

# Development of an automated *in vitro* selection protocol to obtain RNA-based aptamers: identification of a biostable substance P antagonist

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## ABSTRACT

**We have developed an automated SELEX (Systematic Evolution of Ligands by EXponential Enrichment) process that allows the execution of *in vitro* selection cycles without any direct manual intervention steps. The automated selection protocol is designed to provide for high flexibility and versatility in terms of choice of buffers and reagents as well as stringency of selection conditions. Employing the automated SELEX process, we have identified RNA aptamers to the mirror-image configuration (D-peptide) of substance P. The peptide substance P belongs to the tachykinin family and exerts various biologically important functions, such as peripheral vasodilation, smooth muscle contraction and pain transmission. The aptamer that was identified most frequently was truncated to the 44mer SUP-A-004. The mirror-image configuration of SUP-A-004, the so-called Spiegelmer, has been shown to bind to naturally occurring L-substance P displaying a  $K_d$  of 40 nM and to inhibit ( $IC_{50}$  of 45 nM) L-substance P-mediated  $Ca^{2+}$  release in a cell culture assay.**

## INTRODUCTION

*In vitro* selection of nucleic acids, SELEX (Systematic Evolution of Ligands by EXponential Enrichment), is a powerful evolutionary selection method for identifying oligonucleotides with new properties from combinatorial libraries (1). Oligonucleotides with target-binding properties are called aptamers (2). In terms of affinity and specificity, aptamers are comparable with monoclonal antibodies (3,4). The SELEX process involves multiple rounds of alternating selection and amplification steps in order to successively enrich aptamer sequences that show the desired properties. In each round, the nucleic acid library is contacted with the target,

non-binding molecules are discarded, and binders are amplified by PCR; if SELEX is carried out with RNA, additional steps of reverse transcription and enzymatic RNA synthesis are needed to close each SELEX round. Most enzymatic reaction steps as well as the isolation of binding molecules are followed by purification procedures. Typically, between 8 and 20 selection rounds are necessary until no further enrichment of functional nucleic acid species is detectable, making the whole process time-consuming and tedious. The first automated selection protocol based on a Biomek 2000 pipetting robot (Beckman Coulter) was published in 1998 (5), followed by modifications and improvements (6–8). Other suggestions for a parallel processing of selection experiments were made by Drolet *et al.* (9). The reported procedures are primarily designed for the high-throughput generation of aptamers, and for this reason, complex and sophisticated selection protocols are difficult to implement on these automated systems. We have developed an automated selection protocol that is designed to be as close as possible to manual *in vitro* selection. Special emphasis was placed on flexible routines that offer the possibility to adjust stringency or vary incubation times as well as on an online monitoring of the amplification step. Adjustable parameters and online monitoring allow for a very reliable process for the identification of highly affine aptamers.

Substance P served as an appropriate target for demonstrating the robustness of our automated *in vitro* selection process. Substance P is an 11 amino acid peptide that belongs to the tachykinin family. It is released from both the central and peripheral endings of primary afferent neurons and acts as a neurotransmitter. The peptide was first identified by bioassays as early as 1931, and was one of the most extensively studied bioactive substances during the half-century since its discovery. Surprisingly, the amino acid sequence was not determined until 1971 (10–12). Substance P may be considered as the prototype of the tachykinins, neurotransmitters that have been implicated to have a wide variety of biological activities, such as peripheral vasodilation, smooth muscle contraction, pain transmission (nociception), activation of the immune system and neurogenic inflammation (12). Mammalian

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tachykinins known to date include substance P, neurokinin A (neurokinin  $\alpha$ , neuromedin L, substance K) and B (neurokinin  $\beta$ , neuromedin K), and hemokinin 1 as well as the recently discovered endokinins A, B, C and D, which are apparently translated from four splice variants of the human TAC4 gene (13). Neurokinin A is also present in two elongated forms, neuropeptide K and neuropeptide- $\gamma$ . Three tachykinin receptor types could be identified, all of which are G-protein-coupled: NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub>. Substance P, hemokinin 1 and endokinin A and B show preference for NK<sub>1</sub> (13–15), neurokinin A for NK<sub>2</sub> and neurokinin B for NK<sub>3</sub>. However, these tachykinins are not highly selective and can act on all three receptors. Endokinin C and D have only weak activity at the known receptors, indicating that their receptor(s) remain to be elucidated (13).

Substance P has been the target of an *in vitro* selection earlier, resulting in an RNA aptamer with a dissociation constant of 190 nM (16). In order to obtain a potent nuclease-resistant, oligonucleotide-based substance P antagonist with potential therapeutic value, a mirror-image selection process was employed (17,18). For this purpose, first a regular nucleic acid library is used to isolate aptamers that bind to the D-enantiomer of the naturally occurring peptide target. Subsequently, the aptamer sequence is synthesized in its mirror-image configuration, resulting in an L-RNA ligand, a so-called Spiegelmer. Following the principles of chirality, the Spiegelmer binds to the naturally occurring L-peptide target, in this case substance P.

In this paper, we describe the automated generation of a highly affine substance P antagonist that is based on a mirror-image RNA sequence. The antagonist was biophysically characterized and able to effectively inhibit substance P-mediated Ca<sup>++</sup> mobilization in cells expressing the NK<sub>1</sub> receptor.

