STANDARD OPERATING PROCEDURE

APTAMARKER KIT

APTAMARKER PLATFORM

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KIT INFORMATION

The Aptamarker kit is shipped at room temperature. Upon arrival, all components should be stored at 2-8°C to guarantee maximal shelf life. The kit remains stable at 2-8 °C until the expiration date if stored as specified.

Note: Product expires 2 months from the date of shipment.

KIT CONTENTS AND STORAGE

The Aptamarker kit contains the following:

- Aptamarker library plate
- Competitor antisense conjugated to resin solution (Antisense)

  Note: 48 samples/tube
- NGS preparation plate

  Note: Primers for NGS amplification

Note: Store the Aptamarker library plate and the NGS preparation plate at 4°C for up to three months. Store the competitor antisense resin at 4°C for up to two months.

EQUIPMENTS AND REAGENTS REQUIRED BUT NOT SUPPLIED

REGULAR LABORATORY EQUIPMENT

- 1.5 mL microcentrifuge sterile tubes
- 5 mL tube
- Nuclease-free water
- Selection buffer PBS 10X
- PCR plate centrifuge
- PCR Plate Sealing Film, adhesive, optical
- Real-Time qPCR Detection System for characterization of amplification

EQUIPMENT SPECIFIC TO APTAMARKER KIT

- One 96 well plate (200 µL minimum volume)
- Cleanup column : GFX™ PCR DNA and Gel Band Purification Kit : 28903466, 28903470 or 28903471 (Cytiva) One cleanup column per NGS set submission.
- qPCR Master Mix (SsoAdvanced Universal SYBR® Green Supermix : 1725270 to 1725275 (BioRad)

PART A: APTAMARKER PROCESSING

- Procedures within this section are performed at room temperature (RT). Aptamarker library plate may remain open during processing and should be sealed for centrifuging.
- The Aptamarker library plate contains an average of 10,000 copies of each of 268M different Aptamarker probes in each well. Given the need for 20M reads per sample for next generation sequencing analysis, the plate should be considered as two separate sets for Aptamarker processing. Appropriate labelling of the plate is provided in Figure 1.

Figure 1: Aptamarker library plate processing guide.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	46	54	62	70	78	86
B	2	10	18	26	34	42	47	55	63	71	79	87
C	3	11	19	27	35	43	48	56	64	72	80	88
D	4	12	20	28	36	44	49	57	65	73	81	89
E	5	13	21	29	37	45	50	58	66	74	82	90
F	6	14	22	30	38	**C1**	51	59	67	75	83	**C4**
G	7	15	23	31	39	**C2**	52	60	68	76	84	**C5**
H	8	16	24	32	40	**C3**	53	61	69	77	85	**C6**

Example of Sample/well pairings for 90 samples and 6 control libraries.

Numbers refer to Sample number and C1 to C6 refers to library controls.

A.1. PREPARATION OF THE APTAMARKER LIBRARY

1. Briefly centrifuge the plate and unseal it.
2. Add 20 µL of nuclease-free water and 5 µL of 10X selection buffer to each used well of Aptamarker library plate. Pipette up and down once to mix the solutions.
3. Incubate for 30 min at RT.

A.2. APPLICATION OF SAMPLE

1. Add 10 µL of sample (for control libraries, sample is replaced with water) to the 25 µL of prepared Aptamarker library and buffer by pipetting up and down to mix the solution.
2. Incubate for 30 minutes at RT.

3. Add 15 µL of competitor Antisense resin to each tube containing 35 µL of Aptamarker library, sample and buffer solution.

   Important Note: The stock tube should be mixed and vortexed to resuspend resin prior to use.
4. Seal the plate with fresh film.
5. Incubate for 15 minutes at RT agitating the plate manually every five minutes to prevent the Antisense resin from settling.

