

VeriSeq NIPT Solution Package Insert

FOR IN VITRO DIAGNOSTIC USE

Intended Use

The VeriSeq NIPT Solution is an *in vitro* diagnostic test intended for use as a sequencing-based screening test for the detection of fetal aneuploidies from maternal peripheral whole blood samples in pregnant women of at least 10 weeks gestation. VeriSeq NIPT provides information regarding aneuploidy status for chromosomes: 21, 18, 13, X, and Y. This product must not be used as the sole basis for diagnosis or other pregnancy management decisions.

The VeriSeq NIPT Solution includes: the VeriSeq NIPT Workflow Manager for the VeriSeq NIPT Microlab STAR, the VeriSeq NIPT Sample Prep Kits, and the VeriSeq Onsite Server with the VeriSeq NIPT Assay Software.

Summary and Explanation of the Assay

Fetal chromosome abnormalities, specifically aneuploidy, which is an abnormal number of chromosomes, are a common cause of reproductive failure, congenital anomalies, developmental delay, and intellectual disabilities. Aneuploidy affects approximately 1 in 300 live births, with much higher rates associated with miscarriage and stillbirth.^{1,2} Until recently, there have been two types of prenatal tests for these disorders: diagnostic testing or multiple marker screening. Diagnostic testing involves invasive procedures such as amniocentesis or chorionic villus sampling. These testing methods are considered to be the most accurate for detection of aneuploidy. However, they are associated with a risk of pregnancy loss between 0.11% and 0.22%.³ Conventional multiple marker screens have no risk of pregnancy loss as they are noninvasive, but they are less accurate than diagnostic tests. Their detection rates for trisomy 21 vary between 69–96% depending on the particular screen, maternal age, and gestational age at testing.⁴ Importantly, they have false positive rates of approximately 5%, which can lead to invasive diagnostic testing for confirmation and thus, the risk of procedure-related pregnancy loss.⁴

Fetal aneuploidy for chromosomes 21, 18, 13, X and Y can be detected with a high degree of accuracy by noninvasive prenatal testing (NIPT) using whole-genome sequencing of cell-free DNA (cfDNA) obtained from maternal plasma at 10 weeks gestation or later. A recent meta-analysis of multiple clinical studies reported the weighted pooled detection rates and specificities for trisomy 21 and trisomy 18 in singleton pregnancies as follows: trisomy 21 99.2% and 99.91% and trisomy 18 96.3% and 99.87%, respectively.⁵

Given the significant reduction of false positive rates with NIPT compared to conventional multiple marker screening, numerous professional medical organizations have issued opinion statements supporting several indications for the use of NIPT.

Specifically, the International Society for Prenatal Diagnosis, the American College of Obstetricians and Gynecologists (ACOG) /Society for Maternal Fetal Medicine (SMFM), the American College of Medical Genetics and Genomics (ACMG), and the European Society of Human Genetics/American Society of Human Genetics support offering NIPT to all pregnant women.⁶ Pretest counseling, informed consent, and diagnostic testing to confirm a positive cfDNA screening result are recommended.⁷

One study suggests that use of NIPT as a primary screen across all pregnancies could result in an 89% reduction in the number of confirmatory invasive procedures.⁸

The current VeriSeq NIPT Solution is a noninvasive *in vitro* diagnostic (IVD) test that utilizes whole-genome sequencing of cfDNA fragments derived from maternal peripheral whole blood samples from pregnant women of at least 10 weeks gestation to detect fetal aneuploidy of chromosomes 21, 18, 13, X, and Y.

Principles of Procedure

The VeriSeq NIPT Solution is an automated solution for laboratory NIPT testing which consists of automated sample preparation and sequencing data analysis. The VeriSeq NIPT Sample Prep Kits are specialized reagents that are used in conjunction with the VeriSeq NIPT Microlab STAR to prepare 48- or 96-sample batches for next generation sequencing. Whole genome, paired-end sequencing data is analyzed by specialized software, the VeriSeq NIPT Assay Software, and a report is generated.

The workflow consists of the following procedures: sample collection, plasma isolation, cfDNA extraction, library preparation, library quantification, library pooling, sequencing, and analysis which are outlined in detail:

- ▶ **Sample Collection**—7–10 ml of maternal peripheral whole blood is collected in a Streck cell-free blood collection tube, which prevents cell lysis and genomic contamination and stabilizes whole blood at room temperature.
- ▶ **Plasma Isolation**—Within 5 days of collection, or within 10 days of collection if stored at 4°C, plasma is isolated from maternal peripheral whole blood using standard centrifugation techniques. The VeriSeq NIPT Microlab STAR aspirates and dispenses plasma into a 96-well deep-well plate for subsequent processing.
- ▶ **cfDNA Extraction**—Purification of cfDNA from plasma is achieved by adsorption onto a binding plate, washing the binding plate to remove contaminants, and eluting.
- ▶ **Library Preparation**—The purified cfDNA fragments undergo an end repair process to convert 5' and 3' overhangs to blunt ends. Next, a deoxyadenosine nucleotide is added to the 3' ends to create a single base overhang. Indexed adapters containing a single base 3' deoxythymidine overhang are then ligated onto the processed cfDNA fragments. The ligated DNA is purified using solid phase reverse immobilization beads. Each sample in a set of 48 or 96 receives a unique indexed adapter. The adapters serve 2 purposes:
 - ▶ Indexes allow sample identification in subsequent sequencing.
 - ▶ Index adapters contain sequences that allow for library capture on the solid surface of a sequencing flow cell for cluster generation and subsequent sequencing.
- ▶ **Quantification**—The library product is quantified using a fluorescent dye with concentration determined by comparison to a DNA standard curve.
- ▶ **Library Pooling and Sequencing**—The 48-sample batch libraries are pooled together in amounts adjusted to minimize variation in coverage. The 48-sample batch pools are then sequenced using a next-generation sequencer with the following specifications: capable of 2x36 paired-end reads, compatible with index adapters in the VeriSeq NIPT Sample Prep kit, 2 dye-based chemistry, and automatic production of .BCL files (raw data from sequencing instrument). The VeriSeq NIPT Solution does not include sequencing equipment and consumables.
- ▶ **Analysis**—Nucleotide base calls are made directly from signal intensity measurements during sequencing. Secondary analysis consists of:
 - ▶ Demultiplexing the reads using the index sequences
 - ▶ Mapping the sequences to a reference human genome
 - ▶ Calculating the number of unique reads within each of the 100 kb genomic bins
 - ▶ Normalizing the coverage on a subchromosomal level
- ▶ Paired-end read information is used to assess coverage (the number of unique reads aligning to the reference per sample) and the length of the individual fragments within the sample. Fetal fraction within each sample is estimated based on coverage profiling, size distribution, and copy number on chromosome X. Finally, these statistical inputs are used to determine the over or under representation of chromosomes 21, 18, 13, X, and Y. The results are summarized in a report where "aneuploidy detected" or "no aneuploidy detected" is listed for each target chromosome for samples passing quality control metrics. A fetal fraction estimation is also included for each sample as part of the report.

Limitations of the Procedure

- ▶ The VeriSeq NIPT Solution is a screening test and should not be considered in isolation from other clinical findings and test results. Management decisions including termination of pregnancy should not be based on the results of the NIPT screening alone.⁷
- ▶ The assay requires maternal peripheral whole blood samples from pregnant women of at least 10 weeks gestation.
- ▶ The results of the test can be confounded by certain maternal and fetal factors including but not limited to the following:
 - ▶ Recent maternal blood transfusion
 - ▶ Maternal organ transplant
 - ▶ Maternal surgical procedure
 - ▶ Maternal immunotherapy or stem cell therapy
 - ▶ Maternal malignancy
 - ▶ Maternal mosaicism
 - ▶ Confined placental mosaicism
 - ▶ Fetal demise
 - ▶ Disappearing twin
 - ▶ Fetal partial trisomy or partial monosomy
 - ▶ Fetal mosaicism
- ▶ Evidence supporting sensitivity and specificity for the test covers singleton and twin pregnancies. These instructions for use do not provide sensitivity or specificity data for triplets or higher order pregnancies.
- ▶ The VeriSeq NIPT Solution reports on the following:
 - ▶ Over representation of chromosomes 21, 18, and 13
 - ▶ The following sex chromosomal aneuploidies: XO, XXX, XXY, and XYY
- ▶ The VeriSeq NIPT Solution is not intended to detect polyploidy, such as triploidy.
- ▶ The VeriSeq NIPT Solution test looks for specific chromosome abnormalities. Results reported as No Aneuploidy Detected do not eliminate the possibility of chromosomal abnormalities of the tested chromosomes. Additionally, a negative result does not eliminate the possibility that the pregnancy has other chromosomal abnormalities, genetic conditions or birth defects (eg, open neural tube defect).

Product Components

The VeriSeq NIPT Solution consists of the following:

- ▶ VeriSeq NIPT Sample Prep Kit (48 samples) (part # 15066801)
- ▶ VeriSeq NIPT Sample Prep Kit (96 samples) (part # 15066802)
- ▶ VeriSeq Onsite Server (part # 15076164)
 - ▶ VeriSeq NIPT Assay Software, preinstalled on the VeriSeq Onsite Server
- ▶ VeriSeq NIPT Microlab STAR (part# Hamilton Company Reno: 95475-01 (115V) & 95475-02 (230V), Hamilton Company Bonaduz: 806288)
 - ▶ VeriSeq NIPT Workflow Manager, preinstalled on the VeriSeq NIPT Microlab STAR

Reagents

Reagents Provided

Illumina provides the following reagents: VeriSeq NIPT Sample Prep Kit (48 samples) (part # 15066801) and VeriSeq NIPT Sample Prep Kit (96 samples) (part # 15066802). The VeriSeq NIPT Sample Prep Kits are configured for use with the ML STAR, which is provided by Hamilton Company (part # 806288).