## MATERIALS AND METHODS

### Materials

The initial single-stranded DNA (ssDNA) library, primers, truncated aptamers and Spiegelmers were synthesized using standard phosphoramidite chemistry. A combinatorial RNA library of  $\sim 10^{15}$  different molecules was obtained by enzymatic conversion of 3 nmol of a synthetic DNA library (ssDNA pool, 3'-CACCTTGCTGAGTGGACTCGCACG-N<sub>34</sub>-CGT-GCGACGACAACAGATTTCGAGG-5'; forward primer with underlined T7 promoter, 5'-TCTAATACGACTCACTATAG-GAGCTTAGACAACAGCAG-3'; reverse primer, 5'-GTGG-AACCGACTCACCTGAG-3') into double-stranded DNA (dsDNA), which was then transcribed into RNA using T7 RNA polymerase (5'-GGAGCUUAGACAACAGCAGCGU-GC-N<sub>34</sub>-GCACGCUCAGGUGAGUCGGUCCAC-3'). This starting pool was initially labelled with [ $\alpha$ -<sup>32</sup>P]GTP during *in vitro* transcription. All L- and D-peptides were obtained from BACHEM AG, Switzerland (Table 1). For *in vitro* selection, D-substance P with C-terminally attached biotin was used. For capture of D-substance P-biotin•RNA complexes, NeutrAvidin Agarose and Streptavidin Ultralink particles (Pierce) were used. PicoGreen for dsDNA assessment during semi-quantitative PCR was purchased from Molecular Probes (Eugene, OR). The non-functional control Spiegelmer used in isothermal titration calorimetry (ITC) measurements and

**Table 1.** Peptides used in the study

Peptide	Chirality	Sequence
Substance P-biotin	D	RPKPQQFFGLM-biotin
Substance P	L	RPKPQQFFGLM-NH <sub>2</sub>
Substance P1-7	L	RPKPQQF-OH
Substance P5-11	L	QQFFGLM-NH <sub>2</sub>
Neurokinin A	L	HKTDSFVGLM-NH <sub>2</sub>

cell-culture assays had the sequence 5'-UAAGGAAACUCG-GUCUGAUGCGGUAGCGCUGUGCAGAGCU-3'.

### Robot configuration and software

All automated liquid and mechanical manipulations were carried out using a RoboAmp 4200 E instrument (MWG Biotech, Ebersberg, Germany). The robot was augmented with cooled and heated storage racks and workstations, a hotel for storage of microtiter plates and a custom-designed double vacuum manifold for partitioning and RNA purification, respectively. In order to avoid cross-contamination between samples, sample evaporation during prolonged incubation times or storage and dilution of cooled reagents by condensing water, the robot was equipped with the NCC ('non-cross-contamination') system from MWG, which consists of plasticware with individual lids on each well and an automatic lid manipulator. Details are depicted in Figure 1A.

The whole robot with its periphery was placed under a temperature-controlled hood, which allowed performance of the selection process at 37°C (Figure 1B). The script controlling the robot was written in Robomanager version 4.0.8 (MWG Biotech).

### Initial selection rounds

The maximum amount of matrix material for immobilization of biotinylated peptide that can be handled on the automated platform is  $\sim 10$   $\mu$ l, which allows for up to 10  $\mu$ M target concentration in our system. We decided to start the selection with a target concentration of 30  $\mu$ M; accordingly, the initial rounds were performed manually. Manual selection rounds were carried out as outlined below, with the following differences: round 1 was at 25°C; elution was not executed by thermal denaturation, but by incubating the matrix with captured substance P•RNA complexes in 4 M guanidine isothiocyanate twice for 10 min at 37°C and 65°C, respectively. Combined eluates were phenol–chloroform extracted and ethanol precipitated. RT-PCR was run with Superscript II Reverse Transcriptase, and for PCR, *Taq* DNA polymerase was used (Invitrogen, Karlsruhe, Germany). Finally, transcripts were purified by denaturing PAGE.

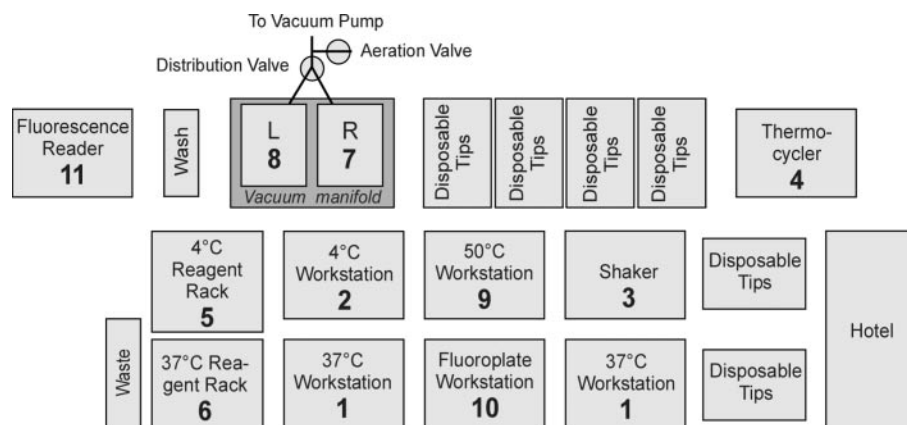
### Process of automated *in vitro* selection

During automated *in vitro* selection, most liquid handling manipulations were performed in 96-well plates with removable lids; partitioning of unbound from bound RNA species was carried out in Mobicol columns (MoBiTec AG, Göttingen, Germany); for automated transcript purification, Microcon ultrafiltration units were employed (Millipore, Eschborn, Germany). Automated fluorescent measurements for

A



B



**Figure 1.** The selection robot. (A) Picture of the RoboAmp robot with accessories under a temperature-controlled hood. (B) Schematic work surface used for the selection; numbers refer to the participation of workstations during the selection as depicted in Figure 2B.

semi-quantitative PCR were processed in black 96-well flat-bottom plates (Costar).

*Input used for the automated selection.* RNA for the first automated selection round (round 4) was obtained after three manual selection rounds. Aliquots containing 250 pmol of this RNA were used as input in the binding reactions of round 4 and 5, 200 pmol for round 6 and 7, and 100 pmol in every further round.

*Denaturation of RNA.* All non-enzymatic selection steps except denaturation of the RNA before contacting with the target molecule (biotinylated D-substance P) were performed in selection buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 0.1% Tween-20). RNA denaturing and re-folding was achieved by incubating at 95°C for 5 min in 57 µl selection buffer without CaCl<sub>2</sub> and

MgCl<sub>2</sub>. After denaturation, the RNA was quickly cooled to 4°C for 2 min and then equilibrated at 37°C. CaCl<sub>2</sub> and MgCl<sub>2</sub> were added to final concentrations of 1 mM each, and the mixture was incubated at 37°C for RNA folding.

