



Plasma-SeqSensei™

Breast Cancer IVD Kit

Instructions for Use

February 2022

ZR150547.R1

IN VITRO TEST/For in-vitro-diagnostics














Glossary of Symbols			
	Manufacturer		Use by
	Catalogue number		Lot number
	Contents sufficient for <n> tests		Caution
	Temperature limit		Consult instructions for use
	In-vitro-diagnostics		Do not re-use
	Keep away from light		Keep dry
	Humidity limit		

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1 Intended purpose

The Plasma-SeqSensei™ (PSS) Breast Cancer IVD Kit is a quantitative next-generation sequencing (NGS) assay intended for the detection and identification of mutations in targeted genes AKT1, ERBB2, ESR1, KRAS, PIK3CA, and TP53 in human circulating cell-free DNA (cfDNA) isolated from blood plasma of breast cancer patients to detect minimal residual disease, recurrence surveillance and (neo-)adjuvant response monitoring in patients.

The PSS Breast Cancer IVD Kit must only be used in conjunction with the PSS IVD Software to achieve its intended use and has to be performed by trained personnel in a professional laboratory environment. The information generated should never be the sole determinant for taking medical decisions.

Note: The PSS Breast Cancer IVD Kit is not intended to be used in screening, for diagnosis of cancer or as a companion diagnostic.

2 Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer deaths among females worldwide (1). During the last years, extensive research regarding curative surgery, (neo-)adjuvant therapy as well as targeted therapy has been conducted, leading to an increase of the survival rate (2).

Tumour cells undergoing apoptosis, necrosis or metabolic secretion release small amounts of their DNA into the blood stream. The tumour-specific fraction of cfDNA is called circulating tumour DNA (ctDNA) and contains the genetic information for the primary tumour and even metastases. A multitude of research studies and trials have demonstrated the clinical application of ctDNA profiling at different stages of cancer treatment, including therapy selection, prognosis, and monitoring (3).

Various NGS-based technologies are available for ctDNA detection. However, due to sequencing and PCR bias/errors most of them are inappropriate for the detection of rare variants. Plasma-SeqSensei™ is a novel NGS-based technology that implements unique molecular identifiers (UID) in the sequencing workflow. This results in a significant error reduction, leading to an ultra-high sensitivity of the PSS technology (4).

3 Test principle

The PSS Breast Cancer IVD Kit detects gene mutations in ctDNA isolated from blood plasma. To enhance the sensitivity of the method, DNA fragments are labelled using UIDs during the first amplification step. This results in the formation of UID families consisting of various copies of each UID assigned. During the second amplification step, each member of a UID family is additionally assigned with a well- and plate-specific barcode (4). For validity reasons, an internal quantification input control (Quantispike) is included in addition to external positive and negative controls in every run.

The workflow includes automated data analysis and report generation using the Plasma-SeqSensei™ IVD software. The software quantifies the cfDNA input and identifies supermutants, that are UID families in which at least 90 % of all PCR fragments contain identical mutations. This concept allows the discrimination of real mutants from PCR or sequencing artifacts present only in a very low number of UID family members. The core process of the PSS technology is shown in Figure 1.

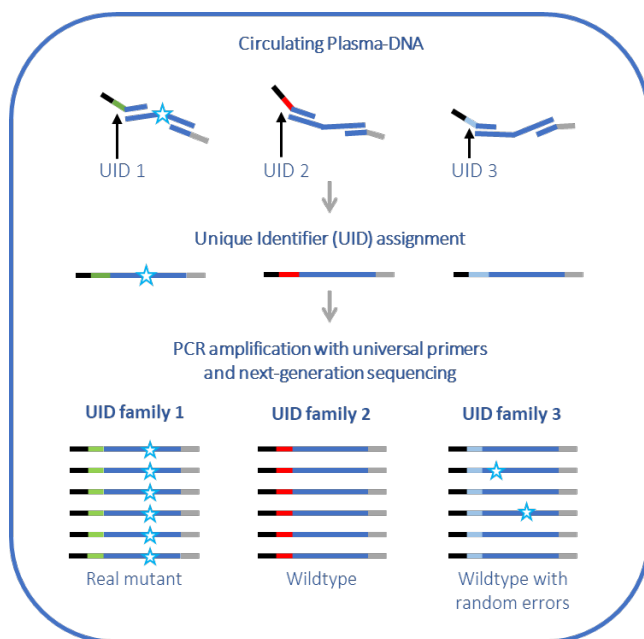


Figure 1: Principle of the PSS technology

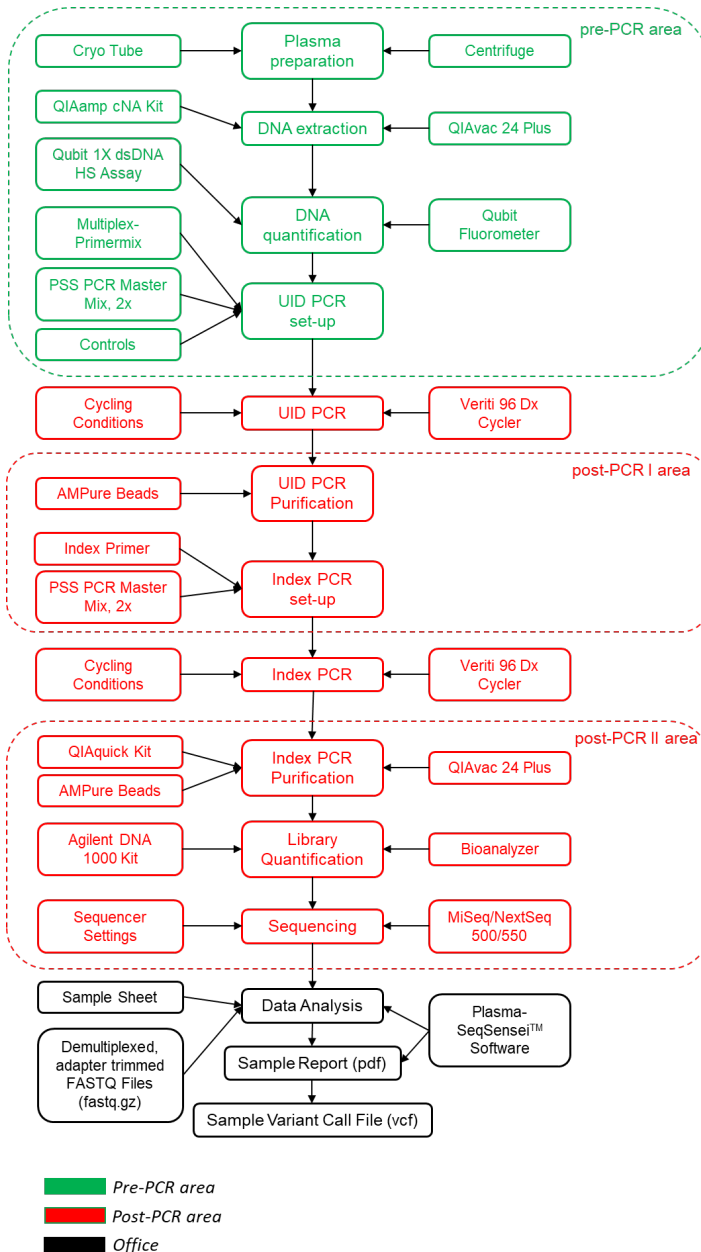


Figure 2: Workflow overview of the Plasma-SeqSensei™ method

4 Covered regions

Table 1: Covered regions with PSS Breast Cancer IVD Kit

Gene	Transcript*	Coding sequence start	Coding sequence end	Amino acid start	Amino acid end
AKT1	ENST00000554581	47	69	17	23
ERBB2	ENST00000269571	907	947	303	315
ERBB2	ENST00000269571	2,258	2,307	754	769
ERBB2	ENST00000269571	2,308	2,360	770	786
ESR1	ENST00000440973	1,108	1,143	370	381
ESR1	ENST00000440973	1,378	1,420	460	473
ESR1	ENST00000440973	1,583	1,614	529	538
KRAS	ENST00000256078	8	43	4	14
PIK3CA	ENST00000263967	254	278	86	92
PIK3CA	ENST00000263967	329	352	111	117
PIK3CA	ENST00000263967	353	367	119	122
PIK3CA	ENST00000263967	1,033	1,058	345	352
PIK3CA	ENST00000263967	1,085	1,115	363	371
PIK3CA	ENST00000263967	1,252	1,264	418	421
PIK3CA	ENST00000263967	1,348	1,387	450	462
PIK3CA	ENST00000263967	1,611	1,659	538	553
PIK3CA	ENST00000263967	2,138	2,184	714	728
PIK3CA	ENST00000263967	3,118	3,169	1,040	1,056
TP53	ENST00000269305	144	232	49	77
TP53	ENST00000269305	293	375	99	125
TP53	ENST00000269305	376	423	126	141
TP53	ENST00000269305	451	537	151	179
TP53	ENST00000269305	574	659	192	219
TP53	ENST00000269305	695	782	233	260
TP53	ENST00000269305	783	856	262	285
TP53	ENST00000269305	888	919	297	306
TP53	ENST00000269305	920	993	308	331
TP53	ENST00000269305	994	1,080	332	360

* Sequence source: Ensemble database

5 Variant result interpretation

The assay is designed to detect somatic mutations in plasma-derived ctDNA. The results of this test may serve as an adjunct to the ordering physician's work-up and as such should be interpreted in the context of clinical findings, tumour pathology and other laboratory data by a qualified healthcare professional.