VeriSeq NIPT Sample Prep, Extraction Box

Table 1 VeriSeq NIPT Extraction Box (48), Part # 15066803

Reagent Name on Label	Number of Containers in Kit	Label Volume	Active Ingredients	Storage
Lysis Buffer	1	100 ml	Guanidine hydrochloride in buffered aqueous solution	15°C to 30°C
Wash Buffer I	1	125 ml	Guanidine hydrochloride and 2-propanol in buffered aqueous solution	15°C to 30°C
Wash Buffer II	1	25 ml	Buffered aqueous solution containing salts	15°C to 30°C
Elution Buffer	1	30 ml	Buffered aqueous solution	15°C to 30°C
Proteinase Buffer	1	35 ml	Glycerol in buffered aqueous solution	15°C to 30°C
Proteinase K	3	75 mg	Lyophilized Proteinase K	15°C to 30°C

Table 2 VeriSeq NIPT Extraction Box (96), Part # 15066807

Reagent Name on Label	Number of Containers in Kit	Label Volume	Active Ingredients	Storage
Lysis Buffer	1	100 ml	Guanidine hydrochloride in buffered aqueous solution	15°C to 30°C
Wash Buffer I	1	125 ml	Guanidine hydrochloride and 2-propanol in buffered aqueous solution	15°C to 30°C
Wash Buffer II	2	25 ml	Buffered aqueous solution containing salts	15°C to 30°C
Elution Buffer	1	30 ml	Buffered aqueous solution	15°C to 30°C
Proteinase Buffer	1	35 ml	Glycerol in buffered aqueous solution	15°C to 30°C
Proteinase K	4	75 mg	Lyophilized Proteinase K	15°C to 30°C

VeriSeq NIPT Sample Prep, Library Prep Box

Table 3 VeriSeq NIPT Library Prep Box (48), Part # 15066809

Reagent Name on Label	Number of Containers in Kit	Label Volume	Active Ingredients	Storage
End Repair Mix	1	2.72 ml	DNA polymerase and dNTPs in buffered aqueous solution	-25°C to -15°C
A-Tailing Mix	1	910 µl	DNA polymerase and dATP in buffered aqueous solution	-25°C to -15°C
Ligation Mix	1	233 µl	DNA ligase in buffered aqueous solution	-25°C to -15°C

Reagent Name on Label	Number of Containers in Kit	Label Volume	Active Ingredients	Storage
Hybridization Buffer	1	12 ml	Buffered aqueous solution	-25°C to -15°C
VeriSeq NIPT DNA Adapter Plate	1	N/A	Oligonucleotides in buffered aqueous solution	-25°C to -15°C

Table 4 VeriSeq NIPT Library Prep Box (96), Part # 15066810

Reagent Name on Label	Number of Containers in Kit	Label Volume	Active Ingredients	Storage
End Repair Mix	1	2.72 ml	DNA polymerase and dNTPs in buffered aqueous solution	-25°C to -15°C
A-Tailing Mix	2	910 µl	DNA polymerase and dATP in buffered aqueous solution	-25°C to -15°C
Ligation Mix	2	233 µl	DNA ligase in buffered aqueous solution	-25°C to -15°C
Hybridization Buffer	1	12 ml	Buffered aqueous solution	-25°C to -15°C
VeriSeq NIPT DNA Adapter Plate	1	N/A	Oligonucleotides in buffered aqueous solution	-25°C to -15°C

VeriSeq NIPT Sample Prep, Accessory Box

Table 5 VeriSeq NIPT Accessory Box, Part # 15066811

Reagent Name on Label	Number of Containers in Kit	Label Volume	Active Ingredients	Storage
DNA Binding Plate	1	N/A	Propylene microplate with modified silicone membrane	2°C to 8°C
Resuspension Buffer	1	35 ml	Buffered aqueous solution	2°C to 8°C
Sample Purification Beads	1	10 ml	Solid-phase paramagnetic beads in buffered aqueous solution	2°C to 8°C
DNA Quantification Reagent	1	294 µl	DNA intercalating dye in DMSO	2°C to 8°C
DNA Quantification Standard	1	110 µl	dsDNA standard, non-specific DNA, and sodium azide in buffered aqueous solution	2°C to 8°C

VeriSeq NIPT Sample Prep, Workflow Tubes and Labels

Table 6 Workflow Tubes and Labels, Part # 15071543

Item Name on Label	Number of Items in Kit	Storage
Label (LBL)–Plate Barcode	9	15°C to 30°C
Label (LBL)–Deep-well Plate Barcode	12	15°C to 30°C
Tube (TB)–Empty Pooling Tube	5	15°C to 30°C

Reagents Not Provided

Reagents Required, Not Provided

- ▶ DNase/RNase-free water

- ▶ Ethanol, 100% (200 proof) for molecular biology
- ▶ Sequencing reagents and consumables required for next-generation sequencing (NGS) system

Reagents Optional, Not Provided

- ▶ Dulbecco's Phosphate-Buffered Saline (DPBS) for no template control (NTC)

Storage and Handling

- 1 Room temperature is defined as 15°C to 30°C.
- 2 All reagents are for one-time use only. After reagents are prepared for use, they should be used immediately.
- 3 If any of the packaging or contents of the VeriSeq NIPT Solution components are damaged or compromised, please contact Illumina Customer Service.
- 4 Reagents are stable when stored as indicated until the specified expiration date on the kit labels. For storage conditions, see the Storage column in the tables in *Reagents Provided on page 4*. Do not use expired reagents.
- 5 Changes in the physical appearance of the reagents provided can indicate deterioration of the materials. If changes in the physical appearance occur (eg, obvious changes in reagent color or cloudiness apparent with microbial contamination), do not use the reagents.
- 6 Adhere to the following best practices when handling Sample Purification Beads:
 - ▶ Never freeze the beads.
 - ▶ Allow the beads to reach room temperature before use.
 - ▶ Immediately before use, vortex the beads until well-suspended and color appears homogeneous.
- 7 Lysis Buffer, Wash Buffer I, Wash Buffer II, Elution Buffer, and Proteinase Buffer can form visible precipitates or crystals. Before use, vortex vigorously, and then visually inspect to make sure that no precipitates are present.
- 8 Never freeze whole blood after collection.
- 9 Sequence libraries as soon as possible after pooling. Pooled libraries are stable for up to 7 days at -25°C to -15°C.

Equipment and Materials

Equipment and Materials Required, Not Provided

Equipment Required, Not Provided

Equipment	Supplier
20 µl single-channel pipettes	General lab supplier
200 µl single-channel pipettes	General lab supplier
1000 µl single-channel pipettes	General lab supplier
Pipette Aid	General lab supplier
Refrigerator, 2°C to 8°C	General lab supplier
Freezer, -25°C to -15°C	General lab supplier
Microcentrifuge	General lab supplier
Vortexer	General lab supplier
Centrifuge and rotor assembly for blood collection tubes	

Equipment	Supplier
Recommended: <ul style="list-style-type: none"> • Allegra 6 Series Centrifuge, 1600 g • Allegra Centrifuge GH-3.8 Rotor with buckets • Allegra Centrifuge Bucket Covers, set of two • Allegra Centrifuge Adapter Assembly, 16 mm, set of four 	<ul style="list-style-type: none"> • Beckman Coulter, item # 366830 (120 V) • Beckman Coulter, item # 360581 • Beckman Coulter, item # 360585 • Beckman Coulter, item # 359150
Equivalent: <ul style="list-style-type: none"> • Refrigerated centrifuge capable of 1600 × g with no-brake option • Swinging bucket rotor with buckets • Bucket inserts, 48 or 96 tube capacity, 76 mm minimum depth • Insert adapters to support 16 x 100 mm blood collection tubes 	General lab supplier
Centrifuge and rotor assembly for microplates	
Recommended: <ul style="list-style-type: none"> • Sorvall Legend XTR Centrifuge • HIGHPlate 6000 Microplate Rotor • One of the following support bases for microplates: <ul style="list-style-type: none"> • MicroAmp 96-Well Support Base • 96-Well PCR Plate Carrier 	<ul style="list-style-type: none"> • Thermo Fisher Scientific, catalog # 75004521 (120 V) or catalog # 75004520 (230 V) • Thermo Fisher Scientific, catalog # 75003606 • Thermo Fisher Scientific, catalog # 4379590 • Thermo Fisher Scientific, catalog # AB-0563/1000
Equivalent: <ul style="list-style-type: none"> • Centrifuge capable of 5600 × g • Swinging plate rotor with 96-well plate carriers, 76.5 mm minimum depth • Support base for microplates 	General lab supplier
One of the following microplate readers (fluorometer) with SoftMax Pro v6.2.2 or higher: <ul style="list-style-type: none"> • Gemini XPS • SpectraMax M2 	<ul style="list-style-type: none"> • Molecular Devices, part # XPS • Molecular Devices, part # M2
SpectraMax High-Speed USB, Serial Adapter	Molecular Devices, part # 9000-0938
Thermal cycler with the following specifications: <ul style="list-style-type: none"> • Heated lid • 4°C to 98°C temperature range • ±2°C temperature accuracy • 2°C per second minimum ramp rate • Compatible with Twin.tec PCR Plate 96-well, full skirt 	General lab supplier
VeriSeq NIPT Microlab STAR	Hamilton, part # 95475-01 (115 V) or part # 95475-02 (230 V)
Next-generation sequencing (NGS) system with the following capabilities: <ul style="list-style-type: none"> • 2 x 36 bp paired-end sequencing • Compatible with VeriSeq NIPT Sample Prep dual index adapters • Automatic production of .BCL files • Two dye-based chemistry • 400 million paired-end reads per run • Compatible with VeriSeq NIPT Assay Software 	Instrument supplier
VeriSeq OnSite Server	Illumina, part # 15076164

Optional Equipment, Not Provided

Equipment	Supplier
Pluggo Decapper System	LGP Consulting, part # 4600 4450
SpectraMax SpectraTest FL1 fluorescence validation plate	Molecular Devices, part # 0200-5060
Tube Revolver/Rotator, 15 ml tubes, 40 rpm, 100–240 V	Thermo Scientific, catalog # 88881001 (US) or catalog # 88881002 (EU)

Materials Required, Not Provided

Consumable	Supplier
1000 µl Conductive Non-Sterile Filter Tips	Hamilton, part # 235905
300 µl Conductive Non-Sterile Filter Tips	Hamilton, part # 235903
50 µl Conductive Non-Sterile Filter Tips	Hamilton, part # 235948
Deep-Well Reservoir	Corning Axygen, product # RES-SW96-HP-SI
MagNA Pure LC Medium Reagent Tub 20, 20 ml	Roche, product # 3004058001
Deep-Well Plate 96, 2 ml	Eppendorf, part # 951033600
Low Volume 384 Well Black Flat Bottom Polystyrene Microplate	Corning, product # 3820
Twin.tec PCR Plate 96-well, full-skirt	Eppendorf, part # 30129512
One of the following seals: • Microseal 'F' Foil • Foil seals	• Bio-Rad, catalog # MSF1001 • Beckman Coulter, item # 538619
Cell-Free DNA BCT CE	Streck, catalog # 218997
Push Caps	Sarstedt, order # 65.802
2 ml Screw-cap tubes	General lab supplier
20 µl filter tips for 20 µl pipettor	General lab supplier
200 µl filter tips for 200 µl pipettor	General lab supplier
1000 µl filter tips for 1000 µl pipettor	General lab supplier
25 ml Serological Pipettes	General lab supplier
10 ml Serological Pipettes	General lab supplier
Recommended: • Deconex® SOLARSEPT • Deconex® 61 DR	Borer Chemie AG
Equivalent: • An alcohol-based rapid disinfectant spray • A solution of disinfecting detergent	General lab supplier

Optional Materials, Not Provided

Consumable	Supplier
Tube, screw cap, 10 ml	Sarstedt, order # 60.551
Tube, screw cap, 50 ml	General lab supplier

Specimen Collection, Transport, and Storage



CAUTION

Handle all specimens as if they are potentially infectious agents.

