



Plasma-SeqSensei™

Breast Cancer IVD Kit

Instructions for Use

December 2023

ZR150547.R3

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™ 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 Plasma-SeqSensei™ Breast Cancer IVD Kit must only be used in conjunction with the Plasma-SeqSensei™ 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 Plasma-SeqSensei™ 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 Plasma-SeqSensei™ technology (4).

3 Test principle

The Plasma-SeqSensei™ 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 Plasma-SeqSensei™ technology is shown in Figure 1.

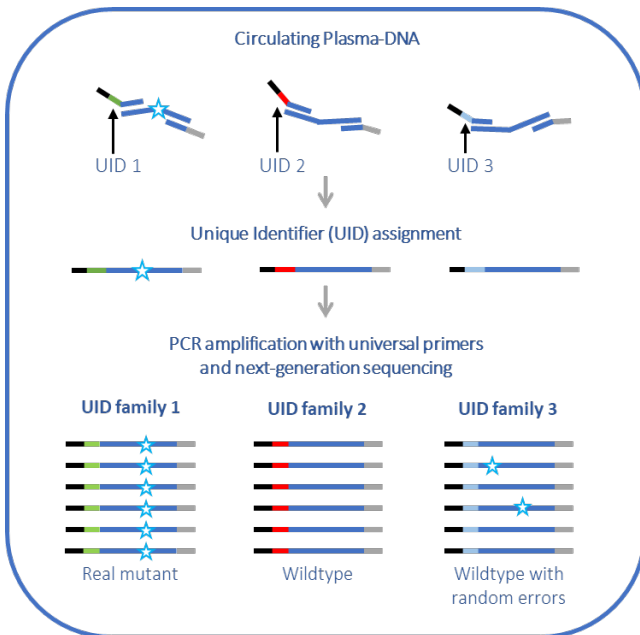


Figure 1: Principle of the Plasma-SeqSensei™ technology

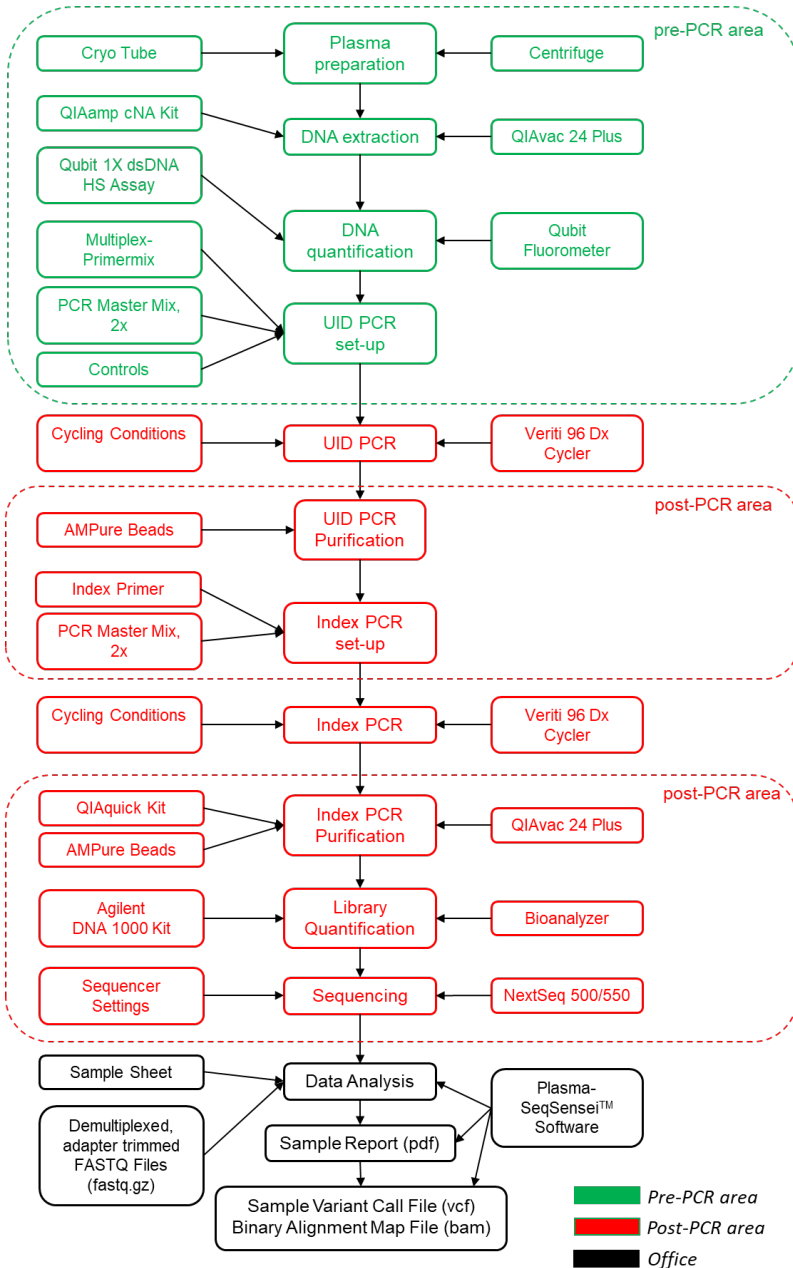


Figure 2: Workflow overview of the Plasma-SeqSensei™ method

4 Covered regions

Table 1: Covered regions with Plasma-SeqSensei™ Breast Cancer IVD Kit

Gene	Transcript*	Coding sequence start	Coding sequence end	Amino acid start	Amino acid end
AKT1	ENST00000554581.1	47	69	17	23
ERBB2	ENST00000269571.5	907	947	303	315
ERBB2	ENST00000269571.5	2,258	2,307	754	769
ERBB2	ENST00000269571.5	2,308	2,360	770	786
ESR1	ENST00000440973.1	1,108	1,143	370	381
ESR1	ENST00000440973.1	1,378	1,420	460	473
ESR1	ENST00000440973.1	1,583	1,614	529	538
KRAS	ENST00000256078.4	8	43	4	14
PIK3CA	ENST00000263967.3	254	278	86	92
PIK3CA	ENST00000263967.3	329	352	111	117
PIK3CA	ENST00000263967.3	353	367	119	122
PIK3CA	ENST00000263967.3	1,033	1,058	345	352
PIK3CA	ENST00000263967.3	1,085	1,115	363	371
PIK3CA	ENST00000263967.3	1,252	1,264	418	421
PIK3CA	ENST00000263967.3	1,348	1,387	450	462
PIK3CA	ENST00000263967.3	1,611	1,659	538	553
PIK3CA	ENST00000263967.3	2,138	2,184	714	728
PIK3CA	ENST00000263967.3	3,118	3,169	1,040	1,056