Mutation frequencies:

Mutation frequencies are reported as both MAF (mutant allele fraction) and absolute number of MM (mutant molecules). MAF is the proportion of mutant ctDNA relative to the total cfDNA. MAF may be used to confirm the presence or absence of mutations. However, it may not reflect the overall tumour burden, as the proportion of ctDNA relative to total cfDNA in a sample may be affected by various factors including tumour anatomic location, tumour cell turnover, vascularity, treatment, blood sampling procedures, sample handling, and patient's clinical characteristics unrelated to tumour status (5). The absolute number of MM detected for a given variant represents the total number of molecules detected in a sample and may provide direct insight into features of tumour biology unique to each patient (5, 6).

Variants reported:

Variants with characterized, likely or predicted functional impact are reported. These are based on publicly available databases such as COSMIC (7) and/or peer-reviewed scientific literature are reported (6, 8, 9). Additionally, variants of suspected germline origin as indicated by an observed MAF between 40 % to 60 % or an observed MAF greater than 90 %, are shown in a separate table on the report.

6 Limitations

Suspected germline mutations are excluded from reporting based on observed MAF values and publicly available information. However, this test cannot definitively determine whether these mutations are of germline origin without the analysis of matched healthy cells. Additionally, mutations reported for certain genes in a small subset of patients may be the result of clonal haematopoiesis and should be adjudicated via analysis of matched blood cells. The detectability of ctDNA depends on various factors including tumour burden, tumour biology, sample collection conditions, sampling heterogeneity, and clinical characteristics. The test has been shown to have low but detectable variations depending on the sequence context, especially in samples with target molecule counts around the cut-off.

The PSS Breast Cancer IVD Kit has been tested to detect the following types of somatic mutations: single-nucleotide variations (SNVs), insertions (up to 27 nucleotides), deletions (up to 48 nucleotides), and deletion/insertion variants (up to 17 nucleotides).

7 Reagents, consumables and equipment

The PSS Breast Cancer IVD Kit contains two sub-boxes and one bag. One box should be stored in the pre-PCR laboratory and the other box as well as the bag containing the PSS Index Primer Plate should be stored in the post-PCR laboratory. It is highly recommended to split the Kit box upon arrival into two separate labs to minimise the risk of reagent contamination. The pre-PCR box is intended to be handled in a laboratory where no amplified DNA is handled. The post-PCR box and the bag are intended to be handled in a laboratory where PCR reaction vials/plates are opened and handled.

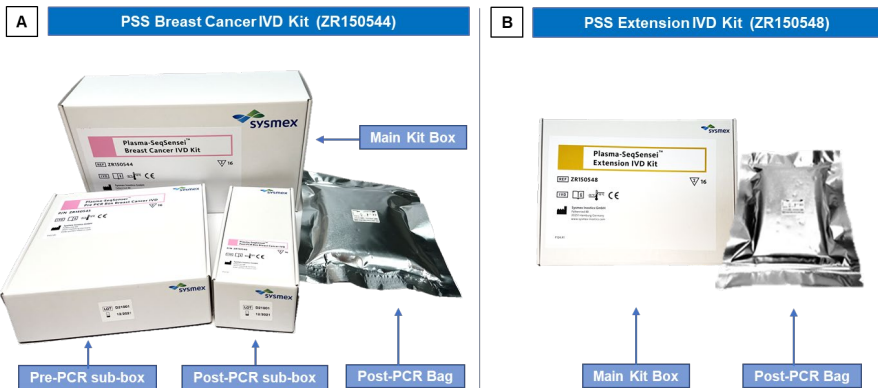
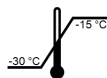


Figure 3: Plasma-SeqSensei™ Breast Cancer IVD Kit boxes with bag (A) and Plasma-SeqSensei™ Extension IVD Kit box and bag (B) with their respective storage locations (pre/post-PCR areas) are shown.

7.1 Supplied material

The supplied material is essential for the assay and cannot be replaced by other products.



The PSS Breast Cancer IVD Kit must be stored at a temperature between -15 °C and -30 °C when not in use.



Once opened, reagents are stable for 30 days or until the expiration date, whichever comes first.

Table 2: Material supplied with PSS Breast Cancer IVD Kit (ZR150544)

Box	Name (cap colour)	Cat No.	Tubes	Freeze-thaw cycles	Storage temperature
Pre-PCR box	PSS Breast Cancer Mpx A (blue)	ZR851013	4	2	-15 °C to -30 °C
	PSS Breast Cancer Mpx B (yellow)	ZR851014	4	2	-15 °C to -30 °C
	PSS Breast Cancer Positive Control (red)	ZR855006	4	2	-15 °C to -30 °C
	PSS No Template Control (transparent)	ZR854002	4	2	-15 °C to -30 °C
	PSS Quantispike (green)	ZR856001	4	2	-15 °C to -30 °C
	PSS PCR Master Mix v2, 2x (black)	ZR850001	4	4	-15 °C to -30 °C
Post-PCR bag	PSS Index Primer Plate IND34 ^{1,2}	ZR852004	1	N/A	-15 °C to -30 °C
Post-PCR box	PSS PCR Master Mix v2, 2x (black)	ZR850001	2	4	-15 °C to -30 °C
	Water, nuclease-free (white)	ZR224006	1	N/A	-15 °C to -30 °C



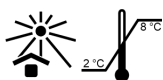
¹ Protect plates from light exposure. After first use store the PSS Index Primer Plate at a temperature between 2 °C and 8 °C.

² PSS Index Primer Plate IND34 is also named Plate A in workflow and PSS IVD Software

In case more than 16 samples are analysed on the same sequencing run, a Plasma-SeqSensei™ Extension IVD Kit must be ordered.

Table 3: Material supplied with PSS Extension IVD Kit (ZR150548)

Box	Name (cap colour)	Cat No.	Tubes	Freeze-thaw cycles	Storage temperature
Post-PCR bag	PSS Index Primer Plate IND35 ^{1,2}	ZR852005	1	N/A	-15 °C to -30 °C



¹ Protect plates from light exposure. After first use store the PSS Index Primer Plate at a temperature between 2 °C and 8 °C.

² PSS Index Primer Plate IND35 is also named Plate B in workflow and PSS IVD Software

Table 4: Composition of supplied material

Name	Composition
PSS Breast Cancer Mpx A	Primer Tris EDTA Buffer
PSS Breast Cancer Mpx B	Primer Tris EDTA Buffer
PSS Breast Cancer Positive Control	Double-stranded synthetic DNA wild-type/mutant Tris EDTA Buffer Carrier RNA
PSS No Template Control	Tris EDTA Buffer
PSS Quantispike	Double-stranded synthetic DNA Tris EDTA Buffer Carrier RNA
PSS Index Primer Plate	Index primers (well-specific) Bromophenol blue
PSS PCR Master Mix v2, 2x	Hot-Start Polymerase PCR Buffer dNTPs
Water, nuclease-free	Nuclease-free water, molecular biology grade



All liquid and dried components of the kit are for single-use only. Each well of the Index Primer Plate is single-use only.

The reagent-containing tubes are multiple-use reagents, as they can be thawed and frozen according to Table 2 to extract liquid for the indicated workflow steps.

7.2 Non-supplied material

Products, where details about manufacturer/vendor and order number are provided in Tables 5 to 7, are essential for the assay and must not be interchanged by products with comparable quality and/or properties.