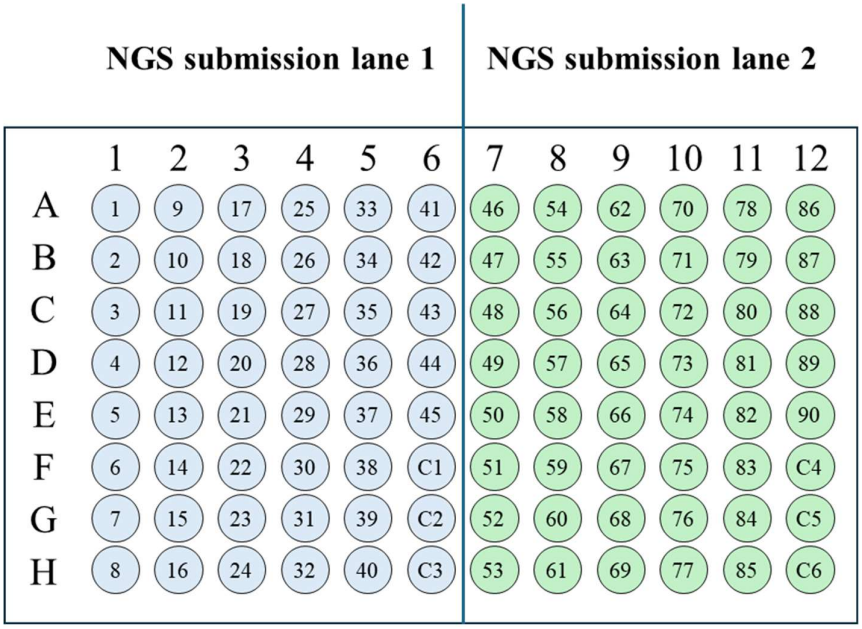
A.3. SEPARATION OF BOUND AND UNBOUND APTAMARKERS

1. Centrifuge the plate at 6000 x g for 10 minutes to pellet Aptamarkers that are hybridized to Antisense on resin.
2. Unseal the plate carefully.
3. Transfer 30 µL of the supernatant to a clean 96-well plate for dilution (this plate is not provided in the kit). It is important not to disturb the resin at the bottom of the tube while collecting the supernatant.
4. Add 120 µL of Nuclease-free water to the collected supernatant and pipette up and down once to mix the solutions.
5. Discard the Aptamarker library plate.
6. Either seal the dilution plate and store the plate at 4°C or proceed directly to NGS preparation.

PART B: NGS PREPARATION

- The NGS preparation plate has different forward primers in each of the first 48 wells. These forward primers are replicated in the second set of 48 wells within this plate. All wells contain the same reverse primer. A guide for the use of this plate is provided in Figure 2.
- Each of the 48 forward primers has a different hex code. The hex codes from columns 1 to 6, are repeated in columns 7 to 12. **Maintain alignment of samples processed in the Aptamarker processing plate with the NGS processing plate.**
- The amplified product from 48 wells is pooled for one NGS submission lane.

Figure 2: Outline of project plan for 90 samples and 3 control libraries per NGS submission lane:



Sample/well pairings for 90 samples and 6 control libraries.

Numbers refer to Sample number and C1 to C6 refers to library controls. The blue colour refers to one NGS submission lane and green to another NGS submission lane.

B.1. PRIMER PREPARATION

1. Add 10 µL of nuclease-free water to each used well within the NGS preparation plate.
2. Incubate for 30 min at RT.

B.2. AMPLIFICATION OF LIBRARIES

1. Pipette 20 µL of SYBR Green master mix into each well of the NGS processing plate

Note: To ensure optimal performance, make sure SYBR Green master mix is thawed and homogenized before use.

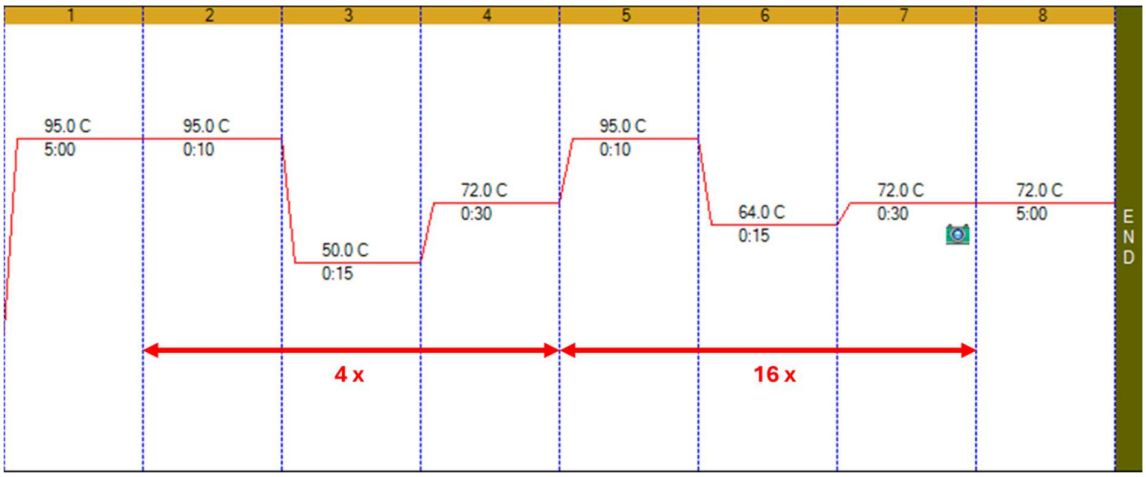
2. Pipette 10 µL of template (supernatant diluted in water obtained in Part A) into each respective well to a final volume of 40 µL.