TP53	ENST00000269305.4	144	232	49	77
TP53	ENST00000269305.4	293	375	99	125
TP53	ENST00000269305.4	376	423	126	141
TP53	ENST00000269305.4	451	537	151	179
TP53	ENST00000269305.4	574	659	192	219
TP53	ENST00000269305.4	695	782	233	260
TP53	ENST00000269305.4	783	856	262	285
TP53	ENST00000269305.4	888	919	297	306
TP53	ENST00000269305.4	920	993	308	331
TP53	ENST00000269305.4	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 characterised, likely or predicted functional impact are reported. These are based on publicly available databases such as COSMIC (7) and/or peer-reviewed scientific literature (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 somatic mutation reporting based on observed MAF values. However, they are listed in a separate table and marked as potential germline mutations, as 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.

This test detects nucleotide changes, and the resulting amino acid changes are depicted in the report. In case of only partially covered amino acid-encoding nucleotide triplets (amplicon borders) the amino acid annotation in the report is made based on the assumption, that the bases not covered by the assay, correspond to the reference sequence.

The Plasma-SeqSensei™ 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 Plasma-SeqSensei™ 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 Plasma-SeqSensei™ 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.

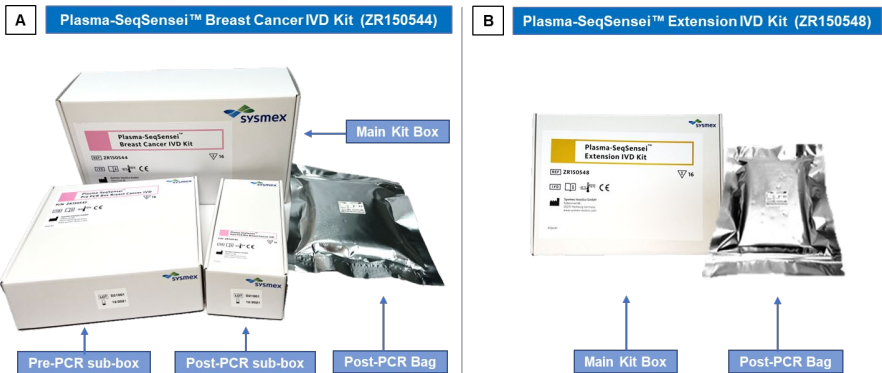
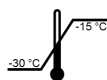


Figure 3: Plasma-SeqSensei™ Breast Cancer IVD Kit boxes with bag (A) and Plasma-SeqSensei™ Extension IVD Kit box and bag (B) with their respective storage locations (pre/post-PCR areas) are shown.

