

Package Insert

FOR IN VITRO DIAGNOSTIC USE.

Intended Use

The VeriSeq™ NIPT Solution v2 is an *in vitro* diagnostic test intended for use as a screening test for the detection of genome-wide fetal genetic anomalies from maternal peripheral whole blood specimens in pregnant women of at least 10 weeks gestation. VeriSeq NIPT Solution v2 uses whole genome sequencing to detect partial duplications and deletions for all autosomes and aneuploidy status for all chromosomes. The test offers an option to request the reporting of sex chromosome aneuploidy (SCA). This product must not be used as the sole basis for diagnosis or other pregnancy management decisions.

The VeriSeq NIPT Solution v2 includes: the VeriSeq NIPT Workflow Manager v2 for the VeriSeq NIPT Microlab STAR, the VeriSeq NIPT Sample Prep Kit and the VeriSeq Onsite Server v2 with the VeriSeq NIPT Assay Software v2. The VeriSeq NIPT Solution v2 is intended to be used with a next generation sequencer.

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 screening. Diagnostic testing involves invasive procedures such as amniocentesis or chorionic villus sampling. These testing methods are considered the gold standard for detection of fetal 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 Ultrasound screens can also detect chromosome abnormalities, but they do so with even less certainty than these other methods.

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 99.7% and 99.96% and trisomy 18 97.9% and 99.96%, respectively.⁵ 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.⁶

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.^{7,8,9} Pretest counseling, informed consent, and diagnostic testing to confirm a positive cfDNA screening result are recommended.⁴

The VeriSeq NIPT Solution v2 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. The test offers two options for types of screening: basic and genomewide. The basic screening provides information on the aneuploidy status of chromosomes 21, 18, 13, X, and Y only. Genomewide screenings provide partial duplications and deletions for all autosomes and aneuploidy status for all chromosomes. Both screening types provide the option for sex chromosome aneuploidy (SCA) reporting with or without fetal sex reporting. The reporting option for SCA can be turned off. If the reporting option for SCA is turned off, fetal sex is not reported either. For more information on the sex reporting options, refer to the *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)*.

Principles of Procedure

The VeriSeq NIPT Solution v2 is an automated solution for laboratory NIPT testing which consists of automated sample preparation and sequencing data analysis. The VeriSeq NIPT Sample Prep Kit are specialized single use reagents that are used in conjunction with the VeriSeq NIPT Microlab STAR to prepare 24-, 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 v2, and a report is generated that provides qualitative results.

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 more detail:

- **Sample Collection**—7–10 ml of maternal peripheral whole blood is collected in a Streck cell-free DNA Blood Collection Tube (BCT), which prevents cell lysis and genomic contamination and stabilizes whole blood.
- **Plasma Isolation**—Within 5 days of collection, 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. In the event that retesting is required, post-processing samples may be recapped and stored at 4°C for an additional 5 days (up to a total of 10 days after blood collection).



CAUTION

Exceeding the aforementioned storage times can negatively impact individual sample failure rates.

- **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 24, 48, or 96 receives a unique indexed adapter. The adapters serve 2 purposes:

**CAUTION**

Take extreme care to avoid cross contamination of the indexes which could lead to incorrect results.

- 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 sample libraries are pooled together into 24- or 48-sample pools in adjusted amounts to minimize variation in coverage. Each pool is then sequenced using a next-generation sequencing system.
- The VeriSeq NIPT Solution v2 does not include sequencing equipment and consumables.
- **Analysis**—For each sample, analysis consists of the following:
 - Identification of library fragments by index sequence and alignment of the paired end reads to a human reference genome.
 - Estimation of the fetal fraction of the library by combining information from the distribution of both the lengths and genomic coordinates of the library fragments.
 - After accounting for known biases, a statistical model detects regions of the genome which are under or overrepresented in the library in a manner consistent with an anomaly at the estimated level of fetal fraction.
 - The NIPT report provides summary results for the selected test menu where ANOMALY DETECTED or NO ANOMALY DETECTED is listed along with a fetal fraction estimate for samples passing QC.
 - The Supplementary Report provides quantitative metrics which characterize each detected anomaly.