Note: To avoid cross-contamination, we strongly recommend pipetting the template last, preferably in a separate work area.

3. Seal the plate with a fresh qPCR appropriate optical adhesive film before proceeding with the real-time qPCR detection steps.
4. Centrifuge the plate to spin down the contents and eliminate air bubbles.
5. Load the reaction plate onto the real-time qPCR instrument and start the qPCR run with Sybr Green setting/mode for a final volume of 40 µL with the protocol defined below:

Figure 3: qPCR protocol

STEP	TEMPERATURE	TIME
Polymerase Activation and Initial Denaturation	95°C	5 minutes
4 Cycles	95°C ***50°C*** 72°C	10 seconds 15 seconds 30 seconds
16 Cycles	95°C ***64°C*** 72°C + Plate Read	10 seconds 15 seconds 30 seconds
Final Extension	72°C	5 minutes
Hold	4-10°C	Infinite



Note : Once the amplification step is performed, keep plate stored at 4°C and keep plate away from the light.

B.3. AMPLIFIED LIBRARY POOLING AND CLEANUP

1. Pipette 10 µL of each amplified library within the first 48 wells into one clear 5 mL tube. Repeat with each amplified library from the second 48 wells into a separate clear 5 mL tube. Each of these tubes represents a different NGS submission.
2. Proceed to cleanup by following the protocol of the Cytiva for the purification of DNA from solution or an enzymatic reaction from the supplier.

   Note: Use a separate Cytiva cleanup column for each amplified library pool.

   Note: Cytiva binding buffer volume varies depending on volume of library pool.

   Note : We have only qualified the Cytiva columns for this application given the relative length of the primers.
3. After the full amplified library pool has been passed through a cleanup column elute the libraries with 150 µL of Nuclease-free water.

PART C: NGS SUBMISSION RECOMMENDATIONS

C.1. DETERMINING NUMBER OF USEABLE READS

We estimate that 64% of the raw instrument output will result in high-quality usable reads for downstream analysis. The Aptamarker library has low genetic diversity, so we must account for data loss required to maintain high sequencing quality. With the low diversity library, we recommend that it be spiked with ~15% PhiX to provide the necessary sequence complexity for NovaSeq sensors.

Factors impacting total useable reads:

Factor	***Value***	***Rationale***
Q30 Quality	90%	A 10% loss it budgeted to ensure we only analyze sequences with base calls with 99.9% accuracy.
PhiX	85%	Essential for low-diversity libraries. Spike with 15% PhiX, leaving 85% of the flow cells for your samples.
Matching	86%	Accounts for reads that are not perfectly aligned with sequences in the Aptamarker library.
Deconvoluting	97%	The expected efficiency of the demultiplexing process (assigning reads to their specific hex codes).
Expected useable reads	64%	Total calculation: 0.90 X 0.85 X 0.86 X 0.97 ~= 64%

Useable reads are calculated as follows:

$$Reads\ per\ sample_{Expected} = \frac{(Total\ Flow\ Cell\ Capacity\ \times\ 0.64)}{Total\ Libraries}$$

Description:

1. Multiply the expected number of total reads for your NGS instrument by the expected number of useable reads (0.64).
2. Divide by the number of libraries included in each NGS submission (samples and technical controls).

This will provide you with the expected number of reads/samples. A minimum of 20 million (M) useable reads per sample is required. This depth is necessary to capture the full complexity of the Aptamarker library and provide statistically significant results during downstream analysis.

C.2. SEQUENCING RUN PARAMETERS

To maximize read depth and efficiency, the run must be configured as follows:

- **Mode:** One-way (Single-End) sequencing
- **Cycles:** 100 cycles

The Aptamarker library was designed and validated around using single-end sequencing. To obtain 20M reads/sample, it is essential to run NGS using the recommended mode.

Users intending to use NovaSeq X 25B flow cells should contact us and order an Aptamarker kit designed for this type of analysis. Kits for NGS analysis with NovaSeq X 25B flow cells contain an NGS preparation plate with 96 different forward primers allowing analysis of 93 samples in one NGS submission.