7.1 Supplied material

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



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

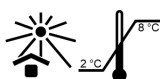


Once opened, reagents are stable for 30 days or until the expiration date, whichever comes first (water excluded).

Table 2: Material supplied with Plasma-SeqSensei™ Breast Cancer IVD Kit (ZR150544)

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

* Names can deviate by the addition of PSS before the name, depending on kit lot.



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

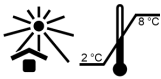
² Index Primer Plate IND34 is also named Plate A in workflow and Plasma-SeqSensei™ IVD Software

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 Plasma-SeqSensei™ Extension IVD Kit (ZR150548)

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

* Name can deviate by the addition of PSS before the name, depending on kit lot.



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

² Index Primer Plate IND35 is also named Plate B in workflow and Plasma-SeqSensei™ IVD Software

Table 4: Composition of supplied material

Name	Composition
Breast Cancer Mpx A	Primers Tris EDTA Buffer
Breast Cancer Mpx B	Primers Tris EDTA Buffer
Breast Cancer Positive Control	Double-stranded synthetic DNA wild-type/mutant Tris EDTA Buffer Carrier RNA
No Template Control	Tris EDTA Buffer
Quantispike	Double-stranded synthetic DNA Tris EDTA Buffer Carrier RNA
Index Primer Plate	Index primers (well-specific) Bromophenol blue
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 Table 5, Table 6 and Table 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 Plasma-SeqSensei™ Breast Cancer IVD Kit

Material	Product
Reagents and kits	Ethanol (EtOH) ≥ 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, #28104 or #28106
	* Buffer PB, QIAGEN, #19066
	* DNA 1000 Kit, Agilent, #5067-1504 <ul style="list-style-type: none"> ■ Microfluidic chips ■ Reagents
	* Qubit™ 1X dsDNA HS Assay Kit, Thermo Fisher, #Q33230 (100 rxns) or #Q33231 (500 rxns)
	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 Plasma-SeqSensei™ 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
	* Qubit™ Assay Tubes, Thermo Fisher, #Q32856
	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, only 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 Plasma-SeqSensei™ 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
	Chip Priming Station, Agilent, #5065-4401
	Illumina NextSeq™ 500/550
	2100 Expert Software, Agilent Technologies
	Pipettes
Multi 8- or 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

7 Reagents, consumables and equipment

Laboratory equipment	Product
	96S Super Magnet Plate, Alpaqua® SKU: A001322
	DynaMag™-2 Magnet, Thermo Fisher, #12321D
	Freezer storage boxes
Miscellaneous	Film applicator
	Stopwatch

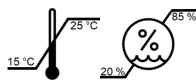
- Equivalency needs to be determined by the user and use of other thermal cycler devices are at the user's own risk.

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 laboratories remain between 15 °C and 25 °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 Plasma-SeqSensei™ 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 48/58).

8.3 Warnings and precautions

This product contains no hazardous material.



Material Safety Data Sheets (MSDS) are available at <https://sysmex-inostics.com/products/kit-specs/>.

In the case of any serious incident that occurs in relation to the Plasma-SeqSensei™ 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-PCR 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-PCR 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 deionised 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 Plasma-SeqSensei™ Breast Cancer IVD Kit utilises 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 23/58, 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.

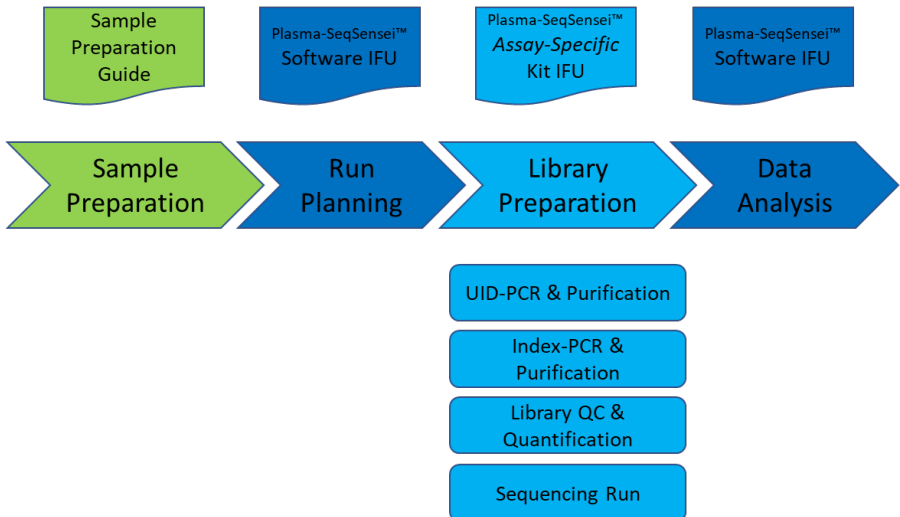


Figure 4: Plasma-SeqSensei™ process, including workflow steps and required documents.