*Partitioning of bound from unbound RNA.* Following denaturation, the RNA was contacted for 15 min at 37°C with 10 µl of the selection matrix (NeutrAvidin agarose or NeutrAvidin UltraLink) without peptide. This so-called pre-selection was designed to remove potential matrix-binding RNA species from the library. The RNA of the supernatant was separated from the matrix by transferring it to fresh wells on the same plate. Different amounts of target were added to the RNA to set up three different binding reactions. A fourth sample of the RNA pool was contacted with binding buffer only and served as a control reaction. After thorough mixing by pipetting up and down, the binding reactions were incubated for 60 min.

Capture of substance P•RNA complexes was achieved by adding biotin capture matrix and shaking the plate for 7 min. The mixture was transferred into column-like plastic tubes with an outlet and a plastic frit, which had been fitted into vacuum manifold A. By applying gentle vacuum, the liquid was separated from the matrix with bound substance P•RNA complexes. The matrix particles were washed with selection buffer, followed by its removal under vacuum. Depending on the selection round, between 2 and 15 wash cycles were performed, which correspond to 18–135 column volumes. The washed matrix particles were resuspended in 120 µl RT-PCR buffer (Qiagen One Step RT-PCR kit) and transferred into fresh wells of the 96-well plate. The RNA was eluted from the matrix by incubating the plate for 15 min at 95°C.

*RT and semi-quantitative PCR.* Prior to the RT reaction, the 96-well plate was transferred to the 50°C workstation to equilibrate the reaction mixture. According to the manufacturer's protocol, RT-PCR enzyme mixture was injected into the wells and incubated for 20 min at 50°C. The plate was then transferred to the thermocycler, incubated for further 10 min at 60°C, and 15 min at 95°C. Thermocycling was processed for 30 s at 95°C, 30 s at 63°C and 30 s at 72°C, with the last polymerization step extended to 2 min. The plate was transferred to the 50°C workstation after cycles 7, 10, 13 and 16. Aliquots containing 3 µl of each reaction were mixed with 180 µl of a 1:400 dilution of PicoGreen (Molecular Probes) in TE buffer, pH 8 (10 mM Tris-HCl and 1 mM EDTA), which was previously filled into a black 96-well microtiter plate. PicoGreen is a fluorescent dye that shows only weak fluorescence when free in solution. If the dye binds to dsDNA, it fluoresces strongly (ex: 485 nm; em: 520 nm). Measurement of fluorescence in comparison with a blank lacking the thermostable polymerase allows a satisfactory estimation of the PCR progress. After reaching the set threshold (fluorescence intensity with polymerase/fluorescence intensity without fluorescence >2), the respective reaction was automatically removed from the plate and pipetted into a storage plate at 4°C before the reaction plate was transferred back to the thermocycler again. Aliquots of the archived dsDNA were used for *in vitro* transcription without further purification.

*In vitro transcription and transcript purification.* Aliquots containing 30 µl of the RT-PCR were added to 116 µl T7 mastermix, which was previously filled into fresh wells of the reaction plate (final concentrations in the T7 reactions, 80 mM HEPES, pH 7.5, 22 mM MgCl<sub>2</sub>, 1 mM spermidine, 10 mM DTT, 4 mM GTP, ATP, CTP and UTP, 80 µg/ml BSA) not accounting for the reagents contained in the RT-PCR aliquot. The reactions were started by the addition of 3 µl 50 U/µl T7 RNA polymerase (Stratagene, Heidelberg, Germany) plus 1 µl RNaseOut RNase inhibitor (Invitrogen) and incubated for 180 min at 37°C. The dsDNA template was then digested with ~1 U DNase I for 15 min at 37°C, followed by heat denaturation for 5 min at 95°C in the thermocycler. The transcription reactions were then transferred to Microcon ultrafiltration units which were fitted onto vacuum manifold B. By application of vacuum, the membrane was then sucked dry. H<sub>2</sub>O was added to remove residual low molecular weight impurities, and after 40 min, the vacuum was switched off.

Finally, the retained RNA was resuspended in 100 µl water. For the next round, 10 µl of the RNA was used, and the rest was archived on the 4°C workstation.

### Pull-down assays

Pools resulting from different selection rounds as well as individual clones were ranked in respect of their binding behaviour to biotinylated D-substance P. For this purpose, the respective dsDNAs were transcribed in the presence of [ $\alpha$ -<sup>32</sup>P]GTP. Aliquots containing 2–5 pmol of the radiolabelled RNAs were denatured and refolded as described above. The oligonucleotides were then incubated in a volume of 1 ml selection buffer for 1 h at 37°C with 30, 100 and 300 nM biotinylated D-substance P. Subsequently, 10 µl of NeutrAvidin agarose was added for immobilization and was incubated under constant shaking for further 10 min. The matrix with bound peptide•RNA complexes was then separated from the supernatant, the matrix was washed with selection buffer, and the radioactivity was determined in both fractions. From the measured values, the no peptide control was subtracted as background.

### Isothermal calorimetry

For calorimetric measurements, a VP-ITC Microcalorimeter (MicroCal, Northampton, MA) was used. The freshly denatured Spiegelmer and the ligand solutions were degassed in selection buffer at 37°C; the Spiegelmer was loaded into the instrument's sample cell at 10 µM (cell volume, 1.4 ml), and the ligands were filled into the injection syringe at 50 µM (syringe volume, 0.25 ml). Each injection of ligand solution was 6 s long, with 5 min between injections for complete equilibration and baseline recovery. The first injection had a volume of 3 µl and the following were 7.5 µl each.