Table 5: Material not provided with the PSS Breast Cancer IVD Kit

Material	Products
Reagents and kits	Ethanol (EtOH) $\geq 99.8\%$, p.a.
	RNase and DNase free distilled water
	* Agencourt® AMPure® XP, Beckman Coulter, #A63881
	* Buffer EB (Elution Buffer), QIAGEN, #19086
	* QIAquick® PCR Purification Kit, QIAGEN, #28106
	* Buffer PB, QIAGEN, #19066
	* DNA 1000 Kit, Agilent, #5067-1504 <ul style="list-style-type: none"> ■ Microfluidic chips ■ Reagents ■ Chip Priming Station, Agilent, #5065-4401
	* DNA 1000 Reagents, Agilent, #5067-1505
	* Qubit™ 1X dsDNA HS Assay Kit, Thermo Fisher, #Q33230 (100 rxns) or #Q33231 (500 rxns)
	* Qubit™ Assay Tubes, Thermo Fisher, #Q32856
	Sodium hydroxide (NaOH), 1 M
	Trizma® hydrochloride solution pH7.0, 1 M
	* NextSeq™ 500/550 Mid Output Kit v2.5 (150 cycles), Illumina, #20024904 Parts of the kit: <ul style="list-style-type: none"> ■ Mid Output Reagent cartridge (150 cycles), #15057940 ■ Mid Output Flow Cell cartridge, #20022409 ■ Buffer cartridge, #15057941 ■ Hybridization Buffer (HT1), #15058251
	* NextSeq™ 500/550 High Output Kit v2.5 (150 cycles), Illumina, #20024907 Parts of the kit: <ul style="list-style-type: none"> ■ High Output Reagent cartridge (150 cycles), #15057931 ■ High Output Flow Cell cartridge, #20022408 ■ Buffer cartridge, #15057941 ■ Hybridization Buffer (HT1), #15058251

* Essential components; must not be interchanged by products with comparable quality and/or properties.

7.3 Consumables

Table 6: Consumables required for the PSS Breast Cancer IVD Kit

Laboratory equipment	Product
Pipette tips/serological pipettes	Aerosol-resistant sterile pipette tips with filters 2, 10, 20, 200, 1000 µl
Reaction tubes	15, 5, 2, 1.5 ml tubes
	* LoBind® DNA tubes 1,5 ml, Eppendorf, #0030108051
	Tube strips plus caps (1.3 ml)
96-well plates	* PCR Plate, 96-well, segmented, semi-skirted, Thermo Scientific, #AB0900 or #AB2400 (required for PCR)
	96-well Multiply® PCR plate without lateral skirt, Sarstedt (optional, for dilutions)
Sealing foil for 96-well plates	Aluminium foil
	Clear adhesive film
Safety equipment	Protective coats, sleeves, eyeglasses, disposable shoe covers, gloves
Miscellaneous	Disposable reagent reservoirs (25 ml)
	* 3 ml Extension tubes for QIAvac vacuum manifolds, Qiagen, #19587
	* VacConnectors (500) for QIAvac vacuum manifolds, Qiagen, #19407

* Essential components; must not be interchanged by products with comparable quality and/or properties.

7.4 Equipment

Table 7: Equipment required for the PSS Breast Cancer IVD Kit

Laboratory equipment	Product
Electronic instruments	Centrifuge for 1.5/2 ml tubes, capable of 20,000 × g, fixed angle rotor
	Centrifuge for 15/50 ml tubes, capable of 7,197 × g, fixed angle rotor
	Centrifuge for 96-well plates, capable of 1,000 × g, fixed angle rotor
	Minicentrifuge capable of ≤ 2,000 × g
	Vortexer with inserts for tubes and 96-well plates
	Vortexer with insert for Agilent DNA Chips, capable of 2,400 rpm
	Freezer, -15 °C to -30 °C
	Refrigerator, 2 °C to 8 °C
	DNA workstation/PCR cabinet
	Fume hood (strongly recommended)
	Class II Biological Safety Cabinets (strongly recommended)
	Qiagen Connecting System
	QIAvac 24 Plus System
	Vacuum pump (230 V, 50 Hz)
	Veriti Dx 96-well Thermal Cycler or equivalent*
	Agilent 2100 Bioanalyzer System
	Illumina NextSeq™ 500/550
	2100 Expert Software, Agilent Technologies
Pipettes	Pipette 1000 µl, 200 µl, 20 µl, 10 µl, 2 µl
	Multi 12-channel pipette 200 µl, 20 µl
	Pipettor 5 to 100 ml
Racks	50 ml, 15 ml, 5 ml, 1.5/2 ml tube rack
	Tube chain rack
	96-well rack
	96S Super Magnet Plate, Alpaqua® SKU: A001322
	DynaMag™-2 Magnet, Thermo Fisher, #12321D
	Freezer storage boxes

Laboratory equipment	Product
Miscellaneous	Film applicator
	Stopwatch

- Equivalency needs to be determined by the user and use of other thermal cycler devices are at the user's own risk.

8 Storage and handling

8.1 Shipping conditions

The product will be shipped on dry ice. Upon arrival, check whether dry ice is still present in the box and the reagents are frozen.

8.2 General handling precautions



Ensure that temperature and humidity within the remain between 15 °C and 30 °C and between 20 and 85 %, respectively (reduce risk of condensation/evaporation).

Do not eat, drink, or smoke in laboratory areas. Perform equipment maintenance according to manufacturer's instructions.

Decontaminate and dispose of all reagents, specimens, and associated supplies in accordance with government regulations applicable in your location. For accurate and reproducible results, it is essential to avoid contamination with foreign DNA, especially PCR products from previously run plates. The amplified products from previous experiments constitute the most common source of DNA contamination.

The supplied reagents appear visually clear and colourless, except for the PSS Index Primer Plate which contains Bromophenol blue in all the wells (blue colour). If any changes in the appearance of the material or suspected degradation due to wrong storage occurs that may affect the performance of the assay, refer to technical assistance (► chapter 10 *Technical Assistance*, page 489/54).

8.3 Warnings and precautions

This product contains no hazardous material.



Material Safety Data Sheets (MSDS) are available at www.sysmex-inostics.com.

In the case of any serious incident that occurs in relation to the PSS Breast Cancer IVD Kit it must be immediately reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

8.3.1 Specific measures

First aid measures

- **General advice:** In case of persisting effects, consult a physician. Remove contaminated clothing and shoes immediately, and launder thoroughly before reusing.
- **If inhaled:** Remove the affected person from the immediate area. Ensure supply of fresh air.
- **In case of skin contact:** Wash off the affected area with soap and plenty of water.
- **In case of eye contact:** Remove contact lenses. Rinse the eye thoroughly under running water keeping eyelids wide open for at least 10 to 15 minutes. Protect the unaffected eye.
- **If swallowed:** Call a doctor immediately. Do not induce vomiting. Never give anything by mouth to an unconscious person.

8.3.2 Handling and storage

General protective and hygiene measures

Do not eat, drink, or smoke in the laboratory and ensure that good hand washing technique is employed before leaving. Do not inhale vapours. Avoid contact with eyes and skin. Remove soiled or soaked clothing immediately.

Precautions for safe handling

Product handling risks must be minimised by taking the appropriate protective measures and preventative actions. The working process should be designed to rule out the release of the hazardous substances or skin contact as far as it is possible.

Advice on protection against fire and explosion

No special measures necessary.

8 Storage and handling

Conditions for safe storage, including any incompatibilities



Keep the container tightly closed in a dry and well-ventilated place. Opened containers must be carefully resealed and kept upright to prevent leakage.

8.3.3 Reagent handling precautions



To ensure proper use and disposal of reagents and to avoid contamination of reagents, follow the precautions listed below:

- Do not use expired or incorrectly stored reagents.
- Prepare reagents according to provided instructions.
- Reagents should be used only with other reagents from the same kit.
- Reagents from different kits or lots must never be pooled or interchanged.
- Record the open date and mark tubes after each use to ensure reagents are not expired or used beyond the recommended number of freeze-thaw cycles.
- Avoid contamination of reagents by changing gloves frequently. Always change gloves between the handling of reagents and specimens.
- Dispose of unused reagents and waste according to country, federal state, and local environmental regulations.

8.3.4 Safety and contamination precautions



Follow the precautions listed below to maintain a laboratory environment free of DNA contamination and to ensure safety of all personnel:

- Separate the workspaces used for pre- and post-PCR and adhere to a unidirectional workflow from 'clean' (pre-amplification) to 'dirty' (post-amplification areas).
- Ensure that dedicated equipment (including pipettes), supplies, reagents, biohazard waste containers, and lab manuals are present

in each working area. Never exchange these materials between pre- and post-PCR work areas. We recommend colour-coding or labelling of equipment, supplies, and reagents to identify those that belong to a particular area.

