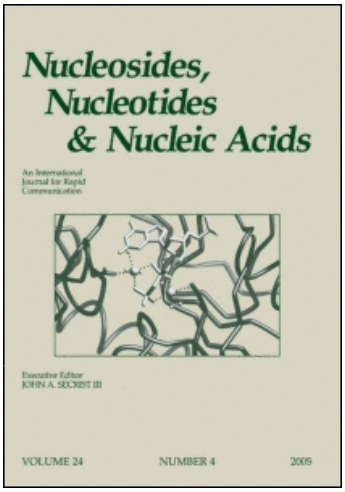

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Fast Rna-Rna Annealing in Vitro: Single Cycle Selection Versus Multiple Cycle Selection

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FAST RNA-RNA ANNEALING *IN VITRO*: SINGLE CYCLE SELECTION VERSUS MULTIPLE CYCLE SELECTION

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ABSTRACT Fast annealing of complementary RNA *in vitro* is related to effective antisense RNA-mediated regulation of gene expression and inhibition of viral replication in living cells. Pools of antisense RNA can be selected for species that anneal fast with a given target strand. In this study, we compare the technical and biological advantages and disadvantages of a single round selection assay with extensive multiple cycle selection for fast-annealing antisense RNA species.

The association of complementary ribonucleic acids (RNA) plays a crucial role in antisense regulation (1). In natural antisense-regulated systems such as plasmid replication in *E.coli* as well as in eukaryotic cells it has been shown that the rate of the annealing between antisense RNA and its target measured *in vitro* is related to efficacy in living cells (2-5). This observation offers a possibility to search for fast-annealing antisense species *in vitro* in order to identify effective antisense inhibitors for the application *in vivo*. Recently, a single round kinetic selection *in vitro* was established and was used to relate annealing rates with the extent of inhibition of the human immunodeficiency virus type 1 (HIV-1). At present, we perform a multiple cycle selection for fast-annealing antisense species out of pools that contain all possible antisense species against the mRNA encoding the chloramphenicol acetyltransferase with respect to size and location of the target sequence (Patzel and Sczakiel, unpublished). Initial pools subjected to multiple cycles of selection contain a substantially larger number of potentially effective antisense species and enable one to perform mutagenic steps resulting in '*in vitro* evolution'. A detailed comparison of both selection strategies will be discussed in the following.

The single cycle selection assay (5) which is schematically depicted in Fig.1. consists of essentially two steps: (i) the generation of a pool of antisense RNA species that are end-labelled at the same position and successively shortend at the other end followed by (ii) separating slow-annealing from fast-annealing species in the course of an annealing reaction by gelelectrophoresis under non-denaturing conditions and the subsequent analysis of both groups by gelelectrophoresis under denaturing conditions (Fig.1). After a single selection step, each antisense species can be unequivocally identified by gel analysis. It is noteworthy that differences in length of only a few nucleotides at the 3' end can dramatically influence the annealing kinetics (5,6).

The multiple cycle selection procedure is based on a pool of antisense species that contains the complete relevant sequence space, i.e. all possible antisense molecules with respect to length and local target position. The selection step is as described above. In addition, the antisense species require non-complementary sequence portions at their 5' and 3' ends for reverse transcription and amplification by PCR. Multiple rounds of selection and amplification can be performed such that mutations are introduced resulting in the creation of new species within the selected pools which is termed *´in vitro* evolution´. In this assay, fast annealing antisense species can only be identified by cloning of selected sequences and sequence determination like in the conventional SELEX protocol.

Both assays for selecting fast-annealing antisense species bear specific advantages and disadvantages that are summarized in Tab. 1. Briefly, the single cycle selection assay can be performed with little expense and does not include additional non-complementary sequences such as linkers or attachment sites for PCR primers which can influence the results of selection. It directly results in sequence information and the correlation between association rates and efficacy in living cells has been shown.

This correlation is also true for the multiple cycle assay (Patzel & Sczakiel, unpublished) but this assay requires significantly more technical skill. More importantly, the starting pools of antisense sequences of the multiple cycle assay contain a high sequence diversity and enable one to include mutagenic steps during amplification of selected sequences, i.e., evolution *in vitro* is possible.