Limitations of the Procedure

Limitations of the Assay

- 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 v2 is not intended to detect polyploidy, such as triploidy.
- The VeriSeq NIPT Solution v2 is not intended to detect balanced chromosome rearrangements.
- The assay requires maternal peripheral whole blood samples from pregnant women of at least 10 weeks gestation.
- For basic screens, the VeriSeq NIPT Solution v2 test looks for specific chromosome abnormalities. Results reported as NO ANOMALY DETECTED do not eliminate the possibility of chromosomal abnormalities of the tested chromosomes. 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).
- For genomewide screens, large deletions and duplications that are less than 75% of the size of the chromosome can be indicative of whole chromosome aneuploidy.
- For genomewide screens, certain regions are excluded from analysis. A list of such blacklisted regions is available on the Illumina Support website. Genomic anomaly detection is only performed on non-excluded regions.
- Fetal sex reporting is not available in all regions due to local regulations governing gender reporting.
- Based on the literature evidence, cell-free DNA-based screening results can be confounded by certain maternal and fetal factors. Some of these are listed below, but are not limited to the following:
 - Recent maternal blood transfusion
 - Maternal prior organ transplant / stem cell transplant
 - Maternal autoimmune disease
 - Maternal neoplasms (benign and malignant)
 - Maternal mosaicism
 - Maternal copy number variations
 - Fetoplacental mosaicism / confined placental mosaicism
 - Fetal demise / vanishing twin

VeriSeq NIPT Solution v2 Reporting

- The VeriSeq NIPT Solution v2 is a screening test and should not be considered in isolation from other clinical findings and test results. Conclusions about the fetal condition and pregnancy management decisions should not be based on the results of the NIPT screening alone.⁷
- The VeriSeq NIPT Solution v2 reports on the following:

- Basic screen tests the overrepresentation of chromosomes 13, 18, and 21.
- Genomewide screen tests under and overrepresentation of all autosomes, including partial deletions and duplications of at least 7 Mb.
- In singleton pregnancies with Yes or SCA selected as the sex reporting option, the following sex chromosomal anomalies: XO, XXX, XXY, and XYY.
- In singleton pregnancies with Yes selected as the sex reporting option, fetal sex is reported.
- The presence of a Y chromosome in twin pregnancies.

Product Components

The VeriSeq NIPT Solution v2 consists of the following sample prep:

- VeriSeq NIPT Sample Prep Kit (24 samples) (part # 20025895)
- VeriSeq NIPT Sample Prep Kit (48 samples) (part # 15066801)
- VeriSeq NIPT Sample Prep Kit (96 samples) (part # 15066802)

The VeriSeq NIPT Solution v2 consists of the following software components:

- VeriSeq NIPT Assay Software v2 (part # 20047024), preinstalled on the VeriSeq Onsite Server v2.
 - VeriSeq Onsite Server v2 (part # 20028403, 20047000, 20101927) or an existing VeriSeq Onsite Server (part # 15076164 or # 20016240) that is upgraded to v2.
- VeriSeq NIPT Workflow Manager v2, (part # 20044988), preinstalled on the VeriSeq NIPT Microlab STAR.
 - VeriSeq NIPT Microlab STAR (part # Hamilton Company Reno: 95475-01 (115 V) & 95475-02 (230 V), Hamilton Company Bonaduz: 806288).
- Local Run Manager VeriSeq NIPT Module (part # 20044989)

Reagents

Reagents Provided

Illumina provides the following reagents: VeriSeq NIPT Sample Prep Kit (24 samples) (part # 20025895), VeriSeq NIPT Sample Prep Kit (48 samples) (part # 15066801), and VeriSeq NIPT Sample Prep Kit (96 samples) (part # 15066802). The VeriSeq NIPT Sample Prep Kit are configured for use with the VeriSeq NIPT Microlab STAR (ML STAR) (part # 95475-01, 95475-02, or 806288), which is provided by the Hamilton Company.

VeriSeq NIPT Sample Prep Kit, Extraction Box

Table 1 VeriSeq NIPT Extraction Box (24) and (48), Part # 20025869 and 15066803

Reagent Name on Label	Number of Containers in Kit	Active Ingredients	Storage
Lysis Buffer	1	Guanidine hydrochloride in buffered aqueous solution	15°C to 30°C
Wash Buffer I	1	Guanidine hydrochloride and 2-propanol in buffered aqueous solution	15°C to 30°C
Wash Buffer II	1	Buffered aqueous solution-containing salts	15°C to 30°C
Elution Buffer	1	Buffered aqueous solution	15°C to 30°C
Proteinase Buffer	1	Glycerol in buffered aqueous solution	15°C to 30°C
Proteinase K	3	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	Active Ingredients	Storