to significantly increase throughput. Although large scale RNAi approaches are currently limited in the bloodstream stage due to the low transfection efficiency, RNAi library screening has been carried out in insect-stage *T. brucei* cells [9]. Thus, a limited number of gene targets could be selected through bioinformatics and used in combination with a 'custom-array' or, limited to the insect-stage at present, a large library could be used for forward genetics by screening for phenotype using a whole genome microarray. In this case non-selected and selected barcode probes would be labelled with different fluorescent dyes. Where assay specificity and sensitivity is found to be insufficient for use with a highly complex culture, pooled screens would be appropriate while screening a whole genome RNAi library (complexity ~10,000) should produce useful data if the selection strategy is sufficiently powerful and the investigator is specifically interested in increased resistance. Cells expressing dsRNA specific for silencing factors may be sufficiently enriched from a highly complex culture by selection for loss-of-silencing for example (see Fig. 1D).

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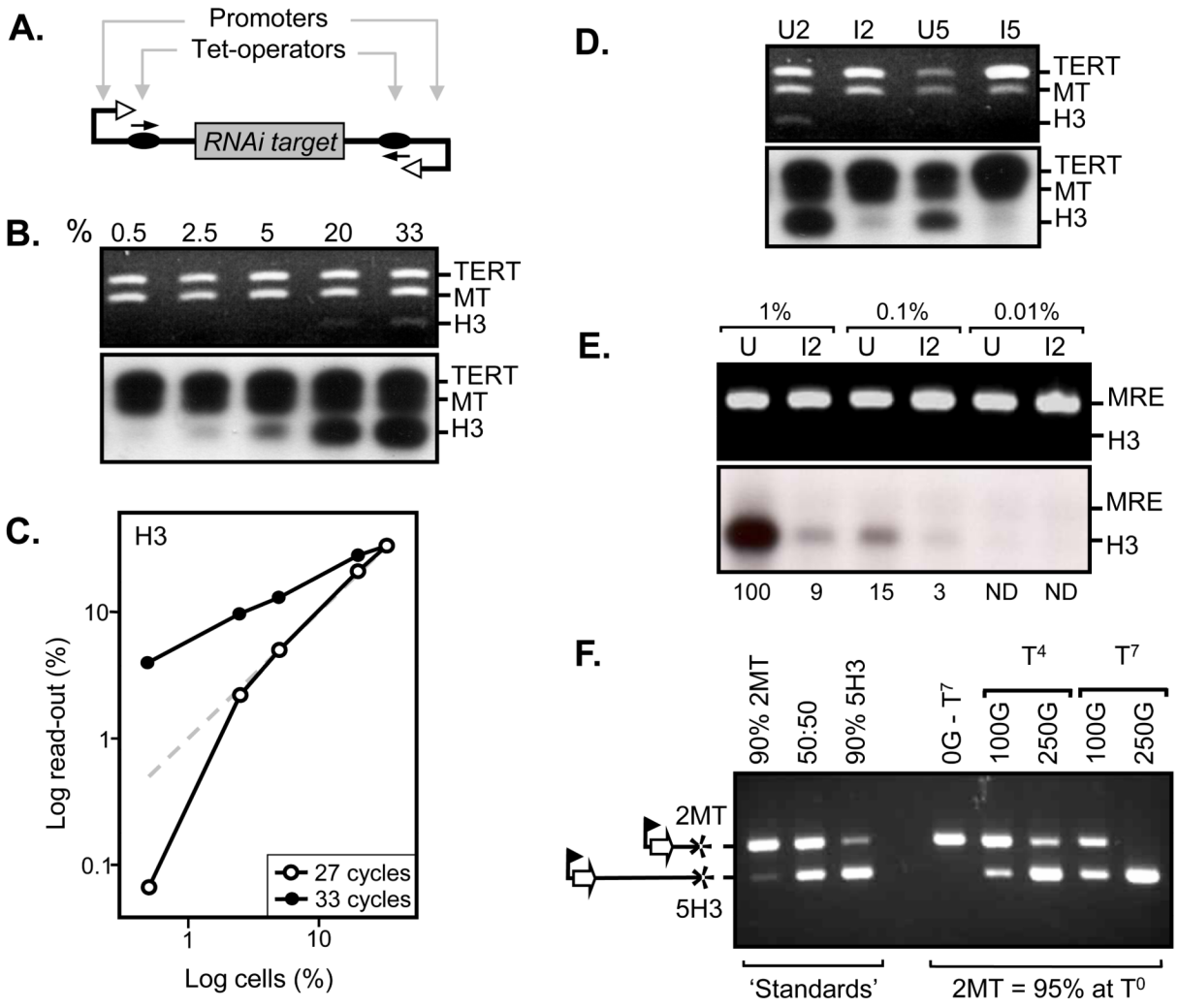

Fig. 1. Multiplex RNAi barcode analysis.