In sum, both kinetic selection assays may lead to improved antisense inhibitors. If one assumes that the annealing between a ribozyme with its target also critically depends

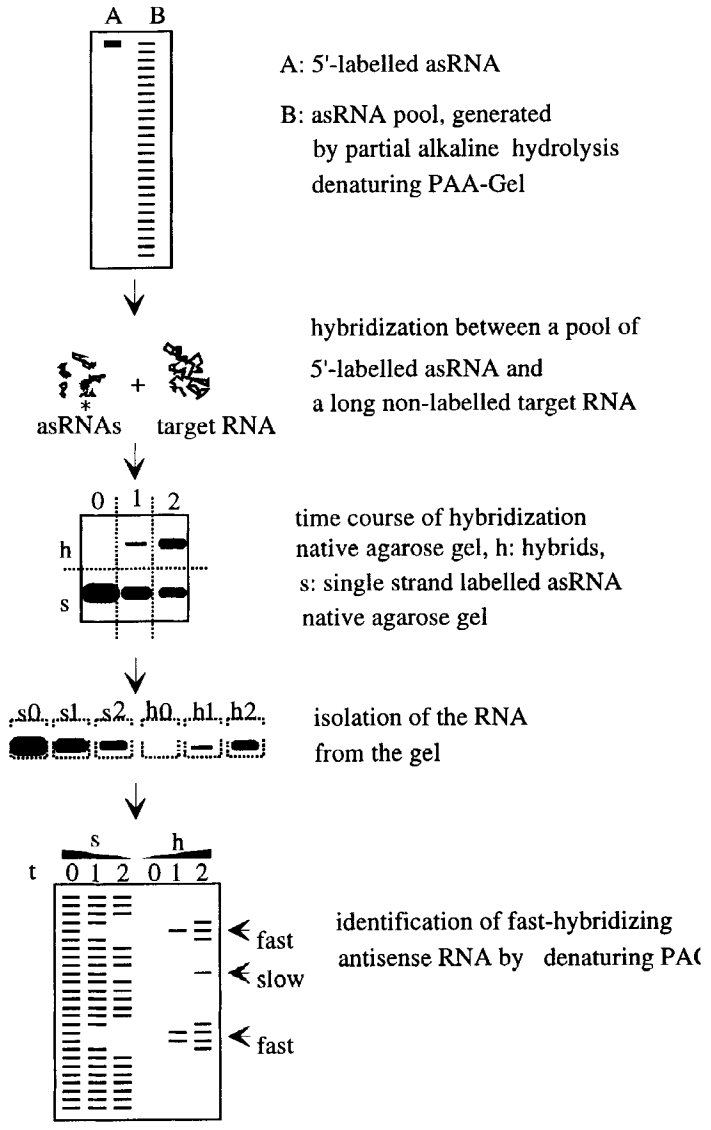

FIGURE 1: Single cycle selection for fast-annealing antisense RNA.

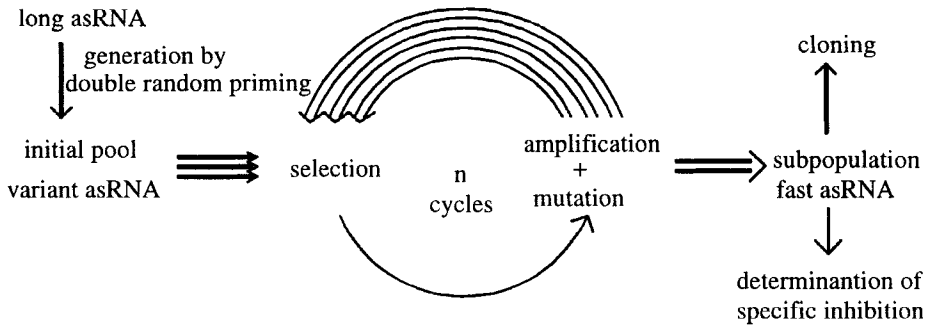

FIGURE 2: Multiple cycle selection for fast-annealing antisense RNA.

TABLE 1: A comparison of characteristics of single versus multiple cycle selection for fast-annealing antisense RNA.

selection protocol	single cycle	multiple cycle
proven correlation between annealing kinetics and efficacy	yes[a)]	yes[b)]
sequence diversity	low, ca 10^2	high, complete antisense sequence space is possible
'in vitro evolution'	not possible	possible
additional linker sequences	no	yes
information on annealing properties of antisense species	annealing data for all species	information only for selected species after cloning
technical complexity	low	very high

footnotes:
a) reference (5).
b) Patzel & Sczakiel, unpublished.

on an efficient (fast) annealing step, these assays can also be used to select for fast-annealing ribozymes (7).

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