Each Plasma-SeqSensei™ 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 Plasma-SeqSensei™ 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 Index Primer Plate **IND35 (Plate B)** from the Plasma-SeqSensei™ Extension IVD Kit instead of the Index Primer Plate IND34 (Plate A) from the original Plasma-SeqSensei™ Breast Cancer IVD Kit.



Warning: *When the same Plasma-SeqSensei™ Index 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).

Note: *Always use the smallest possible sequencing kit. The NextSeq™ High Output kit v2.5 can only be used with 5 or more samples.*

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 Plasma-SeqSensei™ 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. Samples with less than 4.3 ng/116 µl have not been validated and will yield invalid results.

Note: *Qubit measurement of the samples merely represents a rough approximation of input DNA to determine the sample load. The final and possibly differing quantification of the samples will occur during the sequencing of the library using the internal quantifier (Quantispike).*

Recommendation: *To obtain optimal results, we recommend a **DNA input of 43 ng/116 µl** per sample whenever possible, even for samples at or below 86 ng/116 µl.*

Required kits and reagents:

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

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

Preparation:

- All frozen reagents, DNA samples and controls:
 - Thaw
 - Vortex for 5 s
 - Centrifuge for 2 s
- Check samples for total DNA content.
If total DNA content is too high (e.g. > 86 ng/116 µl), dilute sample according to calculation below.
- Label 1.5 ml LoBind® tubes for all samples requiring dilution.
- Clearly label sample tube chains according to the plate layout.

Dilution of DNA:

If DNA concentration exceeds the maximum input of 86 ng/116 µl or is close to the upper limit, we recommend preparing a new tube with the sample diluted to **43 ng/116 µl** according to the following calculations:

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

$$\text{Required eluate volume } [\mu\text{L}] = \frac{135 \mu\text{l}}{\text{dilution factor}}$$

with total eluate volume of 135 μl (for details see ► chapter 4.2 Purification of circulating DNA from plasma of sample preparation guide)

$$\text{AVE buffer volume } [\mu\text{L}] = 135 \mu\text{l} - \text{required eluate volume}$$

$$\text{Diluted sample } [135 \mu\text{l}] = \text{required eluate volume} + \text{AVE buffer volume}$$

Note: AVE buffer for dilution of the sample is part of the QIAamp® Circulating Nucleic Acid Kit (QIAGEN) (for details see ► chapter 4.2 Purification of circulating DNA from plasma of sample preparation guide).

Re-quantification of diluted samples:

For diluted samples re-quantify dilutions using the Qubit™ according to ► chapter 4.3 Sample quantification (Qubit™) of sample preparation guide.

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.

Note: If processing more than 16 samples, always perform UID PCR setup for only one plate at a time.

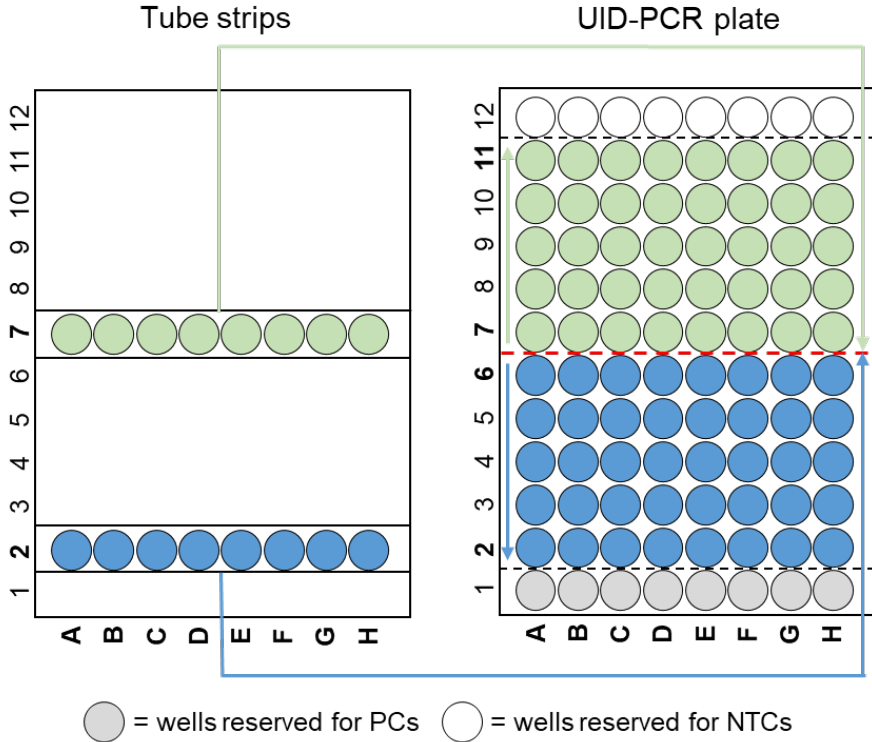


Figure 5: Pipetting scheme used when pipetting from the tube strips into a UID PCR plate