- Wear appropriate personal protective equipment throughout the procedure.
 - Wear a lab coat (preferably disposable) and disposable powder-free gloves at all times when working in the pre-PCR and post-PCR areas.
 - To prevent contamination, change gloves frequently between handling specimens and reagents and after contact of the outside of the gloves with the skin.
 - Wear protective eyeglasses at least during plasma preparation, DNA extraction, and PCR product purification with QIAquick®.
 - Wear disposable shoe covers or change shoes, between pre- and post-PCR laboratories and wear disposable arm protection sleeves (required in the pre-PCR laboratory and recommended in the post-PCR laboratory, especially for UID PCR purification and Index PCR).
- When exiting pre- and post-PCR laboratory areas, remove and discard personal protective equipment.
- Handle all specimens as potentially infectious material. If a spill occurs, it is recommended to clean the affected area first with detergent/disinfectant and water and then with ~0.5 % sodium hypochlorite (bleach) solution prepared using deionized water.

Note: *Commercial liquid household bleach (e.g. Clorox brand) typically contains sodium hypochlorite at a concentration of 5.25 %. A 1:10 dilution of household bleach will produce a 0.5 % sodium hypochlorite solution.*

- Use dedicated PCR cabinets for pipetting steps.
- After use, clean PCR cabinets with quaternary ammonium compounds disinfectant (such as RHEOSEPT-WD plus or equivalent) followed by a product designed for removing nucleic acids and nucleases (such as Roti® Nucleic Acid-Free or equivalent).

8 Storage and handling

- After use, clean PCR workspaces with a product designed for removing nucleic acids and nucleases (such as Roti® Nucleic Acid-Free or equivalent).
- Decontaminate the safety cabinet, PCR workspaces and labware (pipettes, tube racks, or other equipment) with ultraviolet (UV) light after use. To ensure UV radiation is effective, regularly clean UV bulbs from the accumulating residue.
- Use only aerosol-resistant sterile pipette tips with filters (lot certified, RNase-, DNase- and pyrogen-free).
- Use only PCR-grade reagents and tubes.
- Keep only one specimen tube or reagent tube open at a time.
- To prevent contamination of multiple-use reagent solutions, prepare working aliquots according to instructions and avoid direct pipetting.

9 Workflow

The PSS Breast Cancer IVD Kit utilizes quantified cfDNA from plasma to detect ctDNA. Before starting the library preparation workflow (Figure 4), as described in this IFU, ensure that the sample preparation workflow is completed as described in Sysmex Inostics' Sample Preparation Guide.

In addition, the first part of the Plasma-SeqSensei™ IVD Software IFU, the run planning, must be completed. If samples are to be diluted because their DNA content is too high, refer to ► chapter 9.1 *UID PCR (Multiplex PCR)*, page 234/54, of this IFU.

Figure 4 describes the process, including individual workflow steps, as well as which IFU to follow for the entire Plasma-SeqSensei™ process.

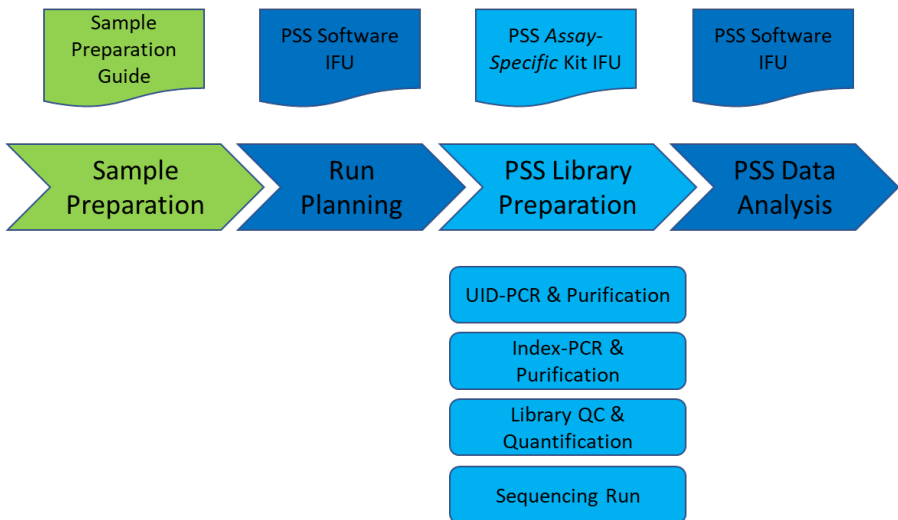


Figure 4: Plasma-SeqSensei™ process, including workflow steps and required documents.



Each PSS Breast Cancer IVD Kit is designed to analyse up to 16 samples on one plate.

If more than 16 samples are to be run on the same sequencing run a second PSS Breast Cancer IVD Kit as well as a Plasma-SeqSensei™ Extension IVD Kit needs to be acquired.

For samples on the second plate (samples 17 to 32) use the PSS Index Primer Plate **IND35 (Plate B)** from the PSS Extension IVD Kit instead of the PSS Index Primer Plate IND34 (Plate A) from the original PSS Breast Cancer IVD Kit.



Warning: When the same PSS Primer Plate (e.g. IND34) is used twice on the same run, results will be not analysable.

If two plates are to be used, always prepare only one plate at a time for each step of the workflow before starting with the other plate. Each plate contains one Positive Control (PC) and one No Template Control (NTC).

9.1 UID PCR (Multiplex PCR)

In the multiplex UID PCR, all target regions are co-amplified while introducing unique molecular barcode sequences. UIDs allow significant background reduction, resulting in ultra-high sensitivity of the Plasma-SeqSensei™ technology.

For the PSS Breast Cancer IVD Kit, samples with a DNA input between 4.3 and 86 ng/116 µl can be analysed. Samples with higher DNA content must be diluted to at least 86 ng/116 µl. Samples with less than 4.3 ng/116 µl have not been validated.

To obtain optimal results, we recommend a **DNA input of 43 ng/116 µl** per sample whenever possible.

Required kits and reagents:

- **PSS Breast Cancer Mpx A** (blue cap), Sysmex Inostics, #ZR851013
- **PSS Breast Cancer Mpx B** (yellow cap), Sysmex Inostics, #ZR851014
- **PSS Breast Cancer Positive Control** (red cap), Sysmex Inostics, #ZR855006
- **PSS No Template Control** (transparent cap), Sysmex Inostics, #ZR854002
- **PSS Quantispike** (green cap), Sysmex Inostics, #ZR856001
- **PSS PCR Master Mix v2, 2x** (black cap), Sysmex Inostics, #ZR850001

Dilution of DNA:

If DNA concentration exceeds the maximum input of 86 ng/116 µl, we recommend diluting the sample to **43 ng/116 µl** according to the following calculations:

$$\text{Dilution Factor} = \frac{\text{Measured concentration in ng/116 } \mu\text{l}}{43 \text{ ng/116 } \mu\text{l}}$$

$$\text{Required eluate volume } [\mu\text{L}] = \frac{130 \mu\text{l}}{\text{dilution factor}}$$

With total eluate volume of 130 µl (for details see ► chapter 4.2 Purification of circulating DNA from plasma of sample preparation guide)

$$\text{AVE buffer volume } [\mu\text{L}] = 130 \mu\text{l} - \text{required eluate volume}$$

The following steps are performed in the sample preparation area in the pre-PCR laboratory.

Preparation:

- All frozen reagents and controls:
 - Thaw
 - Vortex for 5 s
 - Centrifuge for 2 s
- DNA samples to be amplified:
 - Thaw
 - Vortex for 5 s
 - Centrifuge for 2 s
- Check samples for total DNA content.
If total DNA content is too high ($> 86 \text{ ng}/116 \text{ }\mu\text{l}$), dilute sample according to calculation above.
- Label 1.5 ml LoBind® tubes for all samples requiring dilution.
- Clearly label sample tube chains according to the plate layout.
- If processing more than 16 samples, always perform UID PCR setup for only one plate at a time.

