INSTRUCTION SHEET

APTA-BEACON™ (GQ-EXPAR, TMB) DEMONSTRATION KIT

"No Capturing. No Washing. Just READ."

Kit Contents (Store at -20°C or colder) – for 5 Sets of Assays:

Tube	Components†	Total Amount	Amount per Assay Set
1-1	2 mM Caffeine Solution	40 µL	1.5 µL
1-2	2 mM Theophylline Solution	40 µL	1.5 µL
1-3	Theophylline Riboswitch (RS-TFU$_{107}$)	40 µL	4 µL
1-4	RS Buffer	20 µL	2 µL
2-1	Nickase-Polymerase Mix (NPM)	40 µL	7 µL
2-2	EXPAR Mix	350 µL	63 µL
3-1	Hemin solution in DMF	40 µL	4 µL
3-2	TMB solution, ready to use	650 µL	118 µL
0.2 mL	T4 Polynucleotide Kinase (each tube)	10 tubes	1 tube/analyte

Components expire 1 month after resuspension

†Component mixtures and concentrations are proprietary.
MSDS available at www.aptagen.com

Additional Items Needed:

- ☐ Nitrile or Latex gloves
  - – Current version of kit is not optimized for nuclease resistance
- ☐ Pipettors and Pipet Tips (p10/p20, p100/p200, p2.5 if possible)
- ☐ Minifuge
- ☐ Ice bucket or cold block to chill reactions (to ~4°C)
- ☐ Thermocycler (preferred) WITHOUT heated lid or Hot Block (to 55°C)
- ☐ Timer

Introduction:

Aptagen's "No Capturing. No Washing. Just READ" assay is a radical advancement in diagnostic technology. Aptagen, LLC has developed a sensitive diagnostic platform that can detect virtually any target or antigen (from small molecules to protein biomarkers and cells) in a variety of sample matrices. Unlike current diagnostic formats, such as the traditional ELISA method and the more recently popular Luminex® approach, Aptagen's apta-beacon™ assay eliminates the need for multiple target manipulation steps. The apta-beacon™ platform requires neither capturing nor washing of sample(s), streamlining the analysis to quickly provide results with higher sensitivity.

The apta-beacon™ (GQ-EXPAR) is a tripartite system in a one-pot reaction mix. Three components work in concert to produce the results in a fluorescent output or – if desired, for low-resource environments – a colorimetric output. The first component in the system is an allosteric riboswitch, a shape-shifting RNA molecule (ribozyme) that undergoes a self-cleavage event. The allosteric riboswitch contains an aptamer sequence domain and is controlled by the aptamer binding to a unique target or antigen to trigger self-cleavage activity (1). Consequently, the switch is OFF in its natural state, but is turned ON in the presence of target.

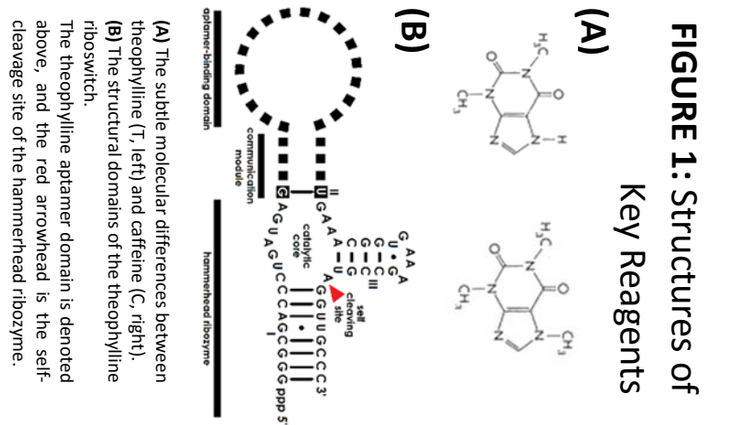
The second component in the system is an exponential amplification reaction (EXPAR), a signal-enhancer that initiates an isothermal amplification reaction upon release of the self-cleaved RNA strand (2). The product of EXPAR has peroxidase activity (3) and forms the third and last component of the system. Peroxidase acting on common substrates produces a fluorescent output that can be measured on a fluorometer; alternatively, using other substrates produces a colored product for visual detection. EXPAR and the subsequent peroxidase activity is a two-stage signal-enhancer strategy with the combined effect of boosting signal output and increasing sensitivity to unprecedented levels compared to conventional strategies (4, 5).