1. Prepare UID PCR Working Mix per plate according to Table 8: 'UID PCR Working Mix'. Mix by pipetting up and down 10 times using a 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
PCR Master Mix v2, 2x [µl]	400	567	734	900	1067	1234	1401	1567
Breast Cancer Mpx A [µl]	39	55	71	87	103	119	135	151
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
PCR Master Mix v2, 2x [µl]	1734	1901	2068	2234	2401	2568	2735
Breast Cancer Mpx A [µl]	167	183	199	215	231	247	263
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

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

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.
7. Seal the plate with adhesive PCR film and spin down at 1,000 x g for 5 s.

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.

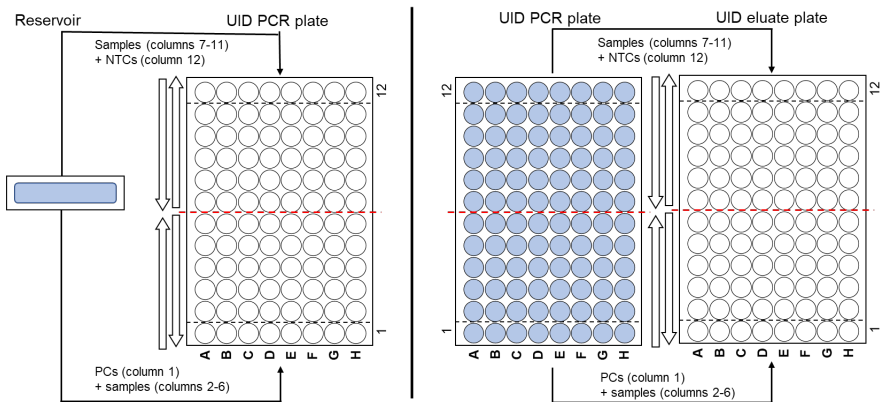


Figure 6: Pipetting scheme used when pipetting from reservoir to UID PCR Plate (left) or UID PCR plate (right) into an UID eluate plate.

2. Add 81 μ l 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 15 °C to 25 °C for 10 min.

- Place the UID PCR plate onto the magnet plate (Alpaqua) and incubate for 5 min.
- Make sure that all beads are bound to the magnet. Carefully remove supernatant by pipetting 134 μ l.

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

- Transfer 70 % EtOH to a reservoir (Table 13).

Table 13: Required volume of 70 % EtOH

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

- Add 100 μ l of 70 % EtOH to each well without resuspending. Incubate for 30 s.
- Keep plate on the magnet. Remove 110 μ l EtOH carefully and discard.
- Add 100 μ l of 70 % EtOH to each well without resuspending. Incubate for 30 s.
- Keep plate on the magnet. Carefully remove 100 μ l EtOH and discard it.
- Remove residual EtOH using the 20 μ l multichannel pipette.
- Remove the UID PCR plate from the magnet and let it dry for 2 min.
- Add required volume of Buffer EB in a reservoir (Table 14).

Table 14: Required volume of Buffer EB

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

- Add 120 μ l Buffer EB to each well to elute the DNA and mix at least 10 times up and down carefully.
- Check visually that all beads are in solution.

16. Incubate the UID PCR plate for 2 min at 15 °C to 25 °C.
17. Place UID PCR plate onto the magnet and incubate for 1 min.
18. Carefully transfer 110 µl of each well eluate into a new UID eluate plate, discard UID PCR plate.
19. 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.
20. 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 Plasma-SeqSensei™ Breast Cancer IVD Kit contains one Index Primer Plate IND34 (Plate A) for up to 16 samples. If more than 16 samples are analysed on one sequencing run, a second Index Primer Plate IND35 (Plate B) of the Plasma-SeqSensei™ Extension IVD Kit must be used.

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

The wells of the Plasma-SeqSensei™ 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:

- **Index Primer Plate IND34** (Plate A), Sysmex Inostics, #ZR852004
- *optional:* **Index Primer Plate IND35** (Plate B), Sysmex Inostics, #ZR852005
- **PCR Master Mix v2, 2x** (black cap), Sysmex Inostics, #ZR850001
- **Water, nuclease-free** (transparent/white cap), Sysmex Inostics, #ZR224006
- **Buffer EB** (Elution Buffer), QIAGEN, #19086

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

Preparation:

- Provide all reagents:
 - Thaw
 - Vortex for 5 s
 - Centrifuge for 2 s
- Label all required plastics (Index PCR Working Mix tube, disposable reservoir, DIL plate, Index PCR plate).
- Place required Buffer EB (Table 15) in a reservoir and cover until use.

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 '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.

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

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.
4. Mix thoroughly by pipetting 70 µl up and down 10 times.
5. 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 Plasma-SeqSensei™ Index Primer Plate:

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

Note: Check that the correct Index Primer Plate (IND34 or IND35 / A or B) was used in the correct orientation.

Preparation of Index PCR:

8. Prepare Index PCR Working Mix according to Table 16. Vortex the mix for 5 s and spin it down for 2 s.

Table 16: Pipetting scheme of the Index PCR Working Mix per plate

Number of samples, with 10 % excess	2	3	4	5	6	7	8	9
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
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 one NTC is already included.

9. Add 20 µl of Index PCR Working Mix per well into Index Primer Plate.

Recommendation: Transfer Working Mix to tube strips with multi-channel pipettes for transfer to plate. Make sure to use fresh pipette tips each time.

10. Add 5 µl of template from the DIL plate to the 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 Plasma-SeqSensei™ Index Primer Plate to avoid sample mix-up.

Note: Check if there are blue dots visible on the bottom of the wells after resuspension. 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.

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

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, #28104 or #28106
- **Buffer PB**, QIAGEN, #19066
- **Ethanol (EtOH) ≥ 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 'Remove Condensate_v1'.
- Label all required plastics (EtOH dilution tube, PB dilution tube(s), spin column(s), QIAquick® eluate tube(s), Index eluate tube(s)).
- Prepare a bin for liquid waste.
- Prepare fresh 70 % EtOH according to the Table 18. Invert 10 times.

Table 18: Preparation of 70 % EtOH

Reagent	Volume
EtOH ≥ 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** by pipetting 2 x 15 µl into an appropriate container using a 20 µl pipette.

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 3 s 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, use extension tube or 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.

9 Workflow

5. Add 50 µl of the Buffer EB to the centre of the QIAquick® membrane and incubate for 1 min at 15 °C to 25 °C before last centrifugation step.

Note: *Do not elute twice.*

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, let the beads equilibrate to 15 °C to 25 °C (~30 min) and resuspend them 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. Do not vortex the bead bottle.

B) When using aliquots of AMPure® beads, let them equilibrate to 15 °C to 25 °C and 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 15 °C to 25 °C for 5 min.
10. Open the tube, place it in the DynaMag-2 and incubate for 2 min at 15 °C to 25 °C.

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 15 °C to 25 °C.
13. Remove supernatant and discard it.
14. Add 500 µl 70 % EtOH and incubate for 30 s at 15 °C to 25 °C. 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 supernatant and discard it. Remove residual EtOH using a 20 µl pipette.
16. Remove the tube from the DynaMag-2 and let it dry for 2 min at 15 °C to 25 °C 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 15 °C to 25 °C.
18. Open the tube, place it in DynaMag-2 and incubate for 1 min at 15 °C to 25 °C.
19. Use a 20 µl pipette, set to 20 µl to transfer the entire 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 labelled 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 always be performed in three replicates.

The Plasma-SeqSensei™ 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:

Allow reagents to equilibrate to 15 °C to 25 °C for 30 min in the dark.

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

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

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 analysed samples in ng/116 μl + 4.3 ng for each positive control (PC)

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

Note: *If dilution factor is <1, do not dilute your Index eluate but use directly for QC measurement, quantification and 2 nM dilution.*

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

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

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.*

Data analysis:

3. Verify that the ladder plot profile looks similar to Figure 8 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 present in every sample read) with a flat baseline (see Figure 8).

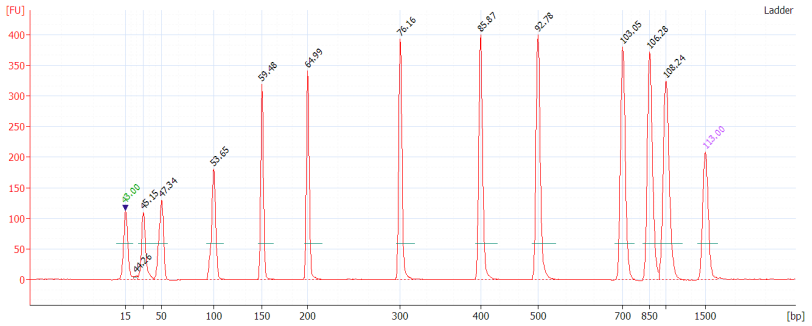


Figure 8: Ladder electropherogram (Bioanalyzer)

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

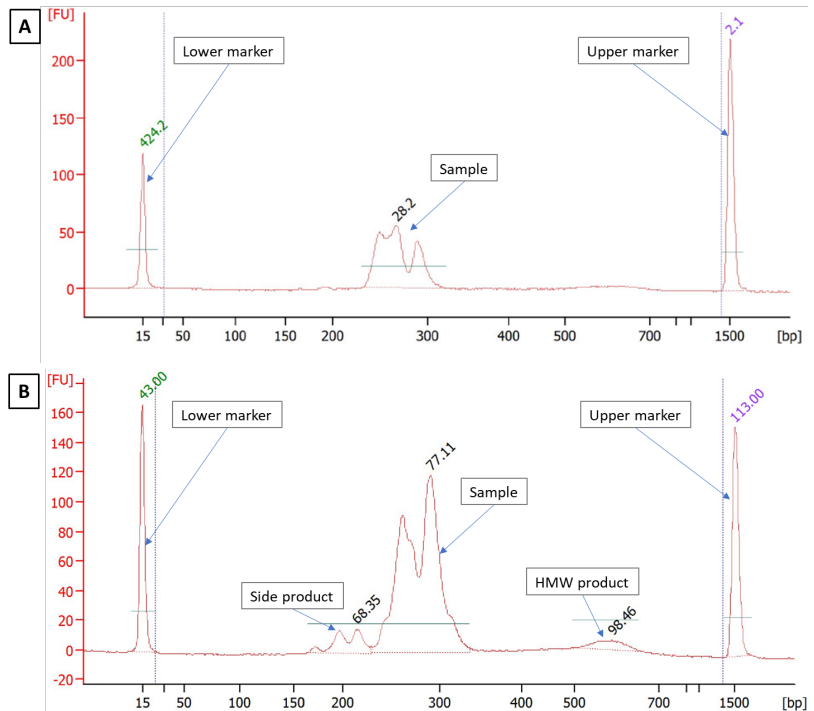



Figure 9: Sample Electropherograms on a Bioanalyzer. (A) An optimal electropherogram without any side products, (B) exemplary electropherogram with side products (e.g. primer dimer and gDNA (HMW product)).

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: Estimate the standard deviation based on a sample.

Note: If the Signal-to-Noise ratio is failing due to an unspecific peak, this value may be excluded from the calculations.

Note: If the CV of the triplicates is > 10 %, the lowest value may be removed from the sample calculations, as long as the other two values are then within the acceptance criteria.

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
≤ 1,304 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 each 2 nM library:

$$\mathbf{Total\ Volume\ [\mu l]} = \frac{3\ \mu l\ BA_DIL * Concentration_{Library\ in\ nM}}{2\ nM}$$

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

$$\mathbf{Volume_{Buffer\ EB}\ [\mu l]} = Total\ volume - 3\ \mu l\ BA_DIL$$

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 using the Plasma-SeqSensei™ Extension IVD Kit pool the two separate 2 nM library dilutions in a final Library Pool Mix of 10 μl according to the following equations:

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

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

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

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 (pooled) library denaturation with freshly prepared 0.2 M NaOH (see Table 20). Vortex for 5 s and centrifuge for 3 s.

Table 20: Required volumes for library denaturation and dilution.

Library	0.2 M NaOH	0.2 M Tris-HCl	HT1 buffer
10 μl	10 μl	10 μl	970 μl

6. Incubate the (pooled) library for 5 min at 15 °C to 25 °C.
7. Add 10 µl of 0.2 M Tris-HCl, pH 7.0 to denatured (pooled) library (see Table 20). Vortex for 5 s and spin down for 3 s.
8. Dilute denatured (pooled) library to 20 pM by adding 970 µl of pre-chilled HT1 buffer (supplied with Illumina Sequencing kit, see Table 20). Vortex for 5 s and spin down for 3 s.
9. Dilute the denatured (pooled) 20 pM library with HT1 buffer in a fresh tube to the optimal final loading concentration depending on chosen sequencing kit and sequencing device (see Table 21). Vortex for 5 s and spin down for 3 s.

