**Table of Content**



## 1. Chemicals and Reagents

**Table 1** — Reagents used in the experimental work (including the purpose of use and brand name or supplier)

| **Reagent** | **Purpose** | **Supplier** |
|---|---|---|
| Oligonucleotides | Cleavage reaction | DnaSynthesis (Moscow, Russia) |
| $H_2O$ RNAse/DNAse Free | Oligonucleotide dilution and buffer preparation | Belbiolab (Moscow, Russia) |
| EDTA | TBE buffer preparation | Helicon (Moscow, Russia) |
| Tris | TBE buffer preparation | Eurogen (Moscow, Russia) |
| Boric Acid | TBE buffer preparation | Helicon (Moscow, Russia) |
| Urea | Stop buffer and PAGE preparation | Diam (Moscow, Russia) |
| Bromophenol | Stop buffer | Chimmed (Moscow, Russia) |
| Acrylamide | Preparation of PAGE | Diam (Moscow, Russia) |
| Bisacrylamide | Preparation of PAGE | Helicon (Moscow, Russia) |
| APS | Preparation of PAGE | Helicon (Moscow, Russia) |
| TEMED | Preparation of PAGE | Helicon (Moscow, Russia) |
| RNase H | Cleavage reaction | TransGen (Beijing, China) |

## 2. Reaction buffers

Commercial 10x RNase H buffer was used (200 mM This-HCL pH 8.3, 150 mM DT, 1 M KCl, 45 mM $MgCl_2$) in cleavage reactions.

**Table 2** – Reaction termination buffer

| **Reagent** | **Concentration** |
|---|---|
| Urea | 7 M |
| TBE | 0.3 M |
| Bromophenol | 0.04 % |

## 2. Oligonucleotides sequences

**Table 3** - Oligonucleotide sequences used in the research

| **Oligo ID** | **Purpose** | **Sequence 5'→ 3'** |
|---|---|---|
| GFP RNA | Cleavage target | FAM-gcc acc uac ggc aag cu g acc cug aag uuc auc ugc acc acc ggc aag cug ccc gug ccc |
| KRAS (DNA) | Cancer marker mRNA fragment (synthesized as DNA) for DNA agents activation | GTT GGA GCT GGT GGC G TA GGC AAG AGT GC |
| miR-17 (RNA) | Cancer marker microRNA for DNA agents activation | caa agu gcu uac agu gca ggu ag |
| miR-20 (RNA) | Cancer marker microRNA for DNA agents activation | uaa agu gcu uau agu gca ggu ag |

| | | |
|---|---|---|
| ASOa | Part of the DNA agents that binds miR-17 | CTA CCT GCA CTG \|HEG\| GGG ACT TC |
| ASOb | Part of the DNA agents that binds miR-17 | A AGT AGA CGT \|HEG\| TAA GCA CTT TG |
| ASOa-Th | Part of the DNA agents that binds miR-17 | AGG TAG CT ACC TGC ACT GTA AGC ACT TTG CTA CCT GCA CTG \|HEG\| GGG ACT TC |
| ASOb-Th | Part of the DNA agents that binds miR-17 | A AGT AGA CGT \|HEG\| TAA GCA CTT TG CTA CCT GCA CTG TAA GCA CTT TG CAA AGT GCT TA |
| ASOa-KRAS | Part of the DNA agents that binds KRAS | GCAC TCT TGC CTA \|HEG\| CT TCA GGG |
| ASOb-KRAS | Part of the DNA agents that binds KRAS | TGC AGA TGA A \|HEG\| C GCC ACC AGC TCC AAC |
| ASOa-Th-KRAS | Part of the DNA agents that binds KRAS | TAG GCA AGA GCA CTC TTG CCT ACG CCA CCA GCT CAC TCT TGC CTA \|HEG\| CT TCA GGG |
| ASOb-Th-KRAS | Part of the DNA agents that binds KRAS | TGC AGA TGA A \|HEG\| C GCC ACC AGC TCC AAC G CAC TCT TGC CTA CGC CAC CAG CTC CAA C GCT GGT GGCG |
| ASO | Positive control | UGC AGA TGA ACT TCA GGG UC |

*N – DNA nucleotides*
*n - RNA nucleotides*
*N – underlined nucleotides with 2'-O-Me modification*
*HEG – hexaethilenglycol-spacer*
*FAM – Carboxyfluorescein*

