Design, in vitro transcription, purification and crystallization of riboswitches.

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ABSTRACT

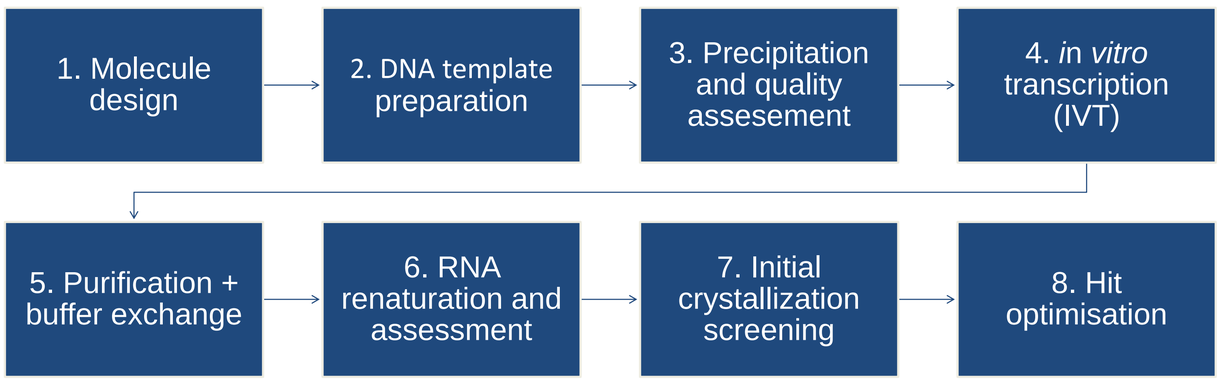
RNA is essential in various biological roles including coding, decoding, regulation and expression of genes. Some of these molecules hold an active role in protein synthesis (tRNAs, rRNAs), catalyze biological reactions (ribozymes), or sense and communicate responses to cellular signals (riboswitches).

Riboswitches are commonly found in 5'-UTRs (5′ untranslated regions also known as leader sequences or leader RNAs) in bacteria and they harness structural changes caused by the binding of small ligands (e.g. metabolites, amino acids or metal ions), which result in switching ON/OFF gene expression. Despite our growing knowledge about riboswitches, their sequence-structure-function relationships are usually vaguely known.

Although it is possible to predict, with high confidence, general structure for many RNA molecules, understanding of molecule's role often requires precise atomistic models obtained through experimental studies. Among various structural techniques, X-ray crystallography remains the major one for RNA structure determination. To obtain well diffracting crystals one requires, however, substantial amounts of pure and homogenous material. Moreover, it is not unusual to test dozens of variants before obtaining the one yielding the target RNA structure.

Here we present the protocol used in our laboratory for efficient and fast production of RNA molecules for crystallization trials. The protocol includes engineering of the RNA molecules (for enhanced crystallizability) based on secondary structure predictions, preparation of DNA templates by assembly PCR (Primerize), in vitro transcription (with secured homogeneity of RNA ends), denaturative UREA-PAGE purification, buffer exchanged, RNA renaturation and finally their crystallization and hits optimization.

Crystallography-grade short RNAs prodution:



1. Selection of homologues and target sequence design:

Modifying elements of structures for enhanced crystalizability - introducing local structural changes while minimalizing global ones. Folding predictions of secondary (RNA structure tools, PETfold) and tertiary structures (simRNA).

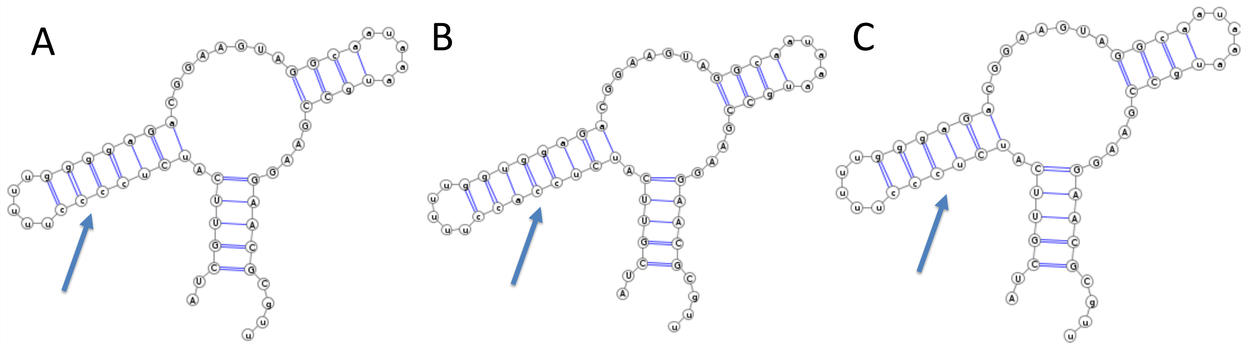


Fig 1. Predicted secondary structure of GlnA riboswitch with changes introduced to one of the non-conserved arms (indicated by an arrow). Consensus structure (A), possible g-quadrouplex structure broken by additional A-U pair (B), or removal of one G-C pair (C). Predictions obtained with IPknot.

2, 3. High homogeneity DNA template preparation:

Primerize (PCR Assembly Primer Design) - web server.

Algorithm creates overlapping F and R primers. Maximum of 6 primers in one reaction is recommended to avoid creating unspecified product. Primers contain modified nucleotides – last primer is 5' ended with two sugar-methylated nucleotides, assuring the homogeneity of the transcript 3' end [1].

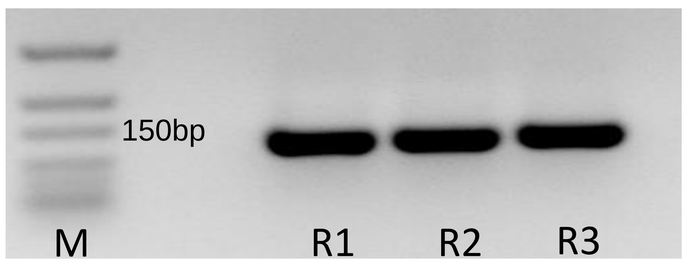
Fig 2. Template arrangement scheme. Primers F and R marked in blue and red respectively. Source: primerize.stanford.edu

Primerize is faster and more precise alternative to standard restriction-ligation. The reaction environment is tighter controlled as with bacterial cloning. Use of modified primers eliminates need of ribozyme sequence addition at the 3' end (such as HDV), an alternative for maintaining homogeneity of RNA's ends.

4, 5, 6. High purity RNA preparation:

- IVT conditions based on [2]:
  - T7 RNA polimerase (Thermo Sientific) – 600 U for 60 mg template DNA.
  - 10x buffer: 400 mM Tris-HCl pH 8.0, 100 mM Dtt, 20 mM spermidine, 0,1% Triton X-100, with additional 25 mM $MgCl_2$, 4 mM NTPs, 8 mM DTT.
  - Overnight at 37°C, optimal vol 1ml/tube (highest yield).
- Product purified using denaturative UREA-PAGE electrophoresis.
- RNA concentration and elution buffer replacement with crystallization buffer, performed as one step on Amicon® Ultra Centrifugal Filters (MERCK).
- RNA renaturation (apo form or with ligand) - 65°C for 5 min, 4°C for 30 min (overnight recommended), brought to room temperature.
- Final product assessment:
  - Spectrophotometric – concentration and contaminations presence.
  - Denaturing PAGE gel – purity.

Fig. 3. Visual assessment of purity and homogeneity of the final RNA product. Three separate GlnA constructs (R1, R2, R3) of increasing length.



7, 8. Sitting drop vapor diffusion crystallization:

- Initial screening:
  - RNA concentration of 4,5-6 g/l (~0,2mM).
  - 96 well crystallization plates with 30 µl well precipitate and drops with both 2:1 RNA:precipitate ratio (0,4 µl RNA) and 1:1 ratio (0,3 µl RNA).
  - Broad Screens: Index, Natrix, Crystal Screen (Hampton Research), Helix, LMB (Molecular Dimensions).

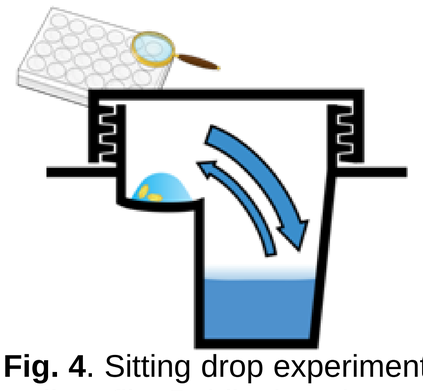


Fig. 4. Sitting drop experiment, well profile. Public domain.

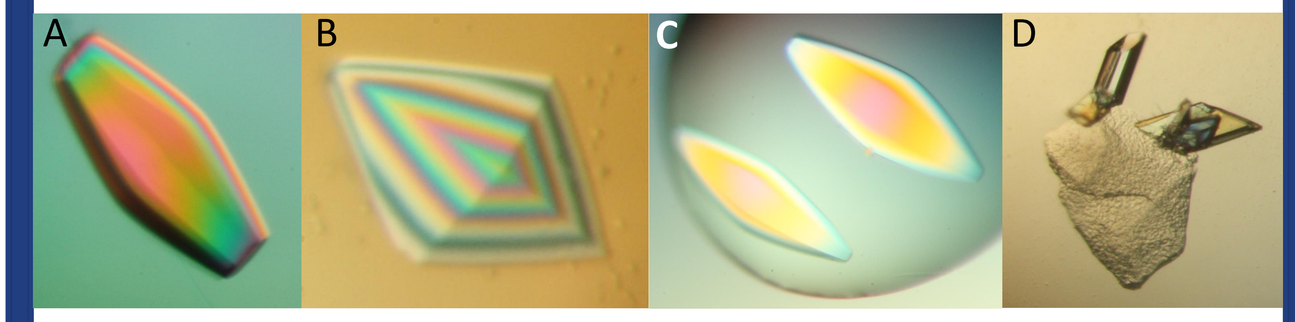


Fig. 5. Examples of in-house grown riboswitch crystals: initial screening results (A,B); crystals grown with no drop precipitate (C); Naomi's nucleants used in conditions previously yielding microcrystals (D).

- Hit optimisation:
  - Directed screens - BCS with PEG smears (Molecular Dimensions) and Ammonium Sulfate Suite (Hampton Research).
  - 2D field extensions, manually set up using high 96 well plates (100 µl of well sollution), ~1 µl total drop vol.
  - Additive screens - Angstrom, Morpheus Additive (Molecular Dimensions) and JBS+ (Jena Bioscience).

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[2] Edwards A. L., Garst A. D., Batey R. T. (2011). "Determining Structures of RNA Aptamers and Riboswitches by X-Ray Crystallography". Methods Mol Biol. 2009; 535: 135–163.

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