### Determination of Spiegelmer inhibitory activity with calcium mobilization assays

AR42J pancreatic cells were seeded with  $6 \times 10^4$  cells per well in a black 96-well plate with clear bottom (Greiner) and cultivated overnight at 37°C and 5% CO<sub>2</sub> in DMEM supplied with 20% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Spiegelmers were incubated with 3 nM substance P in HBSS, containing 20 mM HEPES, 1 mg/ml BSA, 5 mM probenecid (HBSS+), in a 0.2 ml low profile 96-well plate for 15–60 min at room temperature or at 37°C. After washing with 200 µl HBSS+, 50 µl of the calcium indicator dye fluo-4 (10 µM fluo-4, 0.08% pluronic 127 [both Molecular Probes] in HBSS+) were added to the cells and incubated for 60 min at 37°C. Thereafter, cells were washed three times with 180 µl HBSS+. Finally, 90 µl HBSS+ were added per well.

Measurement of fluorescence signals was accomplished at an excitation wavelength of 485 nm and an emission wavelength of 520 nm in a Fluostar Optima multidetection plate reader (BMG, Offenbach, Germany). For parallel measurement of several samples, wells of one (perpendicular) row of a 96-well plate were recorded together. First, three readings with a time lag of 4 s were carried out to determine the base line; the recording was then interrupted and the plate was moved out of the instrument. Using a multi-channel pipette, 10 µl of stimulation solution (L-substance P preincubated with

various concentrations of Spiegelmer) was added to the wells. The plate was then moved back into the instrument and the measurement continued. In total, 30 recordings with time intervals of 4 s were performed. For each well, the difference between maximum fluorescence and base line value was determined and plotted against L-substance P concentration or, in the experiments on the inhibition of L-substance P-induced calcium release by Spiegelmers, against the Spiegelmer concentration.

## RESULTS

### Development of an automated *in vitro* selection protocol

It is well known from the literature that manual selection schemes are able to yield high-quality aptamers. Therefore, the system specifications of the robotic workstation for automated *in vitro* selection were set to be as close as possible to the manual procedures. With this regard, three major problems had to be tackled.

*Prevention of cross-contamination during the selection.* Cross-contamination could be best minimized by using the MWG RoboAmp robot with its NCC system. A special manipulator opens and closes individual lids on the plasticware used. Thus, only one single well on the whole robotic work surface is open at any given time. During thermocycling, no reusable rubber sealing mat (a major source of possible cross-contamination) or sealing foil is necessary. NCC-compatible, lidded reservoir tubes for reagent storage are also available, which prevent liquid loss or, even worse, dilution of cooled enzymes by condensing water.

*Amplification control.* Amplification is the part of an *in vitro* selection round, which optimally just increases the number of selected RNA species to an amount that can be used as input into the next round. In practice, however, the enzymes that are involved in this process exert additional, unwanted selective pressure. This frequently leads to the rise of amplification artefacts that are enriched not owing to their affinity to the target, but because they are good amplification substrates. We have developed a fully automated semi-quantitative PCR protocol, which not only effectively prevents overcycling during PCR, but also supplies the researcher with valuable insight into the status of the selection, thus allowing for optimal adjustment of selection parameters for the following round. For this purpose, thermocycling is interrupted three times after a preset number of PCR cycles (default: 7, 10 and 13 cycles). Assays for quantification of dsDNA with PicoGreen were set up from PCR aliquots; fluorescent analysis was carried out immediately. Whenever the ratio of fluorescence (cycled reactions) and fluorescence (non-cycled blank) exceeded the preset threshold of 2.0, the respective reaction(s) were archived in a safe storage position before the reaction plate was moved back to the thermocycler. Hence, the PCRs were cycled only as long as necessary to obtain enough dsDNA template for the subsequent step of *in vitro* transcription.

*Automated RNA purification.* A robot-accessible and robust procedure had to be developed to remove any spurious substances from the RNA as a prerequisite to link two subsequent selection rounds without manual intervention. This is a feature

that would confer enormous flexibility to the automated process because free choice of buffers for all processes in which the RNA or dsDNA intermediates might be involved is extremely valuable. As we intended to select for aptamers that bind under physiological conditions, low molecular weight compounds that are present in transcription reaction mixtures in large amounts, such as NTPs, MgCl<sub>2</sub>, spermidine or DTT, had to be removed. Therefore, a vacuum manifold was designed and constructed that can host disposable, single Microcon ultrafiltration devices which retain nucleic acids of >60 nt in length. After loading the transcription reactions to the units, applying vacuum to the manifold, and washing with water, only the macromolecules were retained which could be easily resuspended in the desired buffer or reaction mixture.