UID PCR setup:

Note: *Isolated plasma sample DNA is subjected to a Multiplex PCR in 5 replicates/wells. Positive and Negative Control are analysed in single replicates (columns 1 and 12).*

Note: *Samples are added to the UID PCR plate column by column using a multi-channel pipette, as shown in Figure 5 (for contamination prevention). The sample tube strips are to be arranged parallel to the UID PCR plate.*

Note: *Avoid mixing up samples during the workflow.*

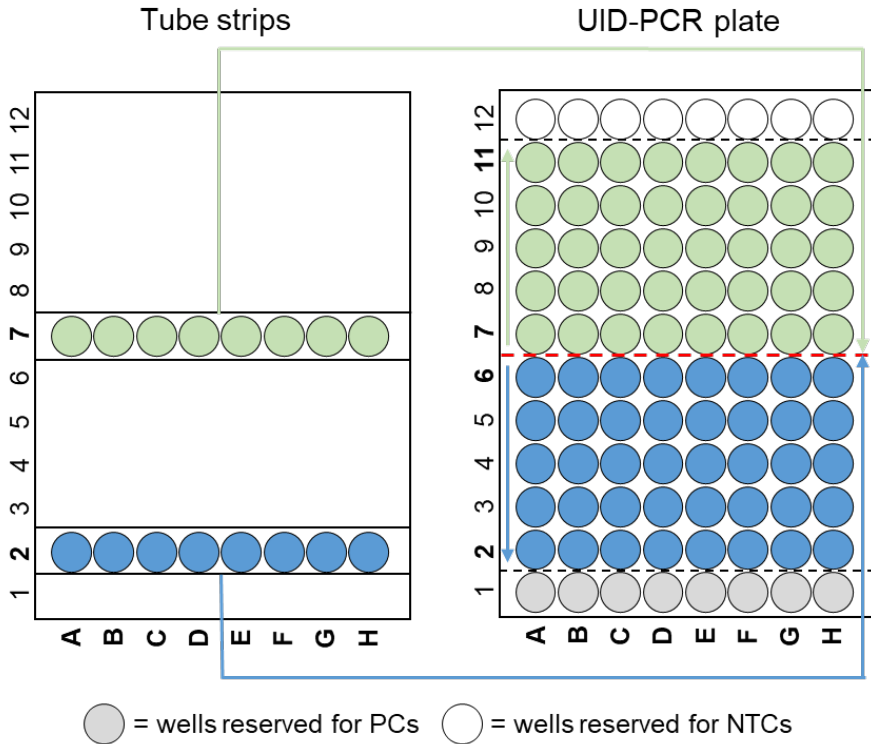


Figure 5: Pipetting scheme used when pipetting from the tube strips into a UID PCR plate

1. Prepare UID PCR Working Mix per plate according to the Table 8: 'UID PCR Working Mix'. Mix by pipetting up and down 10 times using single-channel pipette. The volume of UID PCR Working Mix required for PC and NTC is accounted for in the calculations (see Table 8).

Table 8: Pipetting scheme of the UID PCR Working Mix per plate

Number of samples (1 sample = 5 replicates), with 15 % excess	2	3	4	5	6	7	8	9
PSS PCR Master Mix v2, 2x [µl]	400	567	734	900	1067	1234	1401	1567
PSS Breast Cancer Mpx A [µl]	39	55	71	87	103	119	135	151
PSS Breast Cancer Mpx B [µl]	39	55	71	87	103	119	135	151
Quantspike [µl]	3.0	4.3	5.6	6.8	8.1	9.4	10.6	11.9
Final volume (sum)	481.0	681.3	881.6	1080.8	1281.1	1481.4	1681.6	1880.9

Number of samples (1 sample = 5 replicates), with 15 % excess	10	11	12	13	14	15	16
PSS PCR Master Mix v2, 2x [µl]	1734	1901	2068	2234	2401	2568	2735
PSS Breast Cancer Mpx A [µl]	167	183	199	215	231	247	263
PSS Breast Cancer Mpx B [µl]	167	183	199	215	231	247	263
Quantspike [µl]	13.2	14.4	15.7	17.0	18.2	19.5	20.7
Final volume (sum)	2081.2	2281.4	2481.7	2681.0	2881.2	3081.5	3281.7

2. Add 34.8 µl UID PCR Working Mix to the wells in columns 1 and 12 according to the plate layout.
3. Add 23.2 µl of Positive Control to the well in column 1 according to the plate layout and mix PC by pipetting up and down 10 times.
Add 23.2 µl of Negative Control to the well in column 12 according to the plate layout and mix NTC by pipetting up and down 10 times.
4. Aliquot 187.5 µl of UID PCR Working Mix for each sample in a tube strip.
5. Add 125 µl of sample to the corresponding tube of the tube strip and mix by pipetting up and down 10 times.
6. Using the 200 µl multi-channel pipette, aliquot 58 µl of Sample + Working Mix into 5 wells according to the plate layout.

#	T [°C]	Time [mm:ss]	Go To #	No. Cycles
1	98	02:00	N/A	1
2	98	00:20	N/A	13
3	63	01:30	N/A	13
4	72	00:10	2	13
5	72	02:10	N/A	1
6	4	∞	N/A	1

- **Agencourt AMPure® XP**, Beckman Coulter, #A63881
- **Buffer EB** (Elution Buffer), QIAGEN, #19086
- **Ethanol** (EtOH) ≥ 99.8 %, p.a.
- **RNase and DNase-free distilled water**

- If plate was stored at a temperature between 2 °C and 8 °C run PCR program 'PSS Remove Condensate v1'.

#	T [°C]	Time [mm:ss]	Go To #	No. Cycles
1	4	02:00	N/A	1

Recommendation: *prepare 70 % EtOH during incubation in step 3.*

Table 11: Preparation of 70 % EtOH

	Half plate (8 samples)	Full plate (16 samples)
EtOH ($\geq 99.8\%$, p.a.)	9.1 ml	17.5 ml
Distilled water	3.9 ml	7.5 ml
Total	13 ml	25 ml

- Resuspend beads by rolling the bottle horizontally on the work surface. Roll slowly, pause after each 180 degree turn and wait until the liquid runs down. Repeat until beads are homogenously resuspended and no streaks are visible anymore. Occasionally, invert the bottle.
- Add AMPure® bead solution into a reservoir using a 1 ml pipette.

Table 12: Required volume of AMPure® beads

Half plate (8 samples)	Full plate (16 samples)
4.4 ml	8.3 ml

- If two UID PCR plates are to be used, always perform UID PCR Purification workflow for only one plate at a time.

Purification procedure:

1. Use a multi-channel pipette for the following steps. The UID PCR and UID eluate plates have to be arranged parallel to each other and pipetting is done by column (not by rows, Figure 6).

Note: *Perform all steps by pipetting from left to right.*

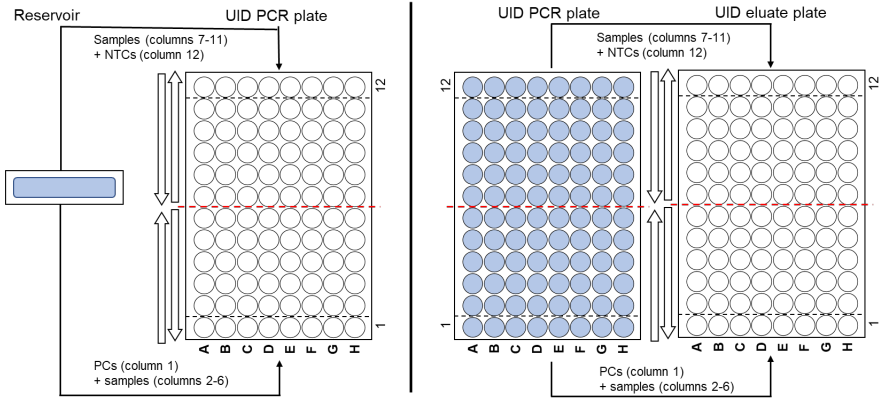


Figure 6: Pipetting scheme used when pipetting from reservoir to UID PCR Plate (left) or UID PCR plate (right) into an UID eluate plate.

2. Add 81 µl AMPure® beads to each well of the UID PCR plate, mix by pipetting slowly 10 times up and down.

Note: Resuspend AMPure® beads 3 times in the reservoir before each aspiration.

Note: Make sure that the beads never dry out.

3. Incubate UID PCR plate at room temperature for 10 min.
4. Put the UID PCR plate onto the magnet plate (Alpaqua) and incubate for 5 min.
5. Make sure that all beads are bound to the magnet. Carefully remove supernatant by pipetting 134 µl.

Note: Do not disturb the ring of separated magnetic beads. Move pipette tip down to the bottom of the well without touching the wall.

6. Transfer 70 % EtOH to a reservoir.

Table 13: Required volume of 70 % EtOH

Half plate (8 samples)	Full plate (16 samples)
13 ml	25 ml

7. Add 100 μ l of 70 % EtOH to each well without resuspending. Incubate for 30 s.
8. Keep plate on the magnet. Remove 110 μ l EtOH carefully and discard.
9. Add 100 μ l of 70 % EtOH to each well without resuspending. Incubate for 30 s.
10. Keep plate on the magnet. Carefully remove 100 μ l EtOH and discard it.
11. Remove residual EtOH using the 20 μ l multichannel pipette.
12. Remove the UID PCR plate from the magnet and let it dry for 2 min.
13. Add required volume of Buffer EB in a reservoir.

Table 14: Required volume of Buffer EB

Half plate (8 samples)	Full plate (16 samples)
7 ml	13 ml

14. Add 120 μ l Buffer EB to each well to elute the DNA and mix at least 10 times up and down carefully.
15. Incubate the UID PCR plate for 2 min at room temperature.
16. Place UID PCR plate onto the magnet and incubate for 1 min.
17. Carefully transfer 110 μ l of each well eluate into the UID eluate plate, discard UID PCR plate.
18. Proceed directly with Index PCR or seal the UID eluate plate. Store it at a temperature between 2 °C and 8 °C up to 7 days or between -15 °C and -30 °C up to 2 months.
19. If processing more than 16 samples, repeat UID PCR purification procedure using the second UID PCR plate starting from step 2.