- 1 Whole blood specimens of 7–10 ml collected in Streck Cell-Free DNA BCT must be used. Do not freeze.
- 2 Store the blood collection tube at 4°C within 5 days of collection and complete plasma isolation within 10 days.
- 3 Transportation of whole blood must comply with all applicable governing regulations for the transport of etiologic agents.

Warnings and Precautions

- ▶ This assay contains Proteinase K. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area, wear protective clothing, avoid breathing dust, and dispose of any containers and unused contents in accordance with applicable governmental safety standards.
- ▶ This assay contains guanidinium chloride. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area, wear protective clothing, and dispose of any containers and unused contents in accordance with applicable local governmental safety standards.
- ▶ This assay contains 2-propanol, a flammable chemical. Keep away from heat and open flames. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Use in a well-ventilated area, wear protective clothing, and dispose of any containers and unused contents in accordance with applicable local governmental safety standards.
- ▶ To prevent the formation of harmful gases, do not dispose of cfDNA extraction waste (contains guanidine thiocyanate) with waste containing bleach (sodium hypochlorite).
- ▶ Handle all specimens as if they contain potentially infectious agents.
- ▶ Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- ▶ Do not use any assay components beyond their stated expiration date on the assay box label. Do not interchange assay components from different assay lots. Assay lots are identified on the assay box label. Store the assay components at the specified temperature.
- ▶ To prevent sample or reagent degradation, make sure that all sodium hypochlorite vapors from cleaning have fully dissipated before starting the protocol.
- ▶ Failure to follow the procedures as outlined can result in erroneous results or significant reduction in sample quality.

Procedural Notes

Avoiding Contamination

- ▶ Use fresh tips and fresh consumable labware.
- ▶ Mix samples with a pipette. Using aerosol-resistant tips reduces the risk of carry-over and sample-to-sample cross-contamination. Centrifuge following vortexing.
- ▶ Due to the potential for contamination, take extreme care to make sure that well contents remain fully in the well. Do not splash contents.
- ▶ Follow applicable governing regulations for proper laboratory practice and hygiene when handling blood and blood derivatives.

VeriSeq NIPT Microlab STAR Deck Cleaning

- ▶ Before use, inspect the deck for cleanliness. At least once a week, conduct the weekly maintenance and follow these cleaning instructions.
- ▶ Clean all carriers with an alcohol-based rapid disinfectant spray (Deconex® SOLARSEPT or equivalent) and leave them to dry. If they are heavily soiled, soak them afterward in a solution of disinfecting detergent (Deconex® 61 DR cleaning liquid or equivalent).
- ▶ Open the front cover and wipe the deck with a cloth saturated with Deconex® SOLARSEPT (or equivalent). The slide blocks in particular must be checked for cleanliness.
- ▶ Remove the CVS manifold and clean manifold, gasket, and interior compartments of CVS with cloth. Empty tip waste for the CORE 96-head and the independent channel.
- ▶ Remove the independent channel tip eject plate of the tip waste station and clean it: spray Deconex® SOLARSEPT (or equivalent) directly onto the surface and wipe. Pull a new plastic bag over the frame and re-attach it. Put the clean tip eject plate back in place.
- ▶ Spray Deconex® SOLARSEPT (or equivalent) directly onto the surface of the CORE 96-head waste box and chute and wipe it clean.
- ▶ Wet a lint-free cloth or cotton swab with 70% ethanol. Swab the laser scanner window of the barcode reader. Using the same cloth or swab, clean each well of the CPAC plate adapter. If using a cloth, press the cloth into each well of the adapter using the back of a pen to assure the interior of the well is properly cleaned.
- ▶ Clean the independent channels:
 - ▶ On the independent channels, clean the tip eject sleeve (outer part of the pipetting channels) with a lint-free cloth soaked in Deconex® SOLARSEPT (or equivalent). (See the *Hamilton Microlab STAR Reference Guide #15070074*.)
 - ▶ Clean the stop disk and the O-rings of the pipetting head (outer part of the pipetting channels) with a lint-free cloth soaked in Deconex® SOLARSEPT (or equivalent).
- ▶ Clean the CORE 96-head:
 - ▶ Using the same lint-free cloth soaked in Deconex® SOLARSEPT (or equivalent), clean the casing of the 96-head and the bottom of the stop disks.
 - ▶ Using the same cloth, or a torn strip of cloth soaked in Deconex® SOLARSEPT (or equivalent), 'floss' the cloth around the sides of the pipette channels of the 96-head in order to clean the o-rings. Repeat this procedure for every pipette channel on the 96-head.
- ▶ Spray the front and side cover with Deconex® SOLARSEPT (or equivalent), and wipe dry.
- ▶ Clean the Autoload protecting ribbon with a cloth soaked in Deconex® SOLARSEPT (or equivalent), and wipe without exerting pressure.



NOTE

Improper cleaning and maintenance of the ML STAR can result in cross-contamination and poor assay performance.

Quality Control

Control material with known performance characteristics may be evaluated to detect differences in processing and technical procedures in the laboratory.



NOTE

Running a reference sample or no template control reduces the total number of unknown maternal samples that can be processed with each sample prep kit.

Do not exceed two NTC samples per batch of 48 samples or four NTC samples per batch of 96 samples.

Instructions for Use

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Barcoding Plates

- Barcodes for full-skirt plates start with PL.
- Barcodes for deep-well plates start with DW.
- Apply barcodes to full-skirt plates and deep-well plates on the side next to column 12.
- Load plates with the barcode facing right to enable automated scanning.

Sealing and Unsealing the Plate

- ▶ Always seal the 96-well plate before the following steps in the protocol:
 - ▶ Centrifuge steps
 - ▶ Thermal cycling steps
- ▶ To seal the plate, apply the adhesive cover to the plate and then seal.
- ▶ Before unsealing:
 - ▶ Briefly centrifuge the 96-well plate at 1000 × g for 20 seconds.
 - ▶ Place the plate on a flat surface before slowly removing the seal.

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- ▶ Before use, perform and document the required maintenance according to the manufacturer instructions.
- ▶ Observe the ML STAR during the automated steps. Monitor the VeriSeq NIPT Workflow Manager software interface for prompts and operator instructions.
- ▶ Keep the front cover in place during operation.
- ▶ Keep the deck clear of all items during operation.
- ▶ During plate vacuum steps:
 - ▶ If prompted by the VeriSeq NIPT Workflow Manager, manually assist forming the seal between the plate and the vacuum manifold.
 - ▶ In the event of an equipment malfunction, manually turn off and on the vacuum when prompted by the Workflow Manager software.
- ▶ Allow the system to dispose of tips from the adapter automatically. Do not manually remove tips.
- ▶ Remove spent reagents and used consumables as prompted by the Workflow Manager.
- ▶ Empty vacuum waste carboys daily. The first carboy should never exceed ½ full. Overflow of vacuum waste can damage the vacuum pump.

Process Blood Samples

Procedure

- 1 Centrifuge barcoded blood samples at 1600 × g for 10 minutes at 4°C with the brake off.
- 2 Wait for the centrifuge to come to a complete stop, and then remove the sample tubes.
After centrifugation, begin plasma isolation within 15 minutes. If more than 15 minutes elapse, repeat the centrifugation procedure.
- 3 Inspect each tube for sample suitability, including verification of the following:
 - ▶ Sample volume is as expected.
 - ▶ Sample separated correctly during centrifugation.

- ▶ Plasma level is at least 1.5 ml above the buffy coat.
- ▶ Sample is not heavily hemolyzed (ie, plasma is not deep red in appearance).
- ▶ Sample is not lipemic (eg, plasma is not cloudy white or milky opaque in appearance).
- ▶ Sample does not have clotting.

**CAUTION**

Samples that have been improperly stored or handled can become unsuitable. If unsuitable samples are processed through the workflow, they can clog the binding plate during extractions, leading to sample overflow events into adjacent wells.

- 4 Uncap tubes and load into the tube carriers. Load all samples and any plasma controls for the batch.

Isolate Plasma

Preparation

- 1 Label 1 deep-well plate Intermediate Plasma and apply a barcode.
- 2 Label 1 deep-well plate Final Plasma and apply a barcode.

**CAUTION**

Make sure to use the correct plate type for the Intermediate Plasma and Final Plasma plates. Use of a deep-well reservoir instead of a deep-well plate leads to sample amalgamation and can produce incorrect results.

Procedure

- 1 Open the AppLauncher, and then click VeriSeq NIPT Method.
- 2 Enter the Batch ID and username, and then click **OK**.
Batch ID has a 26-character limit. Use only numbers, letters, underscores (_), or dashes (-). For example: 2025-10-16_Batch3.
- 3 Click **New Batch**, and after initiation, click **OK** to begin plasma isolation.
- 4 Perform one of the following.
 - To load an existing sample sheet, select the sample sheet associated with the batch, and then click **OK**.
 - To proceed without selecting a sample sheet, click **No Sample Sheet**.

For information about creating a sample sheet, see the *VeriSeq NIPT Solution Software Guide* (document # 100000001949).

**NOTE**

Sample type, singleton or twins, must be accurately recorded for each sample in order to ensure proper data analysis.

- 5 Select the batch size, and then click **OK**.
- 6 Select the number of no template controls (NTCs), and then click **OK**.