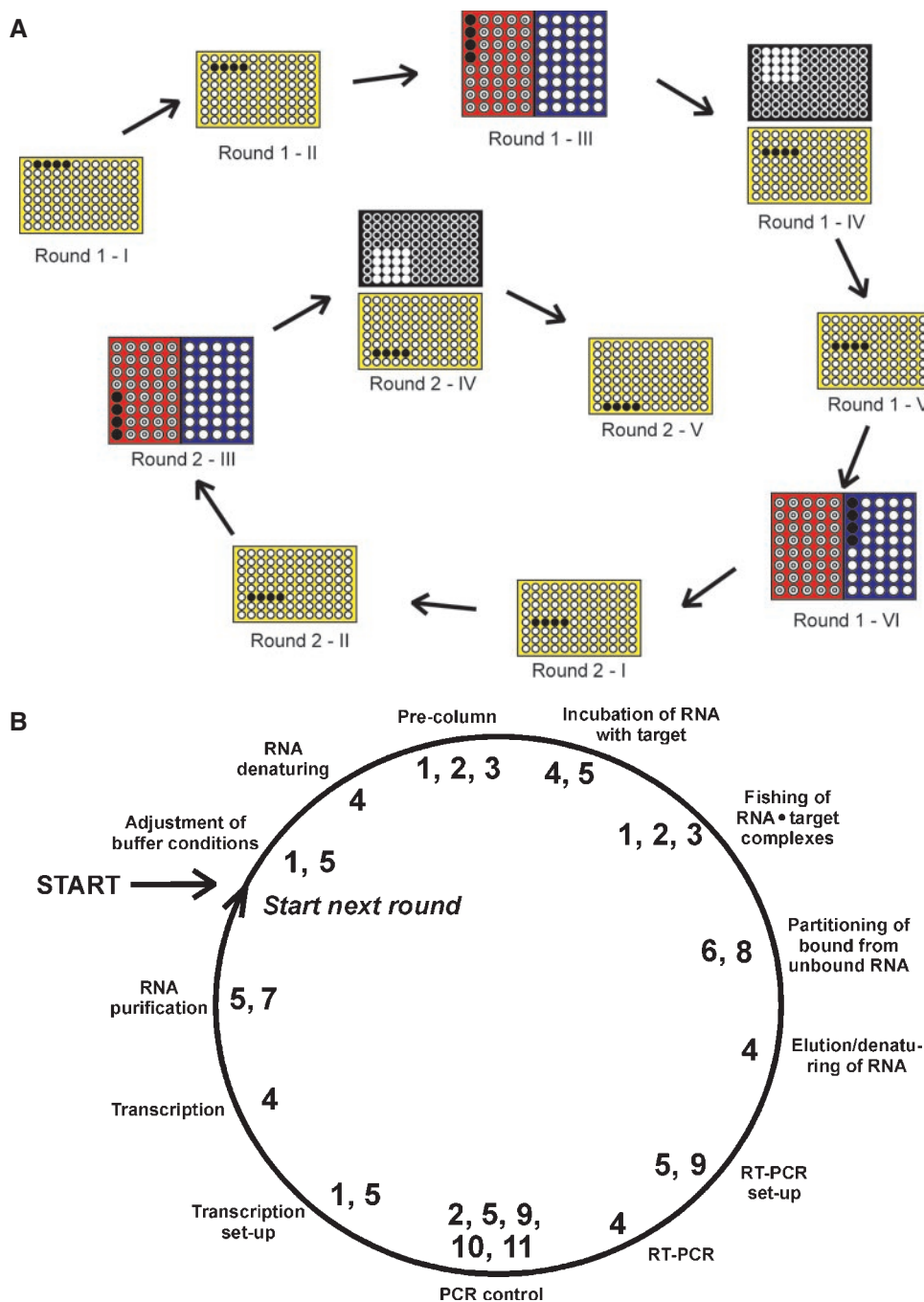
The course of the selected RNA pool and its intermediates through different plasticware during two consecutive selection rounds as well as participation of the workstations in the overall selection process is depicted in Figure 2A and B.

### Automated selection of D-substance P binding aptamers

The amount of material that can be handled in microtiter plates by the robot is limited. To assure that the selection process starts with a huge (>10<sup>14</sup> different molecules) and highly diverse library, initial selection rounds were carried out manually. The target concentrations in the manually performed rounds one and two were 30 μM, in round three 10 μM. The progress of the following automated selection rounds is displayed in Figure 3. In rounds 4 and 5, wash volumes between 18 and 54 column volumes were used with a constant target concentration of 10 μM of biotinylated D-substance P in the binding reaction; beginning with round 6, three different target concentrations were used. In all automated selection rounds, a mock selection lacking target was processed in parallel. After two consecutive rounds, the selection strand with the lowest target concentration which had still exhibited a clear signal/noise ratio in comparison with the mock was gel purified and subjected to the next two selection rounds. dsDNA resulting from round 14 (14 nM) was finally custom cloned and sequenced (Agowa GmbH, Berlin, Germany). The sequences of 24 clones were determined. Nucleotide sequences and frequencies of the clones are summarized in Figure 4A.

### Identification of a minimal binding domain

Spiegelmers are composed of mirror-image nucleotides and, therefore, they have to be synthesized chemically. In order to ensure high-quality syntheses, Spiegelmer sequences should be shorter than ~60 nt in length. The D-substance P-specific aptamer sequences that were obtained from the *in vitro* selection experiment had an overall length of 83 nt. Besides the most frequent sequence A11, only few additional sequences with point mutations had been enriched. dsDNA templates for *in vitro* transcription of different truncated clones were prepared and used to generate radiolabelled truncated transcripts SUP-A-001 (48 nt) to SUP-A-004 (44 nt) (see Figure 4B). Substance P binding of truncated RNA molecules was assessed in pull-down assays. As all examined truncated aptamers had retained substance P binding activity, the shortest sequence



**Figure 2.** The automated selection process. (A) Course of the selected RNA pool and its intermediates through two consecutive selection rounds. Blackened wells indicate the positions where the processed nucleic acids are located during the respective steps. Yellow plates with simple wells: ThermoSprint reaction plates. Black plates: for fluorescent assays. White concentric circles on red plate: plastic columns for partitioning in vacuum manifold L. White circles on blue plate: ultrafiltration devices on vacuum manifold R. Step I: adjustment of buffer conditions, RNA denaturing and re-folding, and pre-selection. Step II: incubation of RNA and target, fishing of RNA•target complexes. Step III: partitioning of bound from unbound RNA. Step IV: semi-quantitative RT-PCR. Step V: *in vitro* transcription. Step VI: purification of RNA. (B) Robot devices involved in each selection step. Numbers refer to workstations as shown in Figure 1B.

SUP-A-004 was chosen for further characterization (Supplementary Figure 2). Accordingly, SUP-A-004 was synthesized in its mirror-image configuration using L-phosphoramidites. Secondary structure predictions (minimum free energy conformations) were made using the Zuker algorithm with default parameter settings for A11, SUP-A-001 and SUP-A-004 (Figure 4C) (19).