This demonstration kit illustrates the utility of the apta-beacon™ (GQ-EXPAR) technology by using theophylline versus caffeine as a test case. Caffeine and theophylline are two small molecules which are difficult to discriminate below millimolar concentrations because of their close structural similarity; they differ by only a single methyl group (Fig. 1A). This version of the kit produces a color output for visual detection of theophylline, with a demonstrated detection limit of 500 nM.

References:

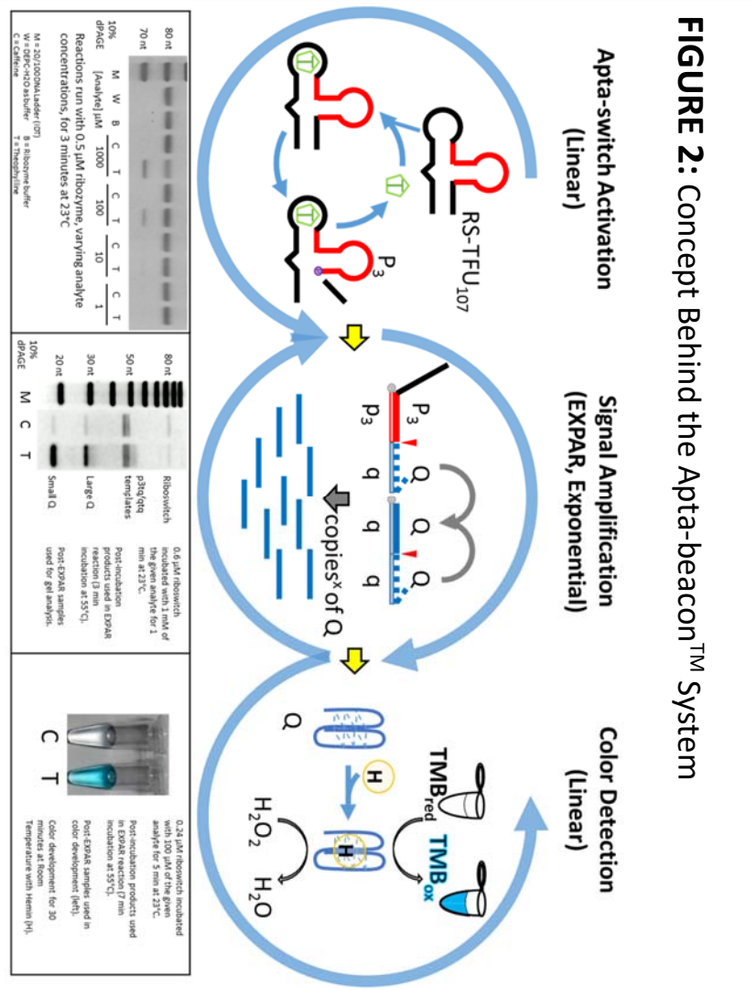
1. Soukup GA, et al. Altering molecular recognition of RNA aptamers by allosteric selection. JMB 298:623-32 (2000).
2. Van Ness J, et al. Isothermal reactions for the amplification of oligonucleotides. PNAS 100(8):4504-9 (2003).
3. Cheng X, et al. General peroxidase activity of G-quadruplex-hemin complexes and its application in ligand screening. Biochemistry 48:7817-23 (2009).
4. Zhao Y, et al. Cleavage-based signal amplification of RNA. Nature Communications 4:1493 (2013).
5. Nie J, et al. Report-triggered isothermal exponential amplification strategy in ultrasensitive homogeneous label-free electrochemical nucleic acid biosensing. ChemComm 50:6211-3 (2014).