- 7 Confirm that all barcodes are affixed, and load the samples, tips, and plates (barcode facing right) onto the carrier. Click **OK** after each load prompt.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Tip	7–12	1000 µl tips	5
	Tube	15	Prepared blood sample tubes 1–24	1–24
	Tube	16	Prepared blood sample tubes 25–48	25–48
	Multiflex	19–24	Empty deep-well plate, Final Plasma – barcoded	4
	Multiflex	19–24	Empty deep-well plate, Intermediate Plasma – barcoded	5
	Reagent	47	[Optional] DPBS for no template control	5
96	Tip	7–12	1000 µl tips	4, 5
	Tube	15	Prepared blood sample tubes 1–24	1–24
	Tube	16	Prepared blood sample tubes 25–48	25–48
	Tube	17	Prepared blood sample tubes 49–72	49–72
	Tube	18	Prepared blood sample tubes 73–96	73–96
	Multiflex	19–24	Empty deep-well plate, Final Plasma – barcoded	4
	Multiflex	19–24	Empty deep-well plate, Intermediate Plasma – barcoded	5
	Reagent	47	[Optional] DPBS for no template control	5

- 8 Make sure that the carriers, labware, and reagents are loaded correctly, and then click **OK** in the Pre-Spin Deck Verification screen.
- 9 Observe the ML STAR perform the automated steps.
- 10 When alerted by the Workflow Manager, make sure that the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers, and then click **Unload** to unload the deck.
- 11 Remove the Intermediate Plasma deep-well plate.
- Visually inspect the plate for consistent volumes in each well (no pipette errors). Expected volume is 1000 µl.
 - Make note of any inconsistencies and record at the end of the Plasma Isolation procedure.
 - Seal the plate, load with balance, and centrifuge at 5600 × g for 10 minutes with the brake off or on the lowest setting.
- 12 Click **Yes** to proceed to final Plasma Preparation.
- 13 Remove the plate seal and reload the plate onto the carrier.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Multiflex	19–24	Intermediate Plasma deep-well plate	5

- 14 Select the **Intermediate Plasma plate has been spun** checkbox, and then click **OK**.
- 15 Observe the ML STAR perform the automated steps.
- 16 When alerted by the Workflow Manager, make sure that the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers, and then click **Unload** to unload the deck.
- 17 When prompted by the Workflow Manager, empty the carriers and deck.
- 18 Remove the Final Plasma deep-well plate.
- 19 Visually inspect the plate for the following:
- ▶ Consistent volumes in each well. Expected volume is 900 µl.
 - ▶ Visible cell pellets
 - ▶ Excessive hemolysis

If you observe a visible cell pellet or excessive hemolysis, invalidate the affected sample at the end of the Plasma Isolation method or use Batch Manager. For more information about Batch Manager, see *VeriSeq NIPT Solution Software Guide (document # 1000000001949)*.

- 20 When prompted by the Workflow Manager, click **OK**.
- 21 Enter comments about affected wells, and then click **OK**.
- 22 Perform one of the following.
 - To continue to cfDNA Extraction, click **Yes**.
 - To stop, click **Exit**.

SAFE STOPPING POINT

If you are stopping, seal the Final Plasma plate and store at 2°C to 8°C for up to 7 days.

Extract cfDNA

Preparation

- 1 Visually examine the Extraction and Accessory Boxes to confirm that the kit is not expired.
- 2 Prepare the following reagents. Label the reservoirs tubs and deep-well reservoirs with the name of the reagents.

Item	Storage	Instructions
Final Plasma deep-well plate from <i>Isolate Plasma</i> on page 12.	2°C to 8°C	If previously stored, let stand for 30 minutes to bring to room temperature. Unseal the Final Plasma deep-well plate before use.
Proteinase K	15°C to 30°C	Slowly add 3.75 ml Proteinase Buffer to each reagent vial. <ul style="list-style-type: none"> • Prepare 3 vials for 48 samples. • Prepare 4 vials for 96 samples. Cap the vial and vortex until resuspended. Pool prepared reagent from all vials into a reagent tub.
Wash Buffer II	15°C to 30°C	Add 100 ml 100% EtOH to each reagent bottle. <ul style="list-style-type: none"> • Prepare 1 bottle for 48 samples. • Prepare 2 bottles for 96 samples. Invert to mix. Mark checkbox on bottle.

- 3 Label 1 new full-skirt plate Intermediate, and apply a plate barcode.
- 4 Label 1 new full-skirt plate cfDNA Elution, and apply a plate barcode.
- 5 Label 1 new deep-well plate Extraction Intermediate, and apply a deep-well plate barcode.
- 6 Apply a plate barcode to the DNA Binding plate.
- 7 Prepare a 70% EtOH cleaning solution (70% EtOH, 30% DNase/RNase-free water) for cleaning of the vacuum system.
- 8 Prepare the vacuum system.
 - a Remove the vacuum manifold and clean with 70% EtOH.
 - b Empty the vacuum waste.
 - c Make sure that the ML STAR vacuum system is on.

Procedure

- 1 Click **OK** to start cfDNA Extraction. If the VeriSeq NIPT Method is not already open:
 - a Open the AppLauncher, and click **VeriSeq NIPT Method**.
 - b Enter the Batch ID and username, and then click **OK**.
- 2 Load tips onto the tip carriers as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Tip	1-6	1000 µl tips	1, 2
		7-12	300 µl tips	1
96	Tip	1-6	1000 µl tips	1, 2, 3, 4
		7-12	300 µl tips	1

- 3 Load counted tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Tip	49-54	1000 µl tips	1
			300 µl tips	2
			50 µl tips	3

- 4 Enter the location of the first and last tips for each tip rack, and then click **OK**.
 5 Scan Extraction Box barcodes.
 6 Enter the user name or reagent preparer initials, and then click **OK**.
 7 Scan Accessory Box barcodes.
 8 Enter the user name or reagent preparer initials, and then click **OK**.
 9 Confirm that barcodes are affixed, unseal the Final Plasma deep-well plate if necessary, and load plates (barcode facing right) onto the plate carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Multiflex	19-24	New full-skirt plate, Intermediate – barcoded	1
			New full-skirt plate, cfDNA Elution – barcoded	2
			New deep-well plate, Extraction Intermediate – barcoded	4
			Final Plasma deep-well plate – barcoded	5

- 10 Confirm that the DNA Binding plate is barcoded, then click **OK**.
 11 For the 48-sample batch size, cut a seal in half width-wise and apply over unused columns 7-12 of the plate before loading onto the vacuum manifold.
 12 Load the DNA Binding plate onto the vacuum manifold with the barcode facing right, and then click **OK**.
 13 Load reagent tubs onto the reagent carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Reagent	47	16 ml Elution Buffer	1
			11 ml Proteinase K	2
96	Reagent	47	16 ml Elution Buffer	1
			15 ml Proteinase K	2

- 14 Transfer the specified reagents into the deep-well reservoirs, and then load the reservoirs onto the deep-well carriers as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Deep-well	39–44	125 ml Wash Buffer II	1
			125 ml Wash Buffer I	2
			60 ml 100% EtOH	3
			100 ml Lysis Buffer	4
			60 ml DNase/RNase-free water	5
96	Deep-well	39–44	200 ml Wash Buffer II	1
			125 ml Wash Buffer I	2
			100 ml 100% EtOH	3
			100 ml Lysis Buffer	4
			100 ml DNase/RNase-free water	5

- 15 Wait for the automated reagent volume check to complete.
- 16 Confirm that vacuum waste is not more than half full (empty recommended), and then click **OK**.
- 17 Confirm the placement of all carriers, labware, and reagents, and then click **OK** in the Extraction Deck Verification screen.
- 18 Observe the ML STAR during the automated steps.
- 19 After the final vacuum step, centrifuge the DNA Binding plate, and then click **OK**.
- Remove the DNA Binding plate and clean the bottom surface with 70% EtOH.
 - Seal any uncovered wells on the DNA Binding plate and place it on the empty Final Plasma deep-well plate.
 - Centrifuge the DNA Binding plate/Final Plasma plate assembly at 5600 × g for 10 minutes with the brake on.
- 20 During DNA Binding plate centrifugation, complete the vacuum cleaning.
- Wait for the automated waste disposal to complete.
 - Clean the vacuum manifold and inside the vacuum system with 70% EtOH, and then replace the vacuum manifold.
 - Select the **Manifold is on Vacuum** checkbox to initiate the elution plate transfer on vacuum manifold, and then click **OK**.
- 21 Remove vacuum manifold, and then click **OK**.
- 22 After centrifugation, unseal the wells containing samples on the DNA Binding plate and place it on the cfDNA Elution plate. The cfDNA Elution plate is on the vacuum manifold. Load the DNA Binding plate with the barcode to the right, and then click **OK**.
- 23 Observe the ML STAR during the automated steps.

**CAUTION**

You must manually invalidate sample overflows not detected by the system prior to the contamination of nearby wells.

- 24 After the incubation step, select the **Plates are assembled as indicated** checkbox, confirming that the DNA Binding/cfDNA Elution plate assembly is on a support base (if required by centrifuge).
- 25 Seal the uncovered wells on the DNA Binding plate and centrifuge at 5600 × g for 2 minutes with the brake on, and then click **OK**.
- 26 Visually inspect the cfDNA Elution plate for consistent volumes in each well. Expected volume is approximately 55 µl.

- 27 Seal and retain the cfDNA Elution plate for library preparation.
- 28 When alerted by the Workflow Manager, make sure that the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers, and then click **Unload** to unload the deck.
- 29 Unload all carriers and clean the ML STAR deck, and then click **OK**.
- 30 Enter comments about affected wells, and then click **OK**.
- 31 Perform one of the following:
 - To continue to Prepare Libraries, click **Yes**.
 - To stop, click **Exit**.

SAFE STOPPING POINT

If you are stopping, seal the cfDNA Elution plate and store at -25°C to -15°C for up to 7 days.

Prepare Libraries

Preparation

- 1 Visually examine the Library Prep and Accessory boxes to confirm that the kits are not expired.
- 2 Prepare the following reagents. Label the reservoir tubs and deep-well reservoirs with the reagent names.

Item	Storage	Instructions
End Repair Mix	-25°C to -15°C	Thaw at room temperature. Vortex to mix.
A-Tailing Mix	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
Ligation Mix	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
Resuspension Buffer	2°C to 8°C	Vortex to mix. Return to storage after use.
Hybridization Buffer	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Return to storage after use.
VeriSeq NIPT DNA Adapter Plate	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Centrifuge at 1000 × g for 20 seconds. Apply a plate barcode.
Sample Purification Beads	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex vigorously before each use. Mix by vortexing or inversion until all beads are in suspension and the mixture is homogenous.
80% EtOH	2°C to 8°C	Prepare fresh. Combine 40 ml 100% EtOH and 10 ml DNase/RNase-free water. Invert to mix.
cfDNA Elution Plate from <i>Extract cfDNA on page 14.</i>	-25°C to -15°C	If previously stored, confirm that the plate was not stored more than 7 days and thaw at room temperature. Vortex at 1500 rpm for 1 minute. Centrifuge at 1000 × g for 20 seconds.

- 3 Label 1 new full-skirt plate Libraries and apply a plate barcode.
- 4 Make sure that the ML STAR thermal control is on.