9.3 Index PCR

Index PCR is performed to amplify purified UID PCR products while introducing indexing tags (well barcodes) and Illumina sequencing adapters. Each PSS Breast Cancer IVD Kit contains one PSS Index Primer Plate IND34 (Plate A) for up to 16 samples. If more than 16 samples are analysed on one sequencing run, a second PSS Index Primer Plate IND35 (Plate B) of the Plasma-SeqSensei™ Extension IVD Kit can be used.

Note: Do **not** use the same plate twice on the same sequencing run. Always use two different plates (IND34 + IND35/Plate A + Plate B).

The wells of the PSS Index Primer Plates are for single use. The positions of the dried Index Primer Plates must match those in the final PCR plate as well as in the plate layout in the Run planning tool of the Plasma-SeqSensei™ IVD Software (Figure 7). Please note which wells are already used. When planning the next run, use remaining index positions/wells and transfer information to the software.

Plate A	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	Sample1				Sample2				NTC		
B		Sample3				Sample4						
C		Sample5				Sample6						
D		Sample7				Sample8						
E												
F												
G												
H												

Figure 7: Plate layout example for Index PCR

Required kits and reagents:

- **PSS Index Primer Plate IND34** (Plate A), Sysmex Inostics, #ZR852004
- *optional:* **PSS Index Primer Plate IND35** (Plate B), Sysmex Inostics, #ZR852005
- **PSS PCR Master Mix v2, 2x** (black cap), Sysmex Inostics, #ZR850001

- **Water, nuclease-free** (transparent cap), Sysmex Inostics, #ZR224006
- **Buffer EB** (Elution Buffer), QIAGEN, #19086

The following steps are performed in the post-PCR laboratory.

Preparation:

- Provide all reagents and let them reach working temperature.
- Label all required plastics (Index PCR Working Mix tube, 1 x reservoir, DIL plate, Index PCR plate).
- Place required Buffer EB in a reservoir and cover until usage.

Table 15: Required volume of Buffer EB

Half plate (8 Samples)	Full plate (16 Samples)
5.5 ml	10 ml

- If plate was stored at a temperature between 2 °C and 8 °C, perform PCR program 'PSS Remove Condensate_v1'.
- If UID eluate plate was stored, spin it down at 1,000 x g for 5 s.
- If processing two UID eluate plates, always perform Index PCR workflow for only one plate at a time.

Preparation of Dilution (DIL) Plate:

Note: Use a multi-channel pipette for all steps of DIL plate preparation.

1. Place UID eluate plate onto the magnet and incubate for 1 min.
2. Add 99 µl of Buffer EB per well to the DIL plate according to the plate layout.
3. Transfer 5 µl per well from the UID eluate plate to the DIL plate, rinse the pipette tip by pipetting up and down 3 times.

Note: If plate was stored, mix each well from UID eluate plate by pipetting up and down 5 times.

4. Mix thoroughly by pipetting 70 µl up and down 10 times.

- Seal the UID eluate plate. Store plate with residual volume at a temperature between 2 °C and 8 °C up to 7 days or between -15 °C and -30 °C for up to 2 months.

Preparation of PSS Index Primer Plate:

- Spin down the PSS Index Primer Plate at 1,000 x g for 5 s.
- Prepare the required well amount of PSS Index Primer Plate by piercing the aluminium foil with 200 µl tips.

Note: Check if the correct plate (IND34 or IND35/A or B) was used in the correct orientation.

Preparation of Index PCR:

- Vortex all reagents for 5 s and spin them down for 2 s.
- Prepare Index PCR Working Mix according to the following table. Vortex the mix for 5 s and spin it down for 2 s.

Table 16: Pipetting scheme of the Index PCR Working Mix

Number of samples, with 10 % excess	2	3	4	5	6	7	8	9
PSS PCR Master Mix v2, 2x [µl]	165	234	303	371	440	509	578	646
Water, nuclease-free [µl]	99	140	181	223	264	305	347	388
Final volume (sum)	264	374	484	594	704	814	925	1034

Number of samples, with 10 % excess	10	11	12	13	14	15	16
PSS PCR Master Mix v2, 2x [µl]	715	784	853	921	990	1059	1128
Water, nuclease-free [µl]	429	470	512	553	594	635	677
Final volume (sum)	1144	1254	1365	1474	1584	1694	1805

Note: Volume for one PC and NTC is already included.

10. Add 20 μ l of Index PCR Working Mix per well into PSS Index Primer Plate.

Recommendation: *Transfer Working Mix to tube strips with multi-channel pipette.*

11. Add 5 μ l of template from the DIL plate to the PSS Index Primer Plate and mix thoroughly by pipetting up and down 10 times until reagents are resuspended. Use a multi-channel pipette. After use discard the DIL plate.

Note: *Visually check the correct orientation of DIL plate and PSS Index Primer Plate to avoid sample mix up.*

Note: *Check if there are blue dots visible on the bottom of the wells. A blue dot is an indication that reagents were poorly resuspended. If blue dots are still visible, repeat resuspension, by pipetting up and down 10 times until no blue dots are visible and the liquid has turned blue.*

12. Seal the PSS Index Primer Plate with adhesive PCR film and spin down at 1,000 x g for 5 s.
13. In case of using only part of the PSS Index Primer Plate, transfer the whole volume of the PSS Index Primer Plate into a new PCR plate.

Note: *Check the correct orientation of the PSS Index Primer Plate and new PCR plate to avoid sample mix up.*

Recommendation: *Use 2x the 20 μ l multi-channel pipette instead of 1x the 200 μ l multi-channel pipette.*

14. Seal the new PCR plate with adhesive PCR film and spin down at 1,000 x g for 5 s.
15. Seal the used wells of PSS Index Primer Plate (only applicable if PSS Index Primer Plate will not be discarded) and store it at a temperature between 2 °C and 8 °C in the dark.
16. Start PCR with the program 'IDX BC PSS_v1' within 15 min.

Table 17: Tt profile of IDX BC PSS_v1

PCR cycler: Veriti

Volume setting: 25 µl

☒ Heating lid

Lid temperature:

96 °C

#	T [°C]	Time [mm:ss]	Go To #	No. Cycles
1	98	00:30	N/A	1
2	98	00:10	N/A	20
3	65	00:10	N/A	20
4	72	00:10	2	20
5	72	05:00	N/A	1
6	4	∞	N/A	1

17. If processing more than 16 samples, repeat Index PCR procedure using the second UID eluate plate starting from step 1.
18. After the PCR spin down the Index PCR plates at 1,000 x g for 5 s. Store plates at a temperature between 2 °C and 8 °C up to 7 days, between -15 °C and -30 °C up to 2 months or proceed directly with Index PCR purification.

9.4 Index PCR purification

Important: This step combines **all sample and control wells of one plate** into **one library**. If two plates were prepared (IND34 and IND35/Plate A and Plate B) combine only the samples and controls from **one plate**, to obtain two sequencing libraries. Additionally, the purification removes dNTPs, primers, primer dimers and salts that would hinder subsequent sequencing.

Required kits and reagents:

- **Agencourt AMPure® XP**, Beckman Coulter, A63881
- **QIAquick® PCR Purification Kit**, QIAGEN, #28106
- **Buffer PB**, QIAGEN, #19066

- **Ethanol (EtOH) \geq 99.8 %, p.a.**
- **RNase and DNase-free distilled water**

The following steps are performed in the post-PCR laboratory.

Preparation:

- If plate was stored at a temperature between 2 °C and 8 °C run PCR program 'PSS Remove Condensate_v1'.
- Label all required plastics (EtOH dilution tube, PB dilution tubes, Spin columns, QIAquick® eluate tubes, Index eluate tubes).
- Prepare a bin for liquid waste.
- Prepare fresh 70 % EtOH according to the table below. Invert 10 times.

Table 18: Preparation of 70 % EtOH

Reagent	Volume
EtOH \geq 99.8 %, p.a. [ml]	2.8
Distilled water [ml]	1.2
Needed volume [ml]	4.0

- Before removing seal, spin down Index PCR plate at 1,000 x g for 5 s.
- Collect the entire liquid of **all wells (samples and controls) from one plate** with a 200 μ l pipette set to 30 μ l into an appropriate container.

Note: *If using a multi-channel pipette, first pool all wells per column in one row of a new PCR plate stripe. Then transfer the content of each well to an appropriate container with a single-channel pipette.*

- If two Index PCR plates are to be used, always perform Index PCR Purification for only one plate at a time.