FIGURE 1: Structures of Key Reagents



(A) The subtle molecular differences between theophylline (T, left) and caffeine (C, right). **(B)** The structural domains of the theophylline riboswitch. The theophylline aptamer domain is denoted above, and the red arrowhead is the self-cleavage site of the hammerhead ribozyme.

FIGURE 2: Concept Behind the Apta-beacon™ System



Apta-beacon™ Kit Detecting Theophylline Versus Caffeine Technical Bulletin

Before you get started:

The kit does not provide microcentrifuge tubes necessary for making dilutions. Thin-wall PCR tubes (0.2 mL) are provided for running reactions. For these purposes, make sure that the tubes and tips used are sterile and nuclease free. Any standard microcentrifuge tube volume, 1.7 ml or smaller, will work with the reaction if the solution needs to be transferred. If one intends to use a multichannel pipettor, strip-tubes or 96-well plates are suitable. A timer will be needed to mark the incubations.

This protocol utilizes 2 analytes at moderate concentration, recommended for first time users of the kit. Experienced users may modify protocols as needed to explore a wider range of parameters, including other analytes and/or negative controls. **The kit provides enough material for 5 sets of 2 assays; final individual assay volume is 100 µl. Each assay should take 45 minutes, including color development.**

Getting Started Prep (READ THOROUGHLY AND COMPLETE FIRST):

General Notes:

- Thaw all components **except the 0.2 mL tubes, tube 2-1, and tube 3-1** completely before use – can take up to 15 minutes; keep all components chilled on ice (4°C)
- **Keep tube 2-1, tube 3-1, and one set of "C" and "T" 0.2 mL tubes on ice.** Store the remaining tubes at -20°C
- If a thermocycler is not available, set hot block (**with water in tube holders**) to 55°C for use during protocol Step 2
- Quick-spin all tubes to be used – material can separate during shipping
- All samples should be mixed by aspirating, followed by a quick spin (Minifuge)
- Prepare all solutions for all steps on ice (4°C) unless other otherwise noted.

Caffeine (1-1) and **Theophylline** (1-2)

Keep stock solutions of theophylline and caffeine on ice when in use, and at 4°C or colder for long-term storage. The protocol as provided will test analytes at 1 mM concentration during Step 1b (analyte stock solutions are 2 mM). Experienced users who want to investigate the effects of analyte concentration on riboswitch cleavage activity should prepare fresh dilutions of analytes prior to each analysis.

Protocol:

Thermocycler preferred. If not available, pre-set hot block to 55°C for Step 2. Make sure all components are thoroughly thawed.

Prepare Step 1 samples on ice (4°C) in 0.2 mL PCR tubes provided with Demo Kit.

Step 1a. Apta-Switch RXN (4°C):	Kit Tube	Assay Tube "C"	Assay Tube "T"
Reagent Tube	0.2 mL tube	0.5 µl enzyme	0.5 µl enzyme
RS Buffer	1-4	1 µl	1 µl
CAF (2 mM stock, or dilution)	1-1	1.5 µl	------
THE (2 mM stock, or dilution)	1-2	------	1.5 µl
Ensure thorough mixing, then add next component LAST and mix QUICKLY (by aspirating, collect by tapping/quick-spin on minifuge) to minimize background.			
$RS\text{-}TFU_{107}$ (600 nM)	1-3	2 µl	2 µl
Total Reaction Mix (4°C)	------	**5 µl**	**5 µl**

Step 1b. Incubate at ROOM TEMPERATURE for 3 minutes (use timer), then chill on ice (4°C) until ready for Step 2a.

Continue Step 2a on ice (4°C) from Step 1 samples (same 0.2 mL PCR tubes).