**3. Results**

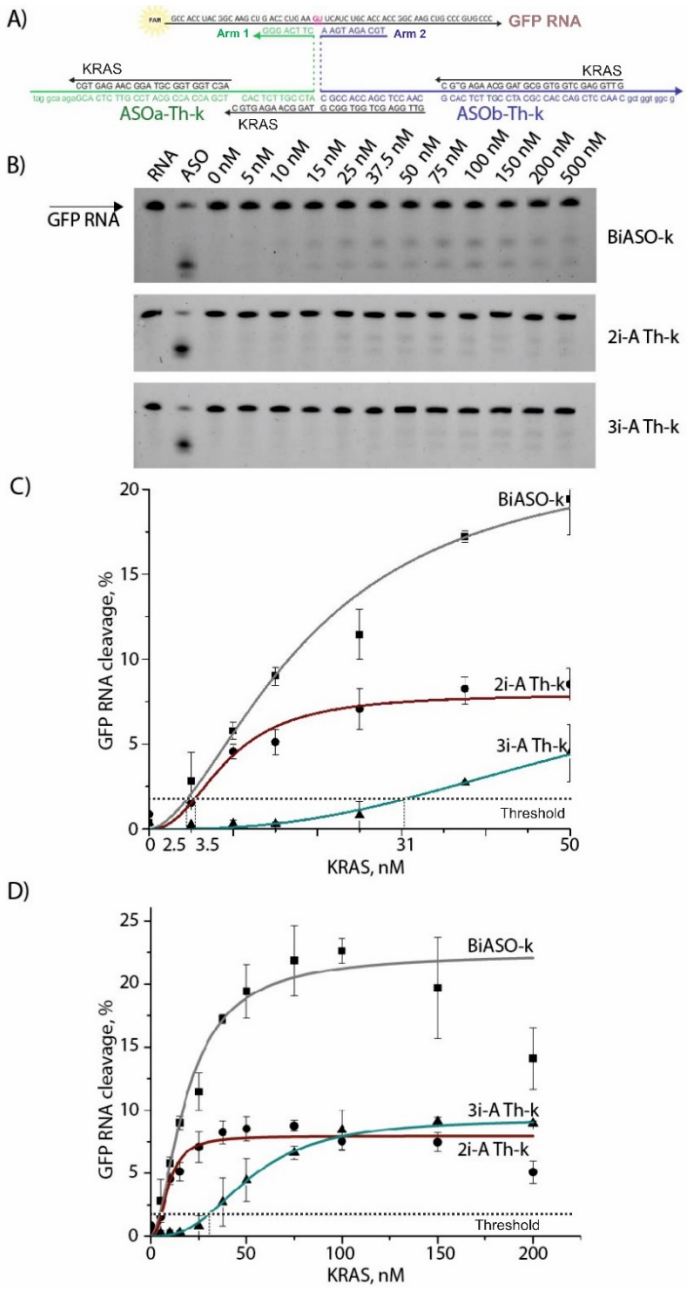


**Figure S1.** DNA thresholder activates RNase H-dependent targeted GFP RNA cleavage only in the presence of high concentrations of KRAS (see details Fig.S3). A)3i-A Th-k in complex with 3 molecules of KRAS and targeted GFP RNA (active form). B) Cleavage of GFP RNA (1000 nM) after incubation BiASO-k, 2i-A Th-k, 3i-A Th-k (50 nM) in the presence of various concentrations of KRAS (0-500 nM) with recombinant RNase H (0,5 unit) in commercial buffer (4,5 Mg2+) for RNase H at 37°C in 20 min. The cleavage was analysed by 15% denaturing PAGE (80V, 120 min). Black arrow indicates initial GFP RNA substrate, below are cleaved products. C) Quantification of three independent cleavage experiments. The threshold line set at 1.75%, which corresponds to three standard deviations above the background average (at 0 nM of KRAS for 3 DNA agents). D) GFP RNA cleavage efficacy (%) using different DNA agents in the presence of miR-17 (0-500 nM). The data are average values of 3 independent experiments with standard deviation.