(A) The RNAi construct. Head to head T7 promoters (open arrows) generate dsRNA. Downstream operators permit tetracyclineinducible RNAi in cells expressing T7 polymerase and tetracycline repressor (T7:Tet^{R}). A single Tet-operator primer (closed arrows, TetOF: ctccctatcagtgatagagatc) is used to amplify RNAi barcodes by PCR. (B) RNAi targets were cloned in the RNAi construct and bloodstream form *T. brucei* T7:Tet^{R} cells [10] were cultured and transformed as described [11]. H3 (histone H3: CAD53128) cells were added in varying proportions to fixed numbers of TERT (putative telomerase) and MT (putative methyltransferase) cells. Genomic DNA was extracted using a DNeasy® Tissue Kit (Qiagen) and eluted in 50μl and PCR, using genomic DNA (1μl) as template, was set up in 50μl according to the manufacturer's instructions. Twenty-seven or 33 cycles were carried out at 94°C for 30s, 55°C for 30s and 72°C for 1min followed by 10min at 72°C. PCR-amplified barcodes were separated in a 1.5% agarose gel with ethidium bromide (upper panel). A Southern blot was hybridised with a mixed probe (lower panel) labelled using the Rediprime^{TM} II Random Prime Labelling System (Amersham). Data generated after 27 cycles is shown. (C) H3 signals were quantified by phosphorimager analysis (Molecular Dynamics) to generate standard curves. (D) DNA templates were from mixed cultures

grown for two or five days un-induced (U) or induced (I) with 1μg/ml tetracycline. PCR, 30 cycles. Other methods as above. (E) DNA templates were from MRE (MRE11: CAD20051) cells 'spiked' with 1-0.01% H3 cells un-induced (U) or induced with 1μg/ml tetracycline for two days (I2). PCR, 35 cycles. The Southern blot was hybridised with an H3 probe. Other methods as above. Phoshorimager values are shown below the blot. ND, not detectable. (F) Cells with *NPT* genes 2 and 5 kbp from *de novo* telomeres formed adjacent to *rDNA* promoters (DH, unpublished) were 'tagged' with MT and H3 constructs to generate 2MT and 5H3 cells respectively. Test cultures (right hand lanes) were grown in 0, 100 or 250μg/ml of G418 (G) for four (T^4) or seven (T^7) days. Genomic DNA extraction and PCR (30 cycles) were carried out as above.

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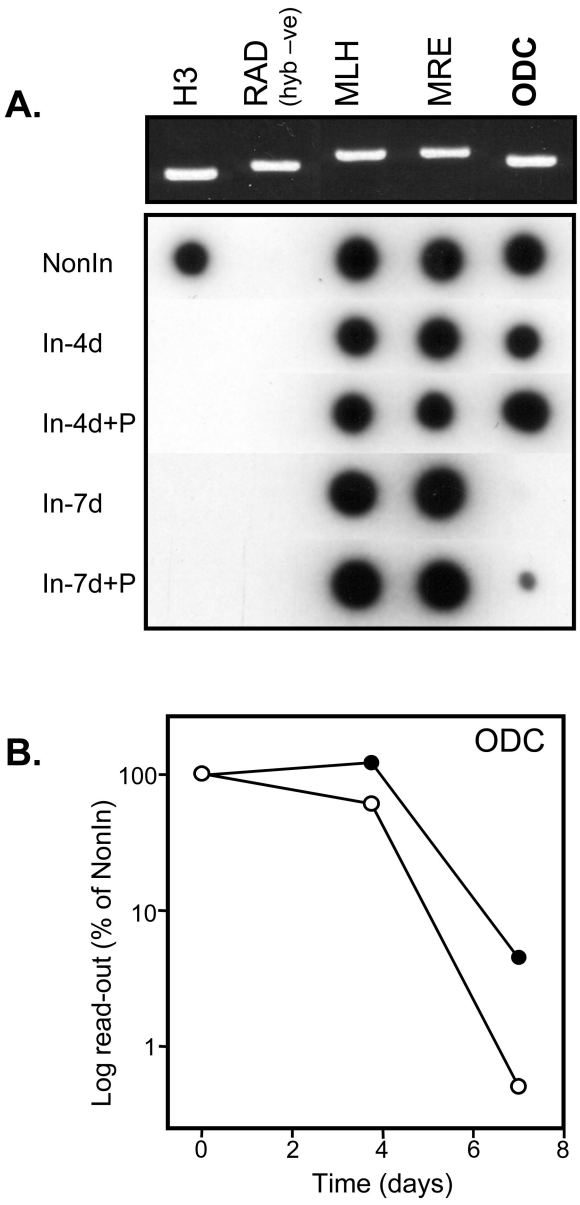

Fig. 2. RNAi barcode analysis of putrescine auxotrophy.

(A) H3, histone H3; RAD, RAD51 (AAD553433, RAD is the 'hybridisation' negative control target and was not present in the mixed culture); MLH, MLH1 (AAK29067); MRE, MRE11; and ODC (DCUTOB). Specific primers to ODC were ODC5: gtgacgaaggtgatccgttt and ODC3: gtttctttgcctgctccaag. The RNAi targets were generated through 30 cycles of PCR (see legend to Figure 1B) using genomic DNA from wild-type *T. brucei* cells as template (upper panel). PCR products were purified using a QIAquick® PCR Purification Kit (Qiagen), cloned in the RNAi vector and introduced into *T. brucei*. They were also used to prepare dot-blots (~100ng per dot) using a standard protocol [12]. One genomic DNA sample was prepared prior to induction (NonIn), a pair following induction for four or seven days (In-4d and In-7d) and a similar pair supplemented with 125μM putrescine (In-4d+P and

In-7d+P). Genomic DNA (0.5μl) was then used as PCR (30 cycles) template as outlined in the legend to Figure 1B except reaction volume was 10μl, dNTP concentration was 20μM and 0.25μl of ^{32}P-dCTP (Redivue #AA0005, Amersham) was added to each reaction. Unincorporated dNTPs were removed using Sephadex G50 (Sigma) gel filtration chromatography and the probes were hybridised to the blots according to a standard protocol [12]. Blots were washed in 0.4x SSC and 0.4% SDS at 65°C and exposed overnight. (B) ODC signals, quantified by phosphorimager analysis, were plotted against induction time. Empty circles, no supplement; filled circles, supplemented with putrescine.

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