### ITC experiments

The dissociation constant of the Spiegelmer L-SUP-A-004•substance P complex was determined to be  $40 \pm 9$  nM by ITC experiments (data from three independent measurements). A typical binding isotherm is shown in Figure 5A. L-SUP-A-004 bound to the N-terminal 7 amino acid peptide

R 1	30 $\mu$ M D-substance P	8 CV		
R 2	30 $\mu$ M D-substance P	15 CV		
R 3	10 $\mu$ M D-substance P	10 CV		
R 4	void	18 CV	36 CV	54 CV
7	1.07	1.58	1.67	1.60
10	1.05	2.92	3.16	2.80
13	1.48	-	-	-
16	3.95	-	-	-
R 5	void	18 CV	36 CV	54 CV
7	1.03	1.92	1.90	2.02
10	1.10	3.33	3.46	-
13	1.66	-	-	-
16	6.54	-	-	-
R 6	void	1.1 $\mu$ M	3.3 $\mu$ M	10 $\mu$ M
7	0.99	0.98	0.89	0.93
10	0.86	0.84	0.84	1.10
13	0.96	0.87	0.87	1.23
16	1.00	0.99	1.17	1.79
R 7	void	1.1 $\mu$ M	3.3 $\mu$ M	10 $\mu$ M
7	0.72	0.97	1.06	0.91
10	0.94	0.84	0.97	1.14
13	1.06	1.02	1.11	1.92
16	1.70	1.60	2.22	5.37
R 8	void	1.1 $\mu$ M	3.3 $\mu$ M	10 $\mu$ M
7	1.05	2.16	1.67	2.24
10	1.14	-	2.80	-
13	1.31	-	-	-
16	3.13	-	-	-
R 9	void	1.1 $\mu$ M	3.3 $\mu$ M	10 $\mu$ M
7	1.12	2.18	2.98	2.74
10	1.23	-	-	-
13	1.72	-	-	-
16	3.38	-	-	-
R 10	void	41 nM	123 nM	370 nM
7	0.93	0.99	1.00	1.21
10	0.90	1.05	1.30	2.13
13	0.93	1.34	2.30	-
16	1.09	2.60	-	-
R 11	void	41 nM	123 nM	370 nM
7	1.08	1.06	1.43	1.81
10	1.01	1.28	1.67	2.03
13	0.98	2.28	3.72	-
16	1.54	-	-	-
R 12	void	14 nM	41 nM	123 nM
7	0.98	0.80	1.07	1.31
10	0.90	1.16	1.33	2.10
13	0.91	1.77	2.32	-
16	0.91	3.43	-	-
R 13	void	14 nM	41 nM	123 nM
7	1.00	0.97	1.17	1.66
10	1.06	1.34	1.73	1.93
13	1.27	2.87	3.55	4.41
16	2.31	-	-	-
R 14	void	4.5 nM	14 nM	41 nM
7	1.05	1.65	2.19	2.85
10	1.08	2.95	-	-
13	0.95	-	-	-
16	1.28	-	-	-
R 15	void	4.5 nM	14 nM	41 nM
7	1.15	1.82	2.63	2.92
10	0.70	3.01	-	-
13	1.69	-	-	-
16	3.05	-	-	-

**Figure 3.** Progress of the *in vitro* selection against substance P. Rounds 1–3 were carried out manually; in rounds 4 and 5, the target concentration was 10  $\mu$ M for all three selection strands while varying the wash volume (CV, column volumes); rounds 6–15, varied target concentrations while washing with a fixed wash volume. Starting with round 4, the ratio of fluorescence intensity with/without polymerase is given.

substance P1-7 with similar affinity, but not to the C-terminal subpeptide substance P5-11 (Supplementary Figure 1A and B). Binding was neither observed to the tachykinin neurokinin A nor to D-substance P (Table 1; Supplementary Figure 1C and D). Likewise, non-functional control Spiegelmer did not bind to L-substance P (Supplementary Figure 1E).

### *In vitro* cell assays

In order to prove biological inhibitory activity of L-SUP-A-004, a cell assay based on substance P sensitive human AR42J

pancreatic cells was carried out. Substance P binding to AR42J cells leads to an intracellular calcium release. After stimulation with different concentrations of substance P, a half-maximum effective concentration of 3 nM ( $EC_{50}$ ) was determined (data not shown). While incubating 3 nM substance P with different concentrations of L-SUP-A-004, the Spiegelmer shows a dose-dependent inhibition of the stimulatory action of substance P. The half-maximum inhibition ( $IC_{50}$ ) was achieved at an L-SUP-A-004 concentration of  $\sim$ 45 nM, whereas a non-functional control Spiegelmer had no effects throughout the concentration range tested (Figure 5B).

### DISCUSSION

Commercially available robotic systems for biochemical or biotechnological applications are mostly designed to facilitate high-throughput analytical pipetting protocols. Thus, they are perfectly suited for the rapid execution of, preferably small volume, biochemical assays. *In vitro* selection, on the other hand, requires accurate succession of various complex procedures as well as preparative steps, such as affinity chromatography, enzymatic reactions and purification protocols. Yields, which are of no importance in qualitative analytical applications, are very important for successful execution of an *in vitro* selection experiment: sufficient amounts of nucleic acid must be generated to allow initiation of the respective following round of selection. At the expense of mere speed, we therefore chose a highly flexible single-pipette robotic platform and equipped it additionally with the necessary periphery to treat the samples almost exactly as a human researcher would do. This instrumentation allows execution strategies for the identification of high-quality aptamers while saving precious reagents at the same time. Throughout the whole automated *in vitro* selection process, physiological buffer and temperature conditions are adjusted and maintained in the binding reactions. The robotic system described performs two selection rounds per day and is, therefore, much slower than the six daily rounds that Cox *et al.* (5–8) achieve with their system. However, in Cox' protocol, time is mainly economized by the reduction of incubation times, which make up substantially more than half of the total time per selection round in our process. In addition, our *in vitro* selection protocol differs significantly in at least two aspects from the previously published automated *in vitro* selection protocol.