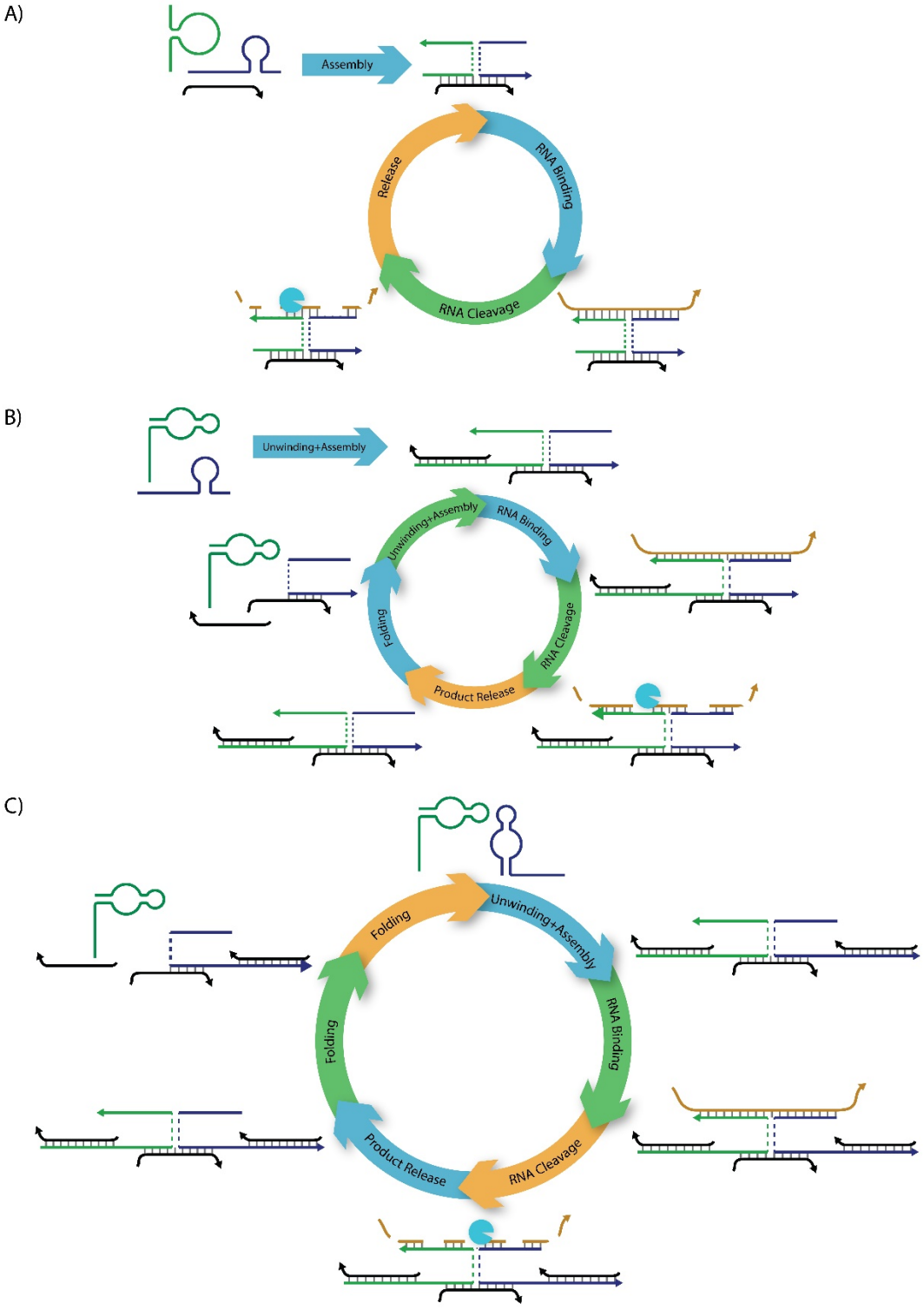


**Figure S2.** Schematic representation of the expected conformational changes of the DNA agents. Complexes of targeted mRNA (brown) and cancer marker RNA (black) with A) BiASO consisting of ASOa (green) and ASOb (blue), B) 2i-A Th consisting of ASOa-Th (green) and ASOb (blue), C) 3i-A Th consisting of ASOa-Th (green) and ASOb-Th (blue).