Dilute Enzymes

1 Combine A-Tailing Mix and Resuspension Buffer in a screw cap tube. Vortex to mix, and then centrifuge briefly.

Sample Batch Size	A-Tailing Mix	Resuspension Buffer
48	900 µl	1200 µl
96	1800 µl	2400 µl

2 Combine Ligation Mix and Resuspension Buffer in a screw cap tube. Vortex to mix, and then centrifuge briefly.

Sample Batch Size	Ligation Mix	Resuspension Buffer
48	230 µl	1713 µl
96	440 µl	3278 µl

Procedure

1 Click **OK** to start Library Preparation. If the VeriSeq NIPT Method is not already open:

- a Open the AppLauncher, and click **VeriSeq NIPT Method**.
- b Enter the Batch ID and username, and then click **OK**.

2 Confirm that the following are prepared as indicated in the Reagent Preparation screen:

- ▶ A-Tailing Mix, Ligation Mix, and 80% EtOH
- ▶ Sample Purification Beads, End Repair Mix, and VeriSeq NIPT DNA Adapter Plate

3 Select the checkboxes, and then click **OK**.

4 Scan Library Prep Box barcodes.

5 Enter the user name or reagent preparer initials, and then click **OK**.

6 Scan Accessory Box barcodes.

7 Enter the user name or reagent preparer initials, and then click **OK**.

8 Load tips onto the tip carriers as follows, and then click **OK** for each carrier.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Tip	1-6	50 µl tips	1, 2
		7-12	300 µl tips	1, 2, 3, 5
96	Tip	1-6	50 µl tips	1, 2, 3, 4
		7-12	300 µl tips	1, 2, 3, 4, 5

9 If you stopped the protocol after the cfDNA Extraction procedure, load counted tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Tip	49-54	1000 µl tips	1
			300 µl tips	2
			50 µl tips	3

10 Enter the location of the first tip for each tip rack, and then click **OK**.

- 11 Confirm that barcodes are affixed, and load plates (barcode facing right) onto the plate carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Multiflex	19–24	cfDNA Elution plate – barcoded	1
			DNA Adapter plate – barcoded	2
			New 96-well full-skirt plate, libraries – barcoded	3
			New 96-well full-skirt plates	4, 5

- 12 Load the deep-well carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Deep-well	39–44	50 ml 80% EtOH in a deep-well reservoir	1
			New 96-well full-skirt plates	2, 3, 4, 5

- 13 Load reagent tubs onto the reagent carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Reagent	47	2.5 ml End Repair Mix	1
			Prepared A-Tailing Mix (total volume)	2
			Prepared Ligation Mix (total volume)	3
			10 ml Sample Purification Beads	4
			12 ml Hybridization Buffer	5

- 14 Make sure that the carriers, labware, and reagents are loaded as indicated, and then click **OK** in the Library Deck Verification screen.
- 15 Wait for the automated reagent volume check to complete.
- 16 Observe the ML STAR during the automated steps.
- 17 When alerted by the Workflow Manager, make sure that the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers, and then click **Unload** to unload the deck.
- 18 Visually inspect the Libraries plate for consistent volumes in each well.
- 19 Seal and retain the Libraries plate.
- 20 Unload the carriers, clean the deck, and then click **OK**.
- 21 Enter comments about affected wells, and then click **OK**.
- 22 Perform one of the following:
- To continue to Quantify Libraries, click **Yes**.
 - To stop, click **Exit**.



NOTE

Proceed immediately with quantification unless storing at safe stopping point.

SAFE STOPPING POINT

If you are stopping, seal the Libraries plate prior to storage. The Libraries plate is stable for up to 7 days cumulative storage at -25°C to -15°C.

Quantify Libraries

Preparation

- 1 Prepare the following reagents:

Item	Storage	Instructions
DNA Quantification Reagent	2°C to 8°C	Protect from light. Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
DNA Quantification Standard	2°C to 8°C	Vortex to mix, and then centrifuge briefly.
Libraries plate from <i>Prepare Libraries on page 17</i>	-25°C to -15°C	If previously stored, confirm that the plate was not stored for more than 7 days and thaw at room temperature. Vortex at to mix. Centrifuge at 1000 × g for 20 seconds.
Resuspension Buffer	2°C to 8°C	Vortex to mix.

- 2 Turn on the fluorometer 10 minutes before use.
- 3 Apply a plate barcode to a new 384-well plate.
- 4 Apply a plate barcode to a new full-skirt plate.

Procedure

- 1 Click **OK** to start quantification. If the VeriSeq NIPT Method is not already open:
 - a Open the AppLauncher, and click **VeriSeq NIPT Method**.
 - b Enter the Batch ID and username, and then click **OK**.
- 2 Scan Accessory Box barcodes.
- 3 Enter the user name or reagent preparer initials, and then click **OK**.
- 4 Load tips onto the tip carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Tip	1-6	300 µl tip rack	1
			50 µl tip rack	2
96	Tip	1-6	300 µl tip rack	1
			50 µl tip rack	2, 3

- 5 Confirm that barcodes are affixed, unseal the Libraries plate, and load plates (barcode facing right) onto the Multiflex carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Multiflex	19-24	New full-skirt plates – barcoded	1
			New 384-well plate – barcoded	2
			Libraries plate – barcoded	3
			New 96-well full-skirt plates	4, 5

- 6 Load reagent tubes without caps into the tube carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Tube	46	DNA Quantification Standard	1
			DNA Quantification Reagent	2

- 7 Load reagent tubs onto the reagent carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Reagent	47	New reagent tubs	1
			16 ml Resuspension Buffer	2

- 8 If you stopped the protocol after the Library Preparation procedure, load counted tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Tip	49-54	1000 µl tips	1
			300 µl tips	2
			50 µl tips	3

- 9 Enter the location of the first and last tips for each tip rack, and then click **OK**.
- 10 Make sure that the carriers, labware, and reagents are loaded as indicated, and then click **OK** in the Quant Deck Verification screen.
- 11 Wait for the automated reagent volume check to complete.
- 12 Observe the ML STAR during the automated steps.
- 13 When alerted by the Workflow Manager, make sure that the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers, and then click **Unload** to unload the deck.
- 14 Unload the Libraries plate.
- Visually inspect the plate for consistent volumes in each well.
 - Seal the Libraries plate and store at room temperature until the fluorometric data analysis is complete.
- 15 Unload the remaining 96-well plates and visually inspect for consistent volumes in each well. Gross errors in volume can indicate an issue with pipetting steps.
- 16 Unload the 384-well plate and visually inspect for liquid in the appropriate wells.
- Seal the plate with a foil seal.
 - Centrifuge at 1000 × g for 20 seconds.
 - Incubate at room temperature for 10 minutes, protected from light.
- 17 Unload all carriers and clean the ML STAR deck, and then click **OK**.
- 18 After incubation, remove the foil seal and load the 384-well plate onto the microplate reader. Make sure that A1 is in the top left corner, and click **Read**.
- 19 Export the data as XML as follows:
- Right-click **Barcode**, select rename, scan the barcode of the Quantification plate, and then click **OK**.
 - At the upper-left corner, click the plate icon, and then select **Export** from the menu.
 - Select the **Expt1** checkbox, set the output format to XML, and click **OK**.
 - Set the output file path and file name, and then click **Save**.



NOTE

Ensure file location is accessible by the Hamilton computer. Do not use spaces in the file name or file path.

Analysis

- On the ML STAR, in the Scanner Information screen, enter the fluorometer ID.
- Enter comments about the fluorometer run, and then click **OK**.
- Navigate to the .XML quantification file that contains the fluorometric data, and then click **OK**.

- 4 Review the standards curve and sample concentration analysis results, and then click **OK**.
- 5 If you need to rerun the plate, click **Rescan**.

**NOTE**

Samples are time and light sensitive. When necessary, perform the Rescan immediately.

- 6 Enter comments about affected wells, and then click **OK**.
- 7 Assess the results and proceed as follows.
 - ▶ If the results pass specification, proceed to Pool Libraries.
 - ▶ If the results fail specification, the system aborts the method. Repeat the quantification procedures beginning with *Preparation on page 19*.
- 8 Perform one of the following:
 - To continue to Pool Libraries, click **Yes**.
 - To stop, click **Exit**.

SAFE STOPPING POINT

If you are stopping, seal the Libraries plate prior to storage. The Libraries plate is stable for up to 7 days cumulative storage at -25°C to -15°C.

Pool Libraries

Preparation

- 1 Prepare the following reagents:

Item	Storage	Instructions
Hybridization Buffer	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Return to storage after use.
Libraries plate from <i>Quantification Procedure on page 20</i> .	-25°C to -15°C	If previously stored, thaw at room temperature. Vortex at 1500 rpm for 1 minute. Centrifuge at 1000 × g for 20 seconds.

- 2 Label an empty pooling tube Pool A. For 96 samples, label a second empty pooling tube Pool B.
- 3 Save the following denature program on the thermal cycler with a heated lid.
 - ▶ Choose the preheated lid option and set to 102°C.
 - ▶ Set the reaction volume to 50 µl.
 - ▶ Set the ramp rate to 4°C per second.
 - ▶ Incubate at 96°C for 10 minutes, and then 0°C for 5 seconds.
 - ▶ Hold at 4°C.

Procedure

- 1 Place the Libraries plate on the preprogrammed thermal cycler and run the denature program.
- 2 Click **OK** to start pool libraries. If the VeriSeq NIPT Method is not already open:
 - a Open the AppLauncher, and click **VeriSeq NIPT Method**.
 - b Enter the Batch ID and username, and then click **OK**.
- 3 Select the pool concentration, and then click **OK**.
The target cluster density is 220–260 k/mm². If necessary, adjust the pooling concentration to achieve the target cluster density.
- 4 When prompted by the Workflow Manager, perform 1 of the following.
 - To load a sample sheet, select the sample sheet associated with the batch, and then click **Load**.
 - To use system default values for remaining sample types or sex reporting, click **Use Default** for each setting.

For information about creating a sample sheet, see the *VeriSeq NIPT Assay Software Guide* (document # 100000001949).

- 5 Click **Start** to begin timer for denaturing plate.
- 6 Load tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Tip	7–12	50 µl filter tips	1

- 7 Load the Denatured Library plate (barcode facing right) onto the Multiflex carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Multiflex	19–24	Denatured Library plate (barcoded)	1

- 8 Load pooling tubes onto the tube carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Tube	46	New 2 ml tube, Pool A	1
96	Tube	46	New 2 ml tube, Pool A	1
			New 2 ml tube, Pool B	2

- 9 Load reagent tubs onto the reagent carrier as follows, and then click **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48	Reagent	47	3 ml Hybridization Buffer	1
96	Reagent	47	3 ml Hybridization Buffer	1

- 10 Load tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
48, 96	Tip	49–54	1000 µl filter tips	1
			300 µl filter tips	2
			50 µl filter tips	3

- 11 Enter the location of the first and last tips for each tip rack, and then click **OK**.
- 12 Make sure that the carriers, labware, and reagents are loaded as indicated, and then click **OK** in the Pooling Deck Verification screen.
- 13 Observe the ML STAR during the automated steps.
- 14 When alerted by the Workflow Manager, make sure that the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers, and then click **Unload** to unload the deck.
- 15 Unload the tube carrier. Cap each pooling tube, vortex, and then centrifuge briefly.
- 16 Sequence libraries as soon as possible after pooling. Store the Libraries plate at -25°C to -15°C for up to 7 days to enable repooling, if necessary. The Libraries plate is stable for up to 7 days cumulative storage at -25°C to -15°C.
- 17 Click **OK**.
- 18 Enter comments about affected wells, and then click **OK**.
- 19 Click **OK** at Pooling Complete screen.

SAFE STOPPING POINT

If you are stopping, cap the pooling tubes and store at -25°C to -15°C for up to 7 days.

Prepare Pool for Sequencing

Preparation

1 Prepare the following reagents:

Item	Storage	Instructions
Pool tubes	-25°C to -15°C	If previously stored, thaw at room temperature. Vortex briefly. Centrifuge briefly.

2 Prepare the next-generation sequencing system with the following settings:

- a Paired-end run with 36 x 36 cycle reads.
- b Dual indexing with 8-cycle index reads.
- c Run Name the same as Pool Name.



NOTE

Incorrect run configurations are rejected by the analysis software and may require resequencing.

The following procedure describes the proper loading of pooled libraries onto a cartridge-based next-generation sequencing instrument.

Procedure

- 1 Add buffer and library pool directly to the sequencer sample cartridge as follows.
 - ▶ 900 µl Hybridization Buffer
 - ▶ 450 µl Pool A
 - ▶ Pipette to mix
- 2 Proceed with sequencing using a next-generation sequencing system according to the manufacturer instructions.
- 3 Confirm correct run configuration when prompted.
- 4 Repeat procedure for Pool B, if necessary.

Sequence Data Analysis

After sequencing is complete, sequencing data is automatically sent to the VeriSeq NIPT Assay Software for analysis and report generation. The report includes classifications for each sample in the batch as well as an assessment of all run QC metrics. The analysis process from sequencing completion to final results takes approximately 4 hours for a 48-sample batch. For detailed information on data analysis and the output file, see the *VeriSeq NIPT Solution Software Guide (document # 100000001949)*.

Interpretation of Results

The VeriSeq NIPT Solution employs an algorithm based on multiple data inputs, including sequencing coverage, sequence read quality and estimated fetal fraction, to determine fetal chromosomal representation.

The VeriSeq NIPT Assay Software automatically generates a result of ANEUPLOIDY DETECTED or NO ANEUPLOIDY DETECTED for chromosomes 21, 18, and 13 for each patient sample. A result of ANEUPLOIDY DETECTED indicates that the sample has screened positive for trisomy of the given chromosome.

Results on the fetal sex chromosome status are automatically generated and optionally reported. When no sex chromosomal aneuploidy is detected the report will state NO ANEUPLOIDY DETECTED appended with the sex classification: XX (female fetal sample) or XY (male fetal sample). Sex chromosome aneuploidies are reported as ANEUPLOIDY DETECTED appended with the particular aneuploidy detected: XXX, XXY, XYY or XO (monosomy X). In rare cases, the sex chromosome values fall outside the reportable range and the system generates a result of SEX CHROMOSOMES NOT REPORTABLE. Results for autosomal aneuploidy may still be reported for these samples.

The VeriSeq NIPT Assay Software uses statistics generated during sequencing to provide a fetal fraction estimation (FFE) for each sample. The FFE is the estimated fetal cfDNA component which is recovered by the assay and reported as a rounded percentage for each sample. The average standard deviation of this estimate across all samples is 1.3%. The FFE is not to be used in isolation to exclude samples when reporting results.

To make chromosomal representation calls, the VeriSeq NIPT Assay Software utilizes the individualized Fetal Aneuploidy Confidence Test (iFACT), a dynamic threshold metric that indicates whether the system has generated sufficient sequencing coverage, given the fetal fraction estimate for each sample. The system makes chromosomal representation calls only if a sample meets the iFACT threshold. If a sample fails to achieve this threshold, the QC assessment displays FAILED iFACT and the system does not generate a result. The iFACT assessment is applied to all samples.

In addition to iFACT, the VeriSeq NIPT Assay Software assesses several other QC metrics during analysis. The additional metrics include assessments of coverage uniformity on reference genomic regions (DATA OUTSIDE OF EXPECTED RANGE) and the distribution of cfDNA fragment lengths (FRAGMENT SIZE DISTRIBUTION OUTSIDE OF EXPECTED RANGE). The QC assessment displays either a QC flag or a QC failure for any metrics outside of the acceptable range. In the case of QC failure, the system does not generate a result for the sample. If a sample fails QC, a second plasma aliquot can be processed provided there is sufficient plasma volume in the blood collection tube.



CAUTION

Check the fetal fraction estimates of all samples. If fetal fraction estimates are similar for all samples within a run, sample amalgamation may have occurred and impacted results. Contact Illumina Technical Support for help troubleshooting.

Performance Characteristics

The following data outlined in the clinical performance and analytical performance sections were generated by using the protocols and materials outlined in the *Instructions for Use* starting with plasma. All sequencing data for this section were generated on an Illumina NextSeq 500/550 sequencing system with the following configurations:

- NextSeq control software v2.1.0.31
- NexSeq 500/550 High Output Kit v2 (75 cycle) sequencing reagents kit
- 2x36 paired-end sequencing run in high output mode

Clinical Study

The clinical accuracy of the VeriSeq NIPT Solution with respect to outcomes determined by a clinical reference standard assessment was demonstrated by evaluating plasma samples from pregnant women with singleton pregnancies undergoing prenatal screening for fetal chromosome aneuploidies. Samples were obtained from de-identified banked plasma samples that were previously processed from peripheral whole blood specimens.

A total of 3,107 samples were tested. Of these samples, 21 (0.68%, 21/3107) samples failed assay QC on first pass during the analysis of the completed sequencing data:

- ▶ 11 failed iFACT
- ▶ 8 had data outside the expected range
- ▶ 2 had fragment size distribution outside of the expected range.

Demographics and Pregnancy Characteristics

Maternal age, ethnicity, gestational age, and trimester of pregnancy are summarized in [Table 7](#).

Table 7 Demographics and Pregnancy Characteristics

(N = 3086)	
Maternal age – years	
Mean	36.8
Standard Deviation	3.6
Median	36.7
25th percentile, 75th percentile	35.3, 38.8
Minimum, maximum	18.2, 51.6
Race/ethnicity – n (%)^a	
White or Caucasian	981 (32%)
Black or African American	231 (7%)
Hispanic or Latino	1,079 (35%)
Asian	706 (23%)
Native American	4 (0.1%)
Multiple	58 (2%)
Unknown ^b	27 (1%)
Gestational age at blood draw - weeks	
Mean	12.2
Standard Deviation	2.8
Median	11
25th percentile, 75th percentile	10, 13
Minimum, maximum	10, 25
Trimester of pregnancy – n (%)	
First (<14 weeks)	2,520 (82%)
Second	566 (18%)
Third (≥ 27 weeks)	0 (0%)

^a This trial was conducted in the United States.
^b Reported as “unknown”.

Clinical Performance in Singleton Pregnancies

All study samples had clinical reference standard outcomes (clinical “truth”) related to aneuploidy status and were based on a medical doctor or genetic counselor’s assessment of cytogenetic test or newborn physical exam results. Samples were eligible for testing if clinical outcomes were recorded for fetal aneuploidy status of chromosomes 21, 18, 13, or fetal sex outcomes including fetal sex chromosome aneuploidy (SCA) (monosomy X, XXX, XXY, or XYY). Within the sample set, 3,057 samples had clinical reference data for autosomal aneuploidies and 3,082 had clinical reference data for SCA. Results as called by the VeriSeq NIPT Solution assay were compared to the clinical reference standard outcomes.

Cross Tabulation of VeriSeq NIPT Solution Result Versus Clinical Reference Standard Outcome for Trisomy 21, Trisomy 18, and Trisomy 13

A cross tabulation of the VeriSeq NIPT Solution results (rows) versus the clinical reference standard outcome (columns) is provided in a series of 2 × 2 tables. There were no instances of cross calling between the autosomal aneuploidies (eg, VeriSeq NIPT Solution did not detect trisomy 18 in a sample that had a trisomy 21 affected outcome).

Table 8 Cross Tabulation of Trisomy 21 Results

VeriSeq NIPT Solution Result	Clinical Reference Standard Outcome ^a		Total
	Trisomy 21 Affected	Trisomy 21 Not Affected	
Chromosome 21 Aneuploidy Detected	90	1	91
Chromosome 21 No Aneuploidy Detected	1	2965	2966
Total	91	2966	3057

^a Clinical reference standard assessment was performed with cytogenetic tests or newborn physical exam.

Table 9 Cross Tabulation of Trisomy 18 Results

VeriSeq NIPT Solution Result	Clinical Reference Standard Outcome ^a		Total
	Trisomy 18 Affected	Trisomy 18 Not Affected	
Chromosome 18 Aneuploidy Detected	18	3	21
Chromosome 18 No Aneuploidy Detected	2	3034	3036
Total	20	3037	3057

^a Clinical reference standard assessment was performed with cytogenetic tests or newborn physical exam.

Table 10 Cross Tabulation of Trisomy 13 Results

VeriSeq NIPT Solution Result	Clinical Reference Standard Outcome ^a		Total
	Trisomy 13 Affected	Trisomy 13 Not Affected	
Chromosome 13 Aneuploidy Detected	8	4	12
Chromosome 13 No Aneuploidy Detected	0	3045	3045
Total	8	3049	3057

^a Clinical reference standard assessment was performed with cytogenetic tests or newborn physical exam.

Sensitivity and Specificity of the VeriSeq NIPT Solution for Detecting Trisomies 21, 18, & 13

Table 11 Sensitivity and Specificity of the VeriSeq NIPT Solution for Detecting Trisomies 21, 18, & 13

	Trisomy 21	Trisomy 18	Trisomy 13
Sensitivity	98.9% (90/91)	90.0% (18/20)	100.0% (8/8)
2-sided 95% CI^a	(94.0%,99.8%)	(69.9%,97.2%)	(67.6%,100.0%)
Specificity	>99.9% (2965/2966)	99.9% (3034/3037)	99.9% (3045/3049)
2-sided 95% CI^a	(99.8%,100.0%)	(99.7%,100.0%)	(99.7%,99.9%)

^a CI based on Wilson's score method.