Important: *Each sequencing device can have a different optimal final loading concentration, which needs to be determined by the user. Start by using our recommended final loading concentration as indicated in Table 21. Increase loading concentration if cluster density is low, decrease loading concentration if runs are overclustered.*

Table 21: Required volumes for recommended final loading concentration for sequencing

	Mid Output Kit	High Output Kit
Recommended final concentration	1.0 pM	1.1 pM
Library input	65 µl	71 µl
HT1 buffer	1,235 µl	1,229 µl

10. Start sequencing run using NextSeq Control if a separate demultiplexing pipeline is implemented at the sequencing site (e.g. bcl2fastq from Illumina). Otherwise, use the Local Run Manager of the NextSeq device to start the sequencing run.
11. Perform the start of the sequencing run according to Illumina's protocol (NextSeq™ 550 System Guide, document # 15069765v06) using following Run Parameter Settings in Table 22:

Table 22: 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.

- When using Local Run Manager, include the following adapter settings in Table 23 (can be copied from the sample sheet) in the 'Advanced Module Settings':

Table 23: Adapter settings for Local Run Manager

Name	Sequence
Adapter	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA

Important: *The cluster density must not exceed the value of 220 K/mm². If cluster density is > 220 K/mm² repeat sequencing run with reduced loading concentration. The recommended range for the cluster density is 150-220 K/mm².*

Next steps

Refer to the Plasma-SeqSensei™ 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: *Sample Preparation Guide, Instructions for Use for the Plasma-SeqSensei™ IVD assay as well as for the Plasma-SeqSensei™ IVD Software are available in different languages online at <https://sysmex-inostics.com/products/kit-specs/>.*

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 mutant molecules (MM).

Analyte (MM)	Hit Rate in % (n=192)	LoD95
20	100	3.81 MM (CI95 3.25 MM – 4.65 MM)
10	100	
5	99.0	
2.5	87.0	
1.25	62.5	
0.625	41.7	

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 Plasma-SeqSensei™ 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 Plasma-SeqSensei™ 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.

11.6 Clinical performance and characteristics

Clinical performance of the Plasma-SeqSensei™ Breast Cancer IVD kit has been determined by testing 76 positive and 78 negative specimens for all target genes. The sensitivity is 97 % (95 % CI: 90.9 % - 99.3 %), and the specificity is 95 % (95 % CI: 87.5 % - 98 %).

11 Performance characteristics

		Reference method: BC_P2 test		
		positive	negative	total
Plasma- SeqSensei™ Breast Cancer IVD Kit	positive	74	4	78
	negative	2	74	76
	total	76	78	154

The overall percentage agreement is 96.1 %

11.7 Limitations

Performance data of samples at the border of the allowed DNA input range may deviate from stated values and can result in lower precision and repeatability of low concentrated samples as well as lower LoD values for high concentrated samples.

12 Glossary and terminologies

Term	Definition
bp	Base pair
BA_Dil	Bioanalyzer dilution
BLAST	Basic local alignment search tool
cfDNA	Cell-free DNA
CI	Confidence interval
CLSI	Clinical & laboratory standards institute
COSMIC	Catalogue of somatic mutations in cancer
ctDNA	Circulating tumour DNA
CV	Coefficient of variation
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
HIV	Human immunodeficiency virus
HMW	High molecular weight
IDX	Index
IFU	Instructions for use
LoD	Limit of detection
MAF	Mutant allele fraction

12 Glossary and terminologies

Term	Definition
MM	Mutant molecules
Mpx	Multiplex primer mix
NaOH	Sodium hydroxide
NGS	Next-generation sequencing
NTC	No template control
PC	Positive control
PCR	Polymerase chain reaction
QC	Quality control
RNA	Ribonucleic acid
SNV	Single nucleotide variant
UID	Unique identifier

13 References

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15 Revision history

Document version	Date	Change description	Section
R3	December 2023	Update of notes about SNR and CV during Bioanalyzer use	9.5
R2	November 2023	Update of detection limits for germline mutations	6
		Information on reporting of incomplete coverage of amino acid-encoding nucleotide triplets	6
		Reduction of upper laboratory temperature limit and specification of room temperature (15 °C to 25 °C)	8.2 9
		Update of download link for IFUs, MSDS	8.3 and 10
		Minimum number of samples per High Output kit included	9
		Update of sample dilution recommendation and quantification with Qubit™	9.1
		Information about AMPure magnetic bead handling	9.2 9.2 step 15 9.4 step 7
		Addition of note about standard deviation	9.5 step 10
		Include extended information about sample denaturation, dilution and sequencing initiation	9.6 steps 5-12
		Update of information on performance characterisation	11.1 11.6 11.7
		Addition of revision history table	15
		Minor corrections, spelling, layout and order changes	
R1	June 2022	N/A	





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