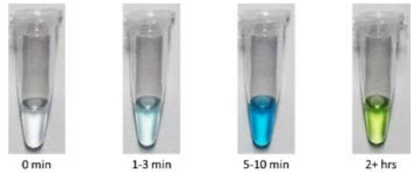
Step 2a. EXPAR RXN (4°C):	From Step	Assay Tube "C"	Assay Tube "T"
Step 1 Caffeine Reaction	1b	5 µl	------
Step 1 Theophylline Reaction	1b	------	5 µl
Nickase-Polymerase Mix (NPM)	2-1	3.5 µl	3.5 µl
EXPAR Mix	2-2	31.5 µl	31.5 µl
Total EXPAR Reaction Mix (4°C)	------	**40 µl**	**40 µl**

Step 2b. Use a timer and incubate all of each Total EXPAR Reaction Mix at 55°C for EXACTLY 5 minutes (thermocycler preferred, no heated lid) THEN chill on ice (4°C) until ready for Step 3a.

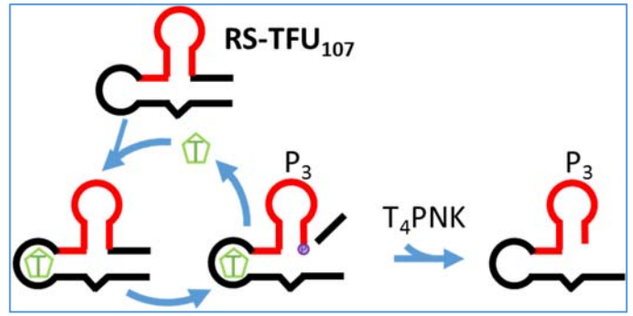
Continue Step 3a at 22°C-25°C (RT) from Step 2b samples (same 0.2 mL PCR tubes).

Step 3a. Color Development (RT):	Kit Tube	Assay Tube "C"	Assay Tube "T"
Step 2b. EXPAR Reaction Product	2b	40 µl	40 µl
25 µM Hemin (Mix Well)	3-1	2 µl	2 µl
TMB solution – **Mix well**	3-2	58 µl	58 µl
Total Color Development Reaction (RT)	------	**100 µl**	**100 µl**

Step 3b. Incubate at ROOM TEMPERATURE for 3-30 minutes to develop color.

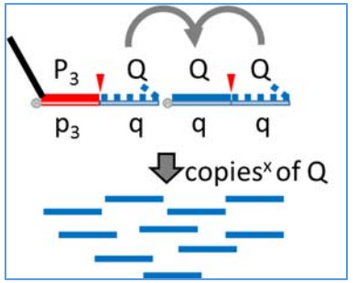


What Is Actually Happening:



Riboswitch RXN (Step 1). Signal generation. $RS\text{-}TFU_{107}$ self-cleaves when bound to its target (theophylline). T_4PNK dephosphorylates the cleaved end to produce a hydroxyl group, allowing P_3 to prime production of Q in Step 2.

EXPAR to Produce GQ (Step 2).

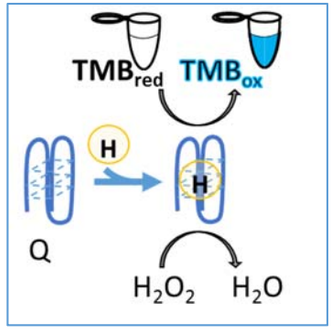


Signal translation/amplification step.

P_3 product from Step 1 is extended to generate the G-quadruplex strand (Q) of the color development complex via Bst 2.0 DNA polymerase. New material is nicked by Nt.BstNBI enzyme between P_3 and Q. Bst 2.0 polymerase has strand-displacement ability, so as new Q is created old Q is released. This allows for isothermal amplification.

Released Q primes exponential amplification from a second template, under the same Bst 2.0/Nt.BstNBI system as above.

Note that due to individual sample preparation and handling, Step 2 may require optimization to identify the best amplification conditions while producing minimal background. For WEAK theophylline color development, increase Step 2b incubation time by 0.5-1 minute. For STRONG caffeine color development, decrease Step 2b incubation time.



Color Development (Step 3).

Signal output step.

Q generated in Step 2 folds into G-quadruplexes in the KCl buffer, and in the presence of Hemin is known to exhibit peroxidase activity.

Once complexes have formed, the color indicators (TMB with H_2O_2) are added and the reaction allowed to proceed.