Note: *Use a single-channel pipette for the following steps in this protocol.*

1st purification with QIAquick®:

1. For purification with QIAquick® PCR Purification Kit refer to the protocol 'QIAquick PCR Purification using a Vacuum Manifold' in the handbook of the manufacturer. Deviations in handling are described below.
2. First, add calculated volume (see Table 19) of the Buffer PB to the respective tube, vortex it for 3s and spin it down at 500 x g for 2 s.

Table 19: Calculation of required volume of Buffer PB

Reagent	Per well	___ x wells
Sample volume [µl]	25	
Buffer PB [µl]	125	
Total volume [µl]	150	

3. Perform following steps of PCR purification according to the instructions described in QIAGEN's handbook.

Note: The maximum loading volume of the column is 800 µl. For pooled sample volumes greater than 800 µl, load again.

Note: Visually check at every step that the correct volume is applied to the column and the complete liquid is passed through the filter.

Note: In case of clogged columns, refer to the troubleshooting guide in QIAGEN's handbook.

4. For DNA elution, place a QIAquick® column into a clean 1.5 ml LoBind® tube.
5. Add 50 µl of the Buffer EB to the centre of the QIAquick® membrane and incubate for 1 min at RT before last centrifugation step.

2nd purification with AMPure® beads:

6. Transfer 45 µl of the eluate into a new LoBind® tube. Discard the previous one.

7. A) When using the original AMPure® bottle, resuspend beads by rolling the bottle horizontally on the work surface. Roll slowly, pause after 180 degrees turn and wait until the liquid runs down. Repeat until beads are homogeneously resuspended.

B) When using aliquots of AMPure® beads, mix the beads by inverting at least 10 times. ensure that the beads are completely resuspended.
8. Add 40 µl AMPure® beads to the eluate, vortex for 10 s and spin down 3 s.
9. Incubate at RT for 5 min.
10. Open the tube, place it in the DynaMag-2 and incubate for 2 min at RT.

The following steps (11 to 15) are performed while tubes are in the magnetic rack:

11. Using a 200 µl pipette, set to 100 µl remove supernatant and discard it.

Note: *Lift the tube ~1 cm and press the bottom completely against the magnet to ensure that all beads are fixed.*
12. Add 500 µl 70 % EtOH and incubate for 30 s at RT.
13. Remove supernatant and discard it.
14. Add 500 µl 70 % EtOH and incubate for 30 s at RT. During incubation, turn the tube around the vertical axis by 180 degrees to ensure efficient mixing. Turn it back slowly at the earliest after 5 s.
15. Remove all the supernatant and discard it. Remove residual EtOH using the 20 µl pipette.
16. Remove the tube from the DynaMag-2 and let it dry for 2 min at RT with open lid.
17. Add 15 µl Buffer EB and completely resuspend bead mix by vortexing 10 s. Spin down for 3 s and incubate for 1 min at RT.
18. Open the tube, place it in DynaMag-2 and incubate for 1 min at RT.

19. Using a 20 µl pipette, set to 20 µl to transfer the whole supernatant to tube 'Index eluate'.

Note: *Lift the tube ~1 cm and press the bottom completely against the magnet to ensure that all beads are fixed.*

20. Discard tube labeled with QIAquick® eluate.
21. Store tube 'Index eluate' at a temperature between 2 °C and 8 °C up to 7 days, at a temperature between -15 °C and -30 °C up to 2 months or proceed directly with Bioanalyzer quantification.
22. If processing more than 16 samples, repeat Index PCR purification procedure using the second Index PCR plate starting from step 2.

9.5 Library QC (Bioanalyzer)

Library QC is performed using a Bioanalyzer to check each library for side products and average size determination. For each library, quantifications should be performed in three replicates.

The PSS Breast Cancer IVD Kit has been developed using Agilent's Bioanalyzer DNA 1000 Kit.

Required kits and reagents:

- **DNA 1000 Kit**, Agilent, #5067-1504
- **Buffer EB** (Elution Buffer), QIAGEN, #19086
- **RNase and DNase-free distilled water**

The following steps are performed in the post-PCR laboratory.

Preparing the Bioanalyzer:

For all steps, refer to the manual of the Agilent Bioanalyzer.

Preparing the Bioanalyzer dilution (BA_DIL):

1. Calculate the **volumes required for BA_DIL**:

$$\text{Dilution factor} = \frac{\text{Total DNA input}}{43}$$

with total DNA input of all analyzed samples in ng/116µl

(measured using Qubit™, see ► chapter 4.3 Sample quantification (Qubit™) of sample preparation guide)

$$\text{Buffer EB } [\mu\text{L}] = (3 * \text{dilution factor}) - 3 \mu\text{L}$$

$$\text{BA_DIL } [\mu\text{L}] = 3 \mu\text{L Index eluate} + X \mu\text{L Buffer EB}$$

2. Dilute Index eluate in a fresh tube according to the calculation. Vortex it briefly and spin down for 3 s. Store the remaining index eluate at a temperature between 2 °C and 8 °C up to 7 days or between -15 °C and -30 °C up to 2 months.

Note: Ensure that at least 10 µl total volume of BA_DIL is available.

Preparing the Labchip:

- For all steps, refer to the manual of the Agilent Bioanalyzer.

Note: The sample measurement should be performed in three technical replicates.

Note: BA_DIL is stable at a temperature between 2 °C and 8 °C up to 7 days or between -15 °C and -30 °C up to 2 months.

Data analysis:

3. Verify that the ladder plot profile looks similar to **Error! Reference source not found.** below and contains 13 peaks with the lowest at 15 bp and the highest at 1,500 bp (these are the markers that will be in every sample read) with a flat baseline (see Figure 8).

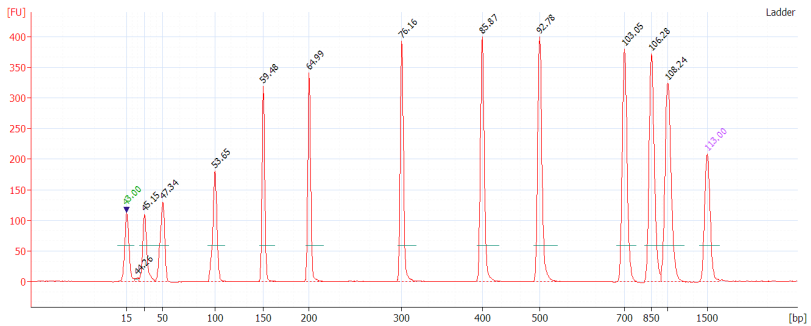


Figure 8: Ladder electropherogram

4. Double-click the electropherogram belonging to Well 1 and select the Peak Table tab (see Figure 9).

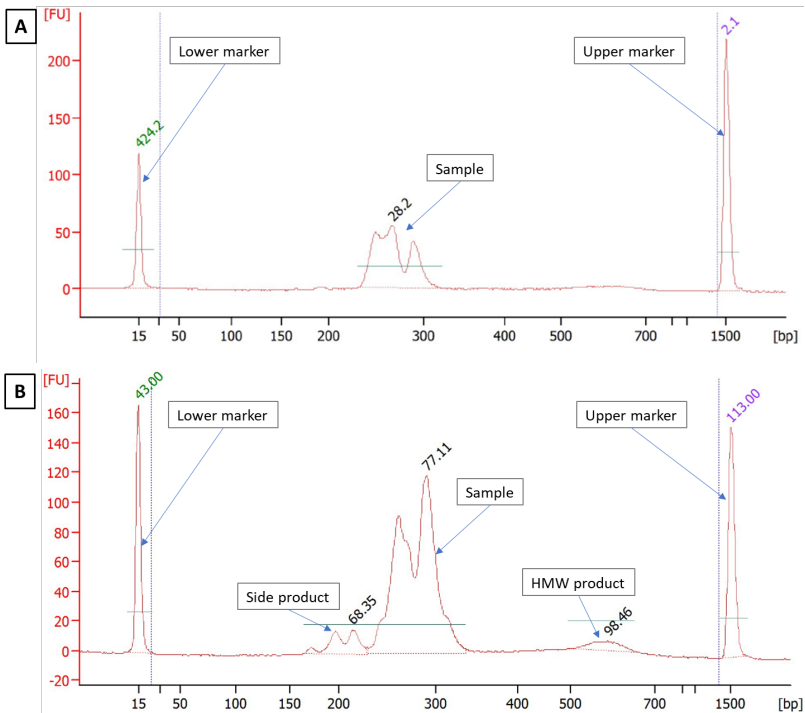




Figure 9: Sample Electropherograms. (A) An optimal electropherogram without any side products, (B) exemplary electropherogram with side products (e.g. primer dimer and gDNA (HMW product)).