**Table 4** – Gibbs energy and Melting temperature of oligonucleotides folding and hybridization predicted by UNA Fold Web Server. Energy rules: 37 °C, [Na+]=165 mM, [Mg2+]= 4,5 mM, concentration – 50 nM.

| Oligonucleotides name | Melting temperature, °C | Gibbs energy, kcal/mol |
|---|---|---|
| *Nucleic acid folding* | | |
| GFP RNA | 52.8 | -5.5 |
| KRAS (DNA) | 46.0 | -1.2 |
| miR-17 (RNA) | 54.4 | -2.6 |
| miR-20 (RNA) | 54.4 | -2.6 |
| ASOa | 34.3 | 0.2 |
| ASOb | 26.6 | 0.8 |
| ASOa-Th | 56.2 | -4.4 |
| ASOb-Th | 67.1 | -10.3 |
| ASOa-KRAS | 44.6 | -0.5 |
| ASOb-KRAS | 42.7 | -0.7 |
| ASOa-Th-KRAS | 69.2 | -8.6 |
| ASOb-Th-KRAS | 71.6 | -13.7 |
| ASO | 29.3 | 0.5 |
| *Nucleic acids hybridization* | | |
| ASOa + miR-17 (RNA) | 43.3 | -12.9 |
| ASOb + miR-17 (RNA) | 33.2 | -10.2 |
| ASOa + miR-20 (RNA) | 40.8 | -12.3 |
| ASOb + miR-20 (RNA) | 32 | -9.9 |
| ASOa-Th + miR-17 (RNA) | 64.6 | -26.4 |
| ASOb-Th + miR-17 (RNA) | 64.6 | -26.4 |
| ASOa-Th + miR-20 (RNA) | 59.2 | -22.5 |
| ASOb-Th + miR-20 (RNA) | 59.2 | -22.5 |
| ASOa-KRAS + KRAS (DNA) | 48.4 | -14.7 |
| ASOb-KRAS + KRAS (DNA) | 61.7 | -21.0 |
| ASOa-Th-KRAS + KRAS (DNA) | 74.5 | -34.6 |
| ASOb-Th-KRAS + KRAS (DNA) | 76.6 | -38.4 |

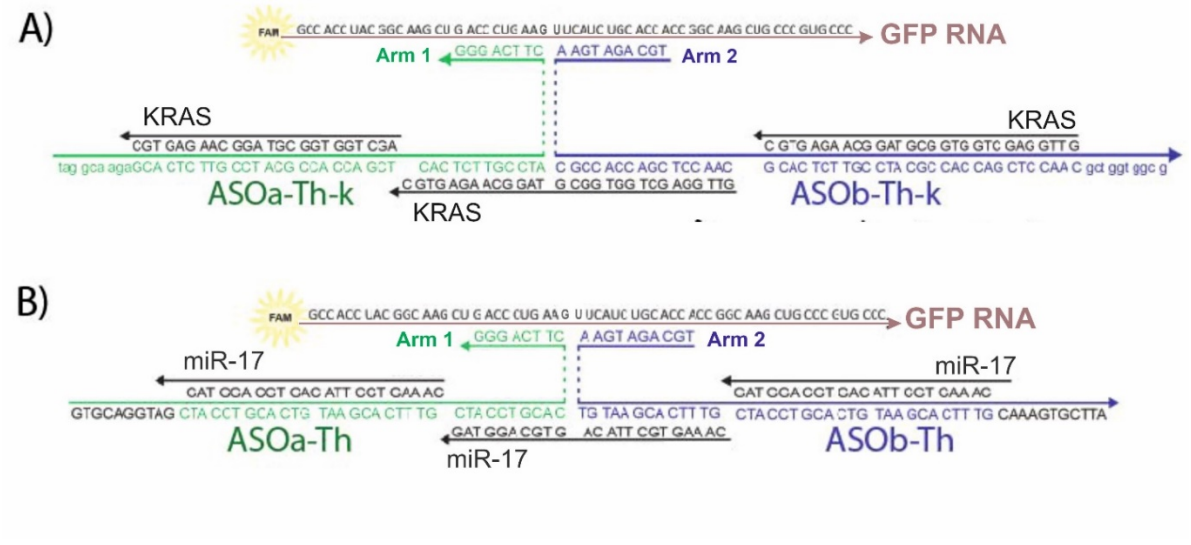


**Figure S3.** 3 input Antisense Thresholder design. (A) 3i-A Th-k in complex with 3 molecules of KRAS and targeted GFP RNA (active form). (B) 3i-A Th in complex with 3 molecules of miR-17 and targeted GFP RNA (active form).