Sensitivity and Specificity of the VeriSeq NIPT Solution in Samples with Fetal Fraction Estimation of $\leq 4\%$ and $> 4\%$

The samples in the performance analyses have estimated fetal fractions ranging from $<1\%$ to 30% . The detection of fetal aneuploidies from maternal cfDNA is, in part, dependent on the fetal fraction for each sample, so the performance of the assay may be decreased at lower fetal fractions. Some NIPT methodologies employ a strict fetal fraction cut-off^{9,10,11,12}, with 4% considered the lower bound of detection.^{9,10,11} The following tables show the performance of the VeriSeq NIPT Solution at fetal fraction estimation of less than or equal to 4% , and greater than 4% . The results of the clinical study demonstrate that VeriSeq NIPT Solution is able to detect fetal aneuploidy at fetal fractions of 4% or less.

Table 12 Sensitivity and Specificity in Samples with Fetal Fraction Estimation $\leq 4\%$

	Trisomy 21	Trisomy 18	Trisomy 13
Sensitivity	90.9% (10/11)	80.0% (4/5)	N/A (0/0)
2-sided 95% CI*	(62.3%, 98.4%)	(37.6%, 96.4%)	N/A
Specificity	99.7% (329/330)	100.0% (336/336)	99.7% (340/341)
2-sided 95% CI*	(98.3%, 99.9%)	(98.9%, 100%)	(98.4%, 99.9%)

*CI based on Wilson's score method

Table 13 Sensitivity and Specificity in Samples with Fetal Fraction Estimation $> 4\%$

	Trisomy 21	Trisomy 18	Trisomy 13
Sensitivity	100.0% (80/80)	93.3% (14/15)	100% (8/8)
2-sided 95% CI*	(95.4%,100.0%)	(70.2%,98.8%)	(67.6%,100.0%)
Specificity	100.0% (2636/2636)	99.9% (2698/2701)	99.9% (2705/2708)
2-sided 95% CI*	(99.9%,100.0%)	(99.7%,100%)	(99.7%,100%)

*CI based on Wilson's score method

Detection of Sex Chromosome Aneuploidies

The VeriSeq NIPT Solution sex chromosome results were compared to the clinical reference standard outcome and are summarized in table below. The percent concordance was calculated for each sex chromosome within each clinical reference standard outcome [classification]. Percent concordance was calculated as the number of samples in which the VeriSeq NIPT Solution sex chromosome call matched the clinical reference standard classification, divided by the total number of samples with the same clinical reference standard classification.

Table 14 Percent Concordance for Fetal Sex Classification

Veriseq NIPT Solution Results For Fetal Sex Classification	Clinical Reference Standard Outcome								
	Newborn Physical Exam Outcome [No Cytogenetic Results]		Cytogenetic Results						
	Female	Male	XX	XY	XO	XXX	XXY	XYY	Other ^a
Percent Concordant	99.9% (1371/1373)	99.9% (1420/1422)	97.4% (147/151)	100.0% (118/118)	100.0% (6/6)	80.0% (4/5)	100.0% (5/5)	100.0% (1/1)	Not Applicable

^a 1 sample was 49, XXXXY classified by the VeriSeq NIPT Solution as "Sex chromosome not reportable"

Positive Predictive Value and Negative Predictive Value of the VeriSeq NIPT Solution for Detecting Trisomies 21, 18, & 13 for a Range of Prevalences

Positive predictive value (PPV) and negative predictive value (NPV) of the test provide information regarding the test's ability to inform clinical decisions based on test sensitivity, specificity, and pretest probability that a fetus is trisomy affected (prevalence). Since PPV and NPV depend on prevalence and the prevalence for these aneuploidies may vary across different subject populations, PPV and NPV were calculated based on the observed sensitivity and specificity seen in the clinical accuracy study for Trisomies 21, 18, and 13. The table below summarizes the PPV and NPV for a range of plausible prevalence values.

Table 15 Positive Predictive Value and Negative Predictive Value of the VeriSeq NIPT Solution for Detecting Trisomies 21, 18, and 13 for a Range of Prevalences

Aneuploidy	Prevalence	PPV	NPV
Trisomy 21	0.05%	59.48%	100.00%
	0.10%	74.60%	100.00%
	0.20%	85.47%	100.00%
	0.50%	93.65%	99.99%
	1.00%	96.74%	99.99%
	1.50%	97.81%	99.98%
	2.00%	98.36%	99.98%
Trisomy 18	0.03%	21.48%	100.00%
	0.05%	31.32%	99.99%
	0.10%	47.71%	99.99%
	0.20%	64.62%	99.98%
	0.30%	73.28%	99.97%
	0.40%	78.54%	99.96%
	0.50%	82.08%	99.95%
Trisomy 13	0.01%	7.09%	100.00%
	0.02%	13.23%	100.00%
	0.05%	27.61%	100.00%
	0.10%	43.29%	100.00%
	0.20%	60.44%	100.00%

NPV = negative predictive value, PPV = positive predictive value

Performance in Twin Pregnancies

Clinical Performance

Due to low prevalence, only a small number of twin samples were available for the clinical study. There were four trisomy 21 twin samples that were tested and all were correctly called for presence of trisomy 21 as well as absence of any other anomaly. However, given the fact that the number of twin samples was too low, the confidence levels for sensitivity and specificity would be too wide to be of practical use. As such, these samples were not included in the overall performance calculations reported in [Table 11](#).

Estimating Trisomy 21, 18, and 13 Performance

To more accurately estimate the performance of VeriSeq NIPT Solution in twin pregnancies, *in silico* models based on observations from clinical samples were used to simulate populations of twin pregnancies consistent with the intended use population. The distribution of fetal fraction was determined from approximately 4,500 twin samples and compared to the distribution from approximately 120,000 singleton samples. The distribution

of fetal fraction conditional on aneuploidy status was determined from singleton putative calls (1,004 trisomy 21, 312 trisomy 18, and 197 trisomy 13). Combining the two distributions allowed for inferences of aneuploidy detection in twins. Sets of dizygotic and monozygotic twins were simulated, and a weighted average representing their prevalence in the intended use population was taken (2 dizygotic: 1 monozygotic) to estimate sensitivity. For specificity, sets of unaffected twins were simulated.

The fraction of each simulated sample affected by the trisomy (ie, the 'affected fraction'), was calculated differently for each sample category:

- For monozygotic twins, the affected fraction of each sample was set to 1.0 because, in this situation, the trisomy affects both twins.
- For dizygotic twins, it was assumed that only one twin was affected (to have both dizygotic twins be affected is extremely rare). Affected fraction values were simulated using the known distribution of fetal fraction ratios as determined from sex discordant clinical twin samples. A conservative approach was taken whereby it was assumed that the affected twin always had the lowest fetal fraction of the two twins. A correction factor was applied for fetal fractions being on average lower in trisomy 13 and 18 pregnancies.
- For unaffected twins, the affected fraction of each sample was set to zero.

For twins affected by either trisomy 18 or 13, the fetal fraction corresponding to the affected fraction of the sample was reduced proportional to the average reduction in fetal fraction observed in clinical data in Trisomy 18 or 13 singletons versus euploid singletons.

Both the overall fetal fraction and the affected fraction of each simulated sample were then used to calculate an aneuploidy score using the standard VeriSeq NIPT Solution algorithm. Sensitivity was calculated by determining how often the aneuploidy scores for the simulated affected twins were above the corresponding aneuploidy cutoff. Correspondingly, specificity was calculated by determining how often the aneuploidy scores for the simulated unaffected twins were below the corresponding aneuploidy cutoff (Table 16). 95% confidence intervals were estimated based on the number of real clinical twin samples in the original data set, which were classified as either affected or unaffected by the relevant trisomy.

Table 16 Estimates for Trisomy 21, 18, and 13 in Simulated Population of Twin Pregnancies

	Trisomy 21	Trisomy 18	Trisomy 13
Sensitivity	97.1%	95.8%	95.1%
2-Sided 95% CI*	(87.9%, 99.2%)	(66.7%, 99.5%)	(67.7%, 99.3%)
Specificity	99.9%	> 99.9%	> 99.9%
2-Sided 95% CI	(99.7%, 99.9%)	(99.9%, > 99.9%)	(99.9%, 99.9%)

*CI based on the Wilson score method

For Table 16, point estimates and estimated 95% confidence intervals for the sensitivity and specificity of VeriSeq NIPT Solution to detect Trisomy 21, 18, and 13 were determined from a simulated population of twin pregnancies consistent with the intended use population. Confidence intervals were estimated based on the number of QC passing clinical twin samples classified as either affected or unaffected by the relevant trisomy. The sensitivity calculation assumes that two thirds of affected twin pregnancies are dizygotic with one affected twin, while one third of affected twin pregnancies are monozygotic with both twins affected.

The estimates listed in Table 16 pertain to twin pregnancies only. Due to even lower prevalence, data for higher-order pregnancies (triplets or higher) were insufficient to establish appropriate statistical models from which accuracy of aneuploidy detection could be estimated.

Analytical Performance

Precision

To assess VeriSeq NIPT Solution precision, two studies were conducted:

- ▶ A multisite internal Reproducibility study that comprised 9 runs across 3 sites using 3 operators and a single lot of reagents

- ▶ A Within-Lab Precision study that comprised 12 runs at a single site using two operators, 2 instrument systems, and 3 reagent lots

A 5% fetal fraction trisomy 21 pool was created by combining cfDNA extracted from maternal plasma from pregnant women (with a trisomy 21 affected fetus) and cfDNA extracted from plasma from nonpregnant women. Pooled cfDNA extracted from maternal plasma from unaffected male (XY fetus) and unaffected female (XX fetus) pregnancies were also tested. Testing was performed over 10 days for a total of 21 runs for the 2 studies combined.

Of the 903 samples included in the analyses across the 2 studies, there was 100% agreement with 84/84 for trisomy 21, 399/399 for XX sex classification, and 420/420 for XY sex classification. Distribution of samples by site were as follows: Site 1 - T21 (12), XX (57), XY(60); Site 2 - T21 (12), XX (57), XY(60); Site 3 - T21 (60), XX (285), XY(300).