5. Select [Manual Integration] () by right-clicking in the electropherogram.
6. Use the blue lines to delineate **all visual peaks**, namely sample product, primer dimer (side product) and high molecular weight (HMW) product **along the zero line** (shown in Figure 9B).

Note: Use the 'Ctrl' key to detach the ends of the blue lines from the red line. If a line is selected, remove it by right-clicking [Remove Peak]. Insert additional blue lines at any position via right-click [Add Peak].

7. Using [Peak Description] () , select [Mol Peak Molarity] to show the respective molarity for each peak.
8. Save the file.
9. Repeat steps 4 to 8 for the remaining wells of each replicate.
10. Calculate mean, standard deviation and coefficient of variation (CV) of molarity sum of all products based on triplicate measurement.

Acceptance and rejection criteria:

- DNA quality check: if the sum of product, primer dimer, and HMW is < 2.0 nmol/l, the DNA concentration is too low for sequencing.
- Signal-to-Noise ratio (SNR) acceptance criterion: ≥ 90 %

$$SNR [\%] = \frac{\text{molarity of specific product}}{\text{sum of specific product, side and HMW product}} * 100$$

- Precision check acceptance criterion: CV of molarity sum of all products ≤ 10 %

$$Coefficient\ of\ Variation\ [\%] = \frac{\text{standard deviation}}{\text{mean}} * 100$$

Note: *If the Signal-to-Noise ratio or CV is failing due to one outlier, that value may be excluded from the sample calculations.*

Note: *If one or more criteria fail prepare a new BA_DIL and repeat the Bioanalyzer run.*

9.6 Sequencing on Illumina NextSeq™ 500/550

Sequencing of libraries is performed using an Illumina NextSeq™ 500 or 550 as described in the Instructions for use provided by Illumina.

Required kits and reagents:

- **NextSeq™ 500/550 Mid Output Kit v2.5 (150 Cycles)**, Illumina, #20024904
≤ 377 ng total DNA input (based on Qubit™ measurement ► chapter 4.3 *Sample quantification (Qubit™)* of Sample preparation guide) OR
- **NextSeq™ 500/550 High Output Kit v2.5 (150 Cycles)**, Illumina, #20024907
≤ 1304 ng total DNA input (based on Qubit™ measurement ► chapter 4.3 *Sample quantification (Qubit™)* of Sample preparation guide)
- **Sodium hydroxide (NaOH)**, 1 M
- **Trizma® hydrochloride solution pH7.0**, 1 M
- **Buffer EB** (Elution Buffer), QIAGEN, #19086
- **RNase and DNase-free distilled water**

The following steps are performed in the post-PCR laboratory.

Preparing the samples (2 nM Starting Library Concentration) for sequencing:

1. Calculate the required total volume of a 2 nM library:

$$\text{Total Volume } [\mu\text{L}] = \frac{3 \mu\text{L BA_DIL} * \text{Concentration}_{\text{Library in nM}}}{2 \text{ nM}}$$

2. Calculate the required **volume of Buffer EB**:

$$\text{Volume}_{\text{Buffer EB}} [\mu\text{l}] = \text{Total Volume} - 3 \mu\text{l BA_DIL}$$

3. Prepare 2 nM library dilution for each library according to the following calculation:

$$2 \text{ nM Library Dilution} = 3 \mu\text{l BA_DIL} + \text{Volume}_{\text{Buffer EB}}$$

Note: Do not pipette < 3 μl .

Note: If 2 nM dilution volume is < 10 μl adjust total volume.

4. *Optional:* If two plates were processed pool the two separate 2 nM library dilutions in a final Library Pool Mix of 10 μl according to the following equations:

$$\text{DNA input}_{\text{total}} = \sum \text{DNA input}_{\text{plateA}} + \sum \text{DNA input}_{\text{plateB}}$$

$$\text{Volume}_{\text{plateA}} = \frac{10 \mu\text{l}}{\text{DNA input}_{\text{total}}} * \text{DNA input}_{\text{plateA}}$$

$$\text{Volume}_{\text{plateB}} = \frac{10 \mu\text{l}}{\text{DNA input}_{\text{total}}} * \text{DNA input}_{\text{plateB}}$$

Note: Only pipette volumes within accepted ranges of available pipettes. If lower volumes are to be pipetted, increase total volume of final Library Pool Mix instead.

5. Perform the following steps (library denaturation and dilution of denatured library to 20 pM) according to Illumina's handbook (NextSeq™ 500 and 550 Sequencing Systems Denature and Dilute

Libraries Guide, protocol A: Standard Normalization Method (document #15048776 v16 or higher).

6. Dilute (pooled) library to loading concentration depending on chosen sequencing kit:

Table 20: Required volumes for sequencing

	Mid Output Kit	High Output Kit
Final concentration	1.0 pM	1.1 pM
Library input	65 µl	71 µl
HT1 buffer	1235 µl	1229 µl

7. Start sequencing run according to Illumina's protocol (NextSeq™ 550 System Guide, document # 15069765v06) using following Run Parameter Settings:

Table 21: Sequencing parameters

Read type	Single read
------------------	-------------

	Read 1	Index 1	Index 2*
Read length	148	10	10

* Index 2 read length will only be included with the use of two plates on the same sequencing run.

Next steps

Refer to the PSS IVD Software IFU (Data Analysis module) to proceed with the sequencing data analysis.

10 Technical assistance

If any problems occur during the workflow of the Plasma-SeqSensei™ Breast Cancer IVD Kit, please contact your local Sysmex support for assistance.



Note: Instructions for Use are available in different languages online at www.sysmex-inostics.com.

11 Performance characteristics

11.1 Analytical Sensitivity

The evaluation of Limit of Detection (LoD) was carried out according to the specifications stated in the guideline *CLSI EP17-A2*.

The analysis included insertions, deletions, substitutions, and deletion-insertions.

The LoD derived cutoff is 6 MM.

Analyte (MM)	Hit Rate in % (n=108)	LoD95
20	100	4.98 (CI95 4.05 MM – 6.56 MM)
10	100	
5	95.4	
2.5	82.4	
1.25	49.1	
0.625	36.1	

11.2 Analytical Specificity

The design was checked in-silico using BLAST analysis against possible cross-reactivity and has been confirmed as highly specific. Off-target sequences included the human genome as well as publicly available DNA sequences of typical blood-borne microorganisms/viruses like *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Aspergillus niger*, Cytomegalovirus, Epstein-Barr-Virus, HIV, and Hepatitis-C Virus.

11.3 Precision/Repeatability

The evaluation for Precision was carried out according to the specifications stated in the guideline *CLSI EP05-A3*.

The qualitative precision is > 99%.

The quantitative repeatability is < 10% (CV max.) and intermediate precision is < 15 % at ≥ 20 MM.

Target MM	Repeatability (CV max in %)	Intermediate precision
500	2.54	13.84
100	5.99	13.10
50	7.76	14.15
20	5.65	14.02

11.4 Measuring Range/Linearity

The determination of the linear range on DNA input was carried out according to the specifications stated in the guideline *CLSI EP06-A*.

The PSS workflow shows linearity within the assay DNA input range (4.3 to 86 ng per sample).

11.5 Interfering Substances

Determination of interfering substances was carried out according to the specifications stated in the guideline *CLSI EP07-A2*.

The PSS workflow has been confirmed as robust against common interfering substances. The presence of hemoglobin (≤ 2 g/l), bilirubin (≤ 200 mg/l), triglycerides (≤ 15 g/l), melanin (≤ 0.2 μ g/l) and ethanol (≤ 86.8 mmol/l) have no impact on test validity and results.

12 Glossary and terminologies

Term	Definition
bp	Base pair
BA_Dil	Bioanalyzer dilution
CDx	Companion diagnostics
cfDNA	Cell-free DNA
COSMIC	Catalogue of somatic mutations in cancer
ctDNA	Circulating tumour DNA
dbSNP	Single nucleotide polymorphism database
dNTP	Deoxyribonucleotide triphosphate
DNA	Desoxyribonucleic acid
EB	Elution buffer
EDTA	Ethylene diamine tetra acetic acid
EtOH	Ethanol
gDNA	Genomic DNA
HMW	High molecular weight
IDX	Index
IFU	Instructions for use
MAF	Mutant allele fraction
MM	Mutant molecules
Mpx	Multiplex primer mix
NaOH	Sodium hydroxide
NGS	Next-generation sequencing
NTC	No template control

Term	Definition
PC	Positive control
PCR	Polymerase chain reaction
PSS	Plasma-SeqSensei™
QC	Quality control
RNA	Ribonucleic acid
RT	Room temperature
SNV	Single nucleotide variant
UID	Unique identifier

13 References

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