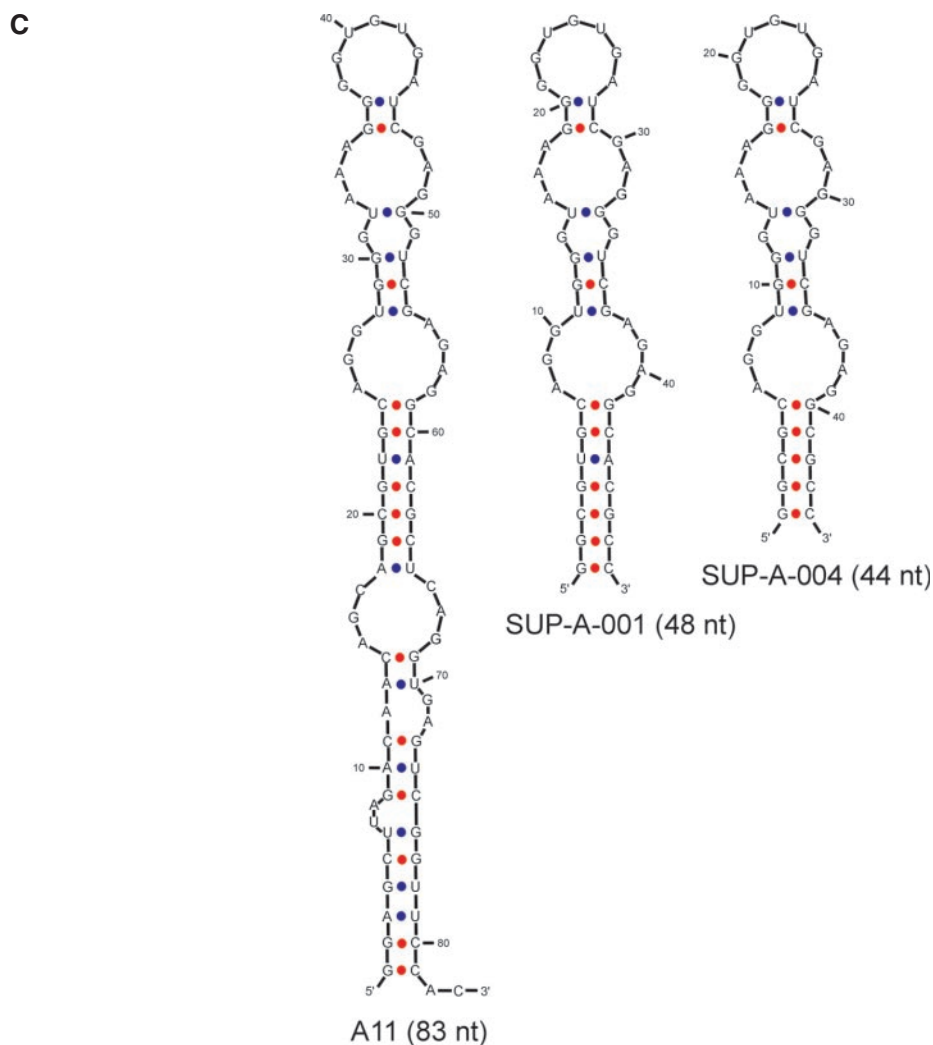
*Online monitoring.* The progress of our automated *in vitro* selection process is controlled through the measurement of the PCR yield. During every selection round, semi-quantitative PCRs are automatically performed, which allow assessment of the amount of nucleic acid that has bound to the target. As this is always carried out for three binding reactions containing different amounts of target plus a non-target control, this facilitates the choice of suited parameters for the respective following round. Just like a human researcher would do, the concentration of target in the binding reaction is kept as low as possible, which increases the chance to select binders with maximal affinity to the target. PCR monitoring, at the same time, helps to control the potential rise of amplification artefacts. Being responsive to the amount of dsDNA present in every PCR, the system automatically terminates further thermocycling of the respective reaction

A	Clone	Occurrence	Sequence
	A11	6x	X-AGCGTGCAGGTGGGTAAAGGGGTGTGATCGAGGGTTCGAGAGGCACGCT-Y
	C9	6x	X-AGCGTGCAGGTGGGTAAAGGGGTGTGATCGAGGGTTCGTGAGGCACGCT-Y
	A10	5x	X-AGCGTGCAGGTGGGTAAAGGGGTGTGATCGAGGGTTCGTAAGGCACGCT-Y
	C10	2x	X-AGCGTGTAGGTGGGTAAAGGGGTGTGATCGAGGGTTCGAGAGGCACGCT-Y
	C11	2x	X-AGCGTGTAGGTGGGTAAAGGGGTGTGATCGAGGGTTCGTGAGGCACGCT-Y
	E10	2x	X-AGCGTGTAGGTGGGTAAAGGGGTGTGATCGAGGGTTCGTAAGGCACGCT-Y

X, GGAGCTTAGACAACAGC (forward primer)

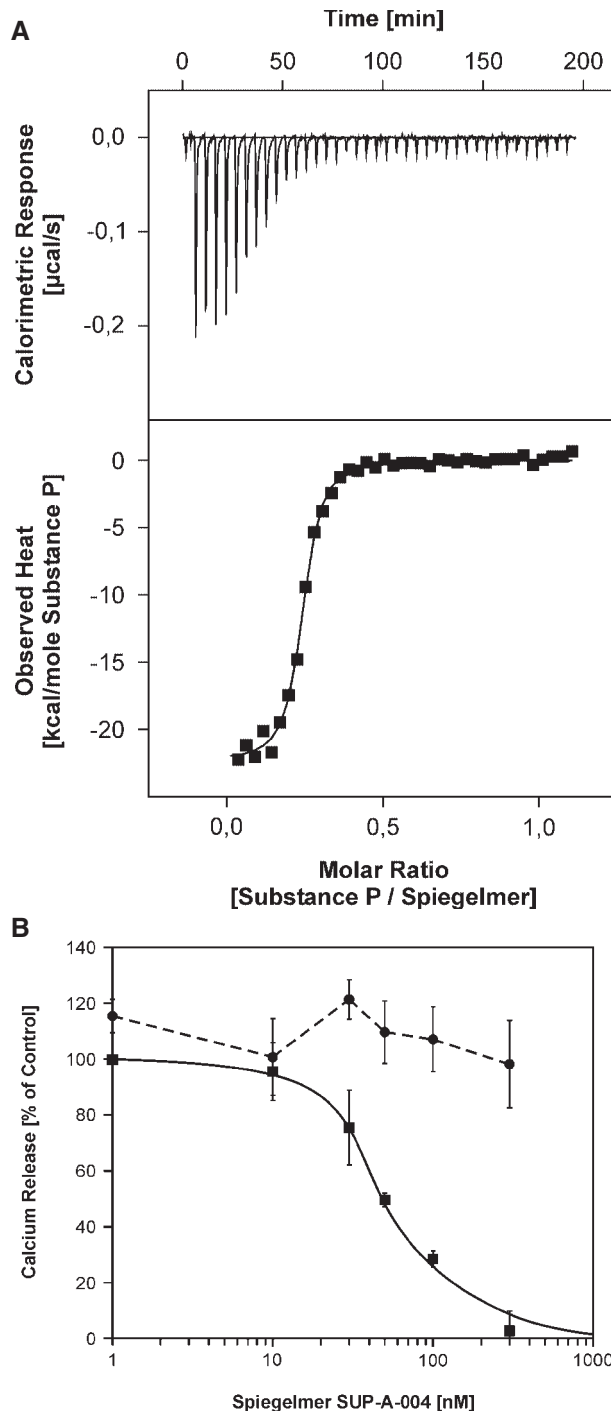
Y, CAGGTGAGTCGGTCCAC (reverse primer)