Table 17 Reproducibility and Within-Lab Precision (Combined Data)

Expected	Attempted	Observed Call				
		T21	T18	T13	XX	XY
T21 (XY)	84	84	0	0	0	84
XX	399	0	0	0	399	0
XY	420	0	0	0	0	420

Cross-Contamination

Cross-contamination was assessed in the VeriSeq NIPT Solution sample preparation workflow. Plasma pools from non-pregnant females (XX) and adult males (XY) were tested in a checkerboard pattern in the 96-well plate format across 4 plates (N=48 each for female and male samples per plate; total of 192 female and 192 male samples). None of the female samples demonstrated chromosome Y coverage that was statistically higher than the estimated background, indicating no cross-contamination from male samples within the same plate. No detectable cross-contamination was observed in the VeriSeq NIPT Solution.

Potentially Interfering Substances

To assess the impact of interfering substances on the VeriSeq NIPT Solution, the performance of the assay was evaluated in the presence of potential interferents.

Albumin, bilirubin, hemoglobin, and triglycerides (endogenous) were each spiked into maternal plasma pools from unaffected female (XX fetus) pregnancies and tested at 2 concentrations for each test substance (n=16 for each). No interference in the performance of the assay was observed.

Table 18 Potentially Interfering Substances (endogenous)

Test Substance	Low Test Concentration (mg/mL)	High Test Concentration (mg/mL)
Albumin	35	50
Bilirubin	0.01	0.15
Hemoglobin	100	200
Triglyceride	1.5	5

Naturally occurring maternal genomic DNA (gDNA) in the plasma can also potentially interfere with assay performance, as it may be extracted along with the fetal cfDNA. Genomic DNA levels at 1.6, 3.3, and 4.9 ng per sample (corresponding to 1, 2, and 3 standard deviations above mean expected gDNA concentration after 7 days of whole blood storage¹³) was added to cfDNA extracted from maternal plasma from unaffected female (XX fetus) pregnancies. Samples were then tested in the VeriSeq NIPT Solution (n=16 for each concentration). No interference in the performance of the assay was observed in the presence of elevated levels of gDNA.

Twenty drug-based potentially interfering substances (exogenous) commonly used or prescribed during pregnancy were tested per EP7-A2 (Interference Testing in Clinical Chemistry; Approved Guideline-Second Edition). The 20 potential interferents were combined into 4 pools, spiked into maternal plasma from unaffected female (XX fetus) pregnancies, and tested in the VeriSeq NIPT Solution (N=16 for each pool). No interference in the performance of the assay was observed in the presence of these exogenous substances.

Table 19 Potentially Interfering Substances (exogenous)

Pool 1	Pool 2	Pool 3	Pool 4
Acetaminophen	Diphenhydramine	Albuterol	Cetirizine
Acetylcysteine	Erythromycin	Bupropion	Dextromethorphan
Bisoprolol	Guafenesin	Caffeine	L-Ascorbic acid
Citalopram	Heparin	Sertraline	Metoprolol
Desloratadine	Lidocaine	Sodium fluoride	Nadolol

Troubleshooting

VeriSeq NIPT Solution Troubleshooting

Failure Mode	Possible Result	Interpretation	Recommended Action	Comments
Insufficient input plasma	Sample QC failure	Insufficient plasma volume	Redraw	Based on visual inspection of plasma volume.
Blood tube failure	No separation of blood into layers	Sample was not centrifuged	Make sure that the centrifuge started and the tube was spun at the correct force. Redraw sample.	
		Improper sample storage or transport (lysis of sample)	Redraw sample.	Frozen samples will not separate.
Sample clog / Slow flow	Plasma contamination	Individual samples may clog the binding plate if there is significant contamination in the plasma sample	Inspect sample, if remaining plasma in tube is red or milky, cancel sample and request redraw. If sample appears normal, retest sample.	
	Sample Overflow	Inadequate visual inspection of each tube for sample suitability.	Invalidate any samples in nearby wells affected by the overflow.	Might indicate an improper transport or storage condition of the sample before processing. You should exclude unsuitable samples from processing.
	Hardware malfunction	Inadequate digestion of material during extraction	Retest sample. If problem persists in well location with other samples, contact Illumina Technical Support.	

Failure Mode	Possible Result	Interpretation	Recommended Action	Comments
Individual Sample Analysis QC failure	Sequencing QC failure	Insufficient genetic input OR Mistransfer during sample handling	Check Sample Annotation. Check for similar performance on previous samples in relative plate position. Retest sample.	Indicates either a bad sample input, or a mistransfer on the ML STAR. Insufficient genetic material can come from insufficient cell-free DNA in the plasma or cell-based DNA causing over-dilution of the sample for sequencing.
	Low FF or NES count	Insufficient data generated to make accurate reporting	Retest from plasma.	
Quantification QC failure	Failed quantification run – Batch median below minimum	Insufficient process yield	Repeat quantification. If repeat also fails, contact Illumina Technical Support.	Passing standards curve metrics indicates issue with library preparation.
	Failed quantification run	Standard curve failure due to bad quantification	Repeat quantification. If repeat also fails, contact Illumina Technical Support.	
Pooling Failure	Failure to complete sample pooling	Pooling Analysis is unable to calculate proper pool volumes	Reevaluate target pool concentration, rerun pooling analysis.	

VeriSeq NIPT Microlab STAR Troubleshooting

Process Step	Error Code	Error Dialog	Description	User Resolution
Batch Creation	EM0044	The Batch ID entered contains forbidden characters.	The VeriSeq NIPT Solution only accepts numbers, letters, underscores, and dashes for all data fields.	Rename batch using a name that does not contain any special text characters.
Batch Creation	EM0051	The Batch ID is greater than 26 characters in length.	The VeriSeq NIPT Solution limits the length of batch names to 26 characters or less.	Rename batch using a name that is fewer than 26 characters.
Batch Creation	EM0076	Unable to connect to VeriSeq Onsite Server	The VeriSeq Onsite Server is not responding to data requests from the Workflow Manager.	Make sure that: <ol style="list-style-type: none"> 1. The ML STAR is connected to the network. 2. The VeriSeq Onsite Server is on. 3. The ML STAR can connect to the VeriSeq Onsite Server (via ping request). 4. If above steps do not resolve the issue, email Illumina Technical Support.
Batch Creation	EM0118	This batch has been failed and cannot be further processed.	The specified batch has already been failed and cannot be processed any further.	The batch record on the VeriSeq Onsite Server indicates that the selected batch has been failed. No further processing is allowed. Create another batch with the desired samples.

Process Step	Error Code	Error Dialog	Description	User Resolution
Batch Creation	N/A	This batch has already completed processing. Would you like to repool?	The indicated batch has been processed through pooling. The only allowable processing is repool.	To repool, click Re-Pool . OR Abort method and double check Batch Name.
Plasma Isolation	WP0087	Duplicate sample barcodes loaded.	Samples with identical barcodes have been loaded onto the system.	1. Follow the Workflow Manager prompts to identify which samples are duplicates. 2. Remove those samples and either relabel or replace them. 3. Reload samples.
Plasma Isolation	EP0102	Samples specified in the Sample Sheet were not loaded.	Samples included in the sample sheet were not included in the loaded barcodes.	1. Follow the Workflow Manager prompts to identify missing samples. 2. Add missing samples to the batch and reload samples OR Abort method, modify the sample sheet as needed, restart method.
Plate Load	N/A	Venus Barcode Mask Error	The Workflow Manager enforces correct plate-to-batch association using Venus barcode masks.	1. Check plate placement to confirm that the plate layout is correct. 2. Make sure that the plate loaded is the correct plate for the indicated batch.

Process Step	Error Code	Error Dialog	Description	User Resolution
cfDNA Extraction	WE0150	Pressure in the vacuum chamber is too low.	The Workflow Manager will not proceed if the resting vacuum line pressure sense is < 400 Torr.	<ol style="list-style-type: none"> 1. Check for kinks or other obstructions in the vacuum line. 2. Open the waste line release clips, allow for pressure to release, fully close the release line clips. 3. Make sure that the vacuum controller and pump are turned on. 4. If the problem persists, contact Illumina Technical Support.
	WE0153	Pressure in the vacuum chamber is too high.	If the measured vacuum pressure is too high prior to starting the pressure control, the system may be malfunctioning.	On the back of the controller, make sure that all vacuum connections and lines are secure.
	WE0996	Vacuum failed to seal.	The system fails to create a vacuum seal on the Binding Plate.	<p>NOTE Do not select OK until the seal failure is completely resolved.</p> <ol style="list-style-type: none"> 1. Make sure that the binding plate is flush against the vacuum manifold. With a gloved hand, forcibly press down on the binding plate. 2. Click OK to continue with cfDNA Extraction. 3. If this error message is displayed more than three times in a run, e-mail Illumina Technical Support.
	WM0219	If Vacuum is on, manually rest the pump.	The vacuum may stay on after a method abort during extraction.	<ol style="list-style-type: none"> 1. On the Vacuum Controller, press the Power button to shut off the vacuum. 2. Wait 10 seconds, and then press the Power button again to turn on the vacuum.
	EE0477	An error has occurred while moving a plate. (iSWAP error)	If an iSWAP error is encountered (plate drop, failed to pick up, and so on) the system will prompt the user to complete the plate move manually.	<p>Make sure that the plate can be recovered (no spilled material).</p> <ul style="list-style-type: none"> - If not, abort run. - If so, follow the displayed instructions to complete the plate transfer manually.
	EE0519	Scanned barcode does not match binding plate barcode on record.	The loaded Binding plate does not match the barcode of the plate removed.	Make sure that the plate being loaded matches the recorded barcode (see the trace log for the expected barcode).

Process Step	Error Code	Error Dialog	Description	User Resolution
API	EA0372	Unable to connect to the data server.	The VeriSeq Onsite Server is not responding to data requests from the Workflow Manager.	Make sure that: 1. The ML STAR is connected to the network. 2. The ML STAR can connect to the VeriSeq Onsite Server (via ping request). 3. The VeriSeq Onsite Server is on.
	EA0774	Connection Error The API server connection failed to validate.	The VeriSeq Onsite Server has stopped responding to data requests from the Workflow Manager.	Make sure that: 1. The ML STAR is connected to the network. 2. The ML STAR can connect to the VeriSeq Onsite Server (via ping request). 3. The VeriSeq Onsite Server is on.
	EA0780	403: Invalid Request The current transaction is not valid.	The data sent violates the system workflow logic.	Consult the error details for more information. Common causes involve inputs that are too long or violate the acceptable character list.

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Revision History

Document	Date	Description of Change
Document # 1000000001856 v08	August 2021	<ul style="list-style-type: none">• Added Revision History table.• Updated EU Authorized Representative address.

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