B	Name	length (nt)	Sequence
	SUP-A	48	AGCGUGCAGGUGGGUAAAAGGGGUGUGAUCGAGGGUCGAGAGGCACGCU
	SUP-A-001	48	GGCGUGCAGGUGGGUAAAAGGGGUGUGAUCGAGGGUCGAGAGGCACGCC
	SUP-A-002	46	-GGGUGCAGGUGGGUAAAAGGGGUGUGAUCGAGGGUCGAGAGGCACCC-
	SUP-A-003	46	-GGGUGCAGGUGGGUAAAAGGGGUGUGAUCGAGGGUCGAGAGGCGCC-
	SUP-A-004	44	--GGGUGCAGGUGGGUAAAAGGGGUGUGAUCGAGGGUCGAGAGGCCC--



**Figure 4.** Aptamer/Spiegelmer sequences. (A) DNA sequences that were obtained after cloning and sequencing the 14th round of selection. (B) Truncated versions of the most frequent clone A11. (C) Secondary structure (minimum free energy conformations) as predicted by mfold (19).





**Figure 5.** Binding and inhibition of substance P by Spiegelmer L-SUP-A-004. (A) Calorimetric profile of Spiegelmer L-SUP-A-004 binding to substance P (representative data from one ITC experiment). The dissociation constant was determined to be  $\sim 40$  nM. (B) Inhibition of substance P-induced AR42J cell stimulation by L-SUP-A-004 (filled squares) and non-functional control Spiegelmer (filled circles). The  $\text{IC}_{50}$  was determined to be  $\sim 45$  nM at a stimulatory substance P concentration of 3 nM.

as soon as sufficient material for the following reaction has been generated.

In contrast, Cox *et al.* tried to avoid overamplification in PCR by performing a defined number of 20 cycles in rounds

1–10 and 16 cycles in the following rounds; although this procedure helps to reduce the risk of unwanted PCR side reactions, it does not deliver data about the progress of the selection. Therefore, it is difficult to determine the right time point to increase the selection stringency by either lowering the target concentration or intensifying the washing procedure. Moreover, uncertainty about the selection progress leads to an increased risk to enrich matrix binders. This can be partly reduced by constantly enhancing the stringency with increasing concentrations of salt in the selection buffer (7). Nevertheless, online monitoring of the PCR provides a valuable opportunity to react flexibly on the progress of the selection itself.

*Versatility.* Introduction of ultrafiltration into the purification protocols of an automated SELEX process allows robust and highly effective purification of RNA from low molecular weight compounds, such as NTPs, salts or detergents. Moreover, ultrafiltration allows a free choice of buffer conditions in every reaction or incubation step. This is a prerequisite for linking two selection rounds together, especially when the desired binders have to bind under clearly defined conditions or if special solvents and reagents are incompatible with subsequent steps of an *in vitro* selection cycle. This is an issue if denaturing reagents are used to elute the target-bound RNA or if a competitive elution scheme using the target itself is introduced. By ultrafiltration, even high concentrations of urea can be successfully separated from the isolated nucleic acids so that subsequent steps are not negatively affected. Furthermore, even highly sophisticated selection protocols that involve multiple changes of buffers and conditions (20) are compatible with our robotic system and have already been realized (see Supplementary Material). However, a drawback of every automated process is its restriction to the volume range, which is determined by the used tools and labware. In order to maintain the maximum library complexity that is practically accessible and to make sure not to lose a single potential good binding sequence, we started the selection process with a manual selection round. We decided to continue the selection with two additional manual rounds because we wanted to be sure that the selection runs stably before fully relying on the new automated process. Since the process turned out to be very robust, we are convinced that it could be started in an earlier round or even right at the beginning. In our opinion, the latter is only recommendable if the target is judged to be a ‘good’ SELEX target (e.g. heparin binding protein) and if it can be expected to obtain satisfactorily binding aptamers even from libraries with lower complexities.

The identified Spiegelmer L-SUP-A-004 binds substance P with a dissociation constant of  $\sim 40$  nM at  $37^\circ\text{C}$ . This result is a satisfactory proof that our automated *in vitro* selection process is capable of yielding aptamers with equal, if not better, qualities than reported for manual *in vitro* selection schemes. Nieuwlandt *et al.* (16) reported an RNA aptamer to substance P that was isolated after two consecutive selection schemes and eventually showed a  $K_d$  of 190 nM (at room temperature). L-SUP-A-004 not only binds to substance P but also inhibits substance P action in a relevant cell assay. With an  $\text{IC}_{50}$  of 45 nM, L-SUP-A-004 will serve as a lead molecule for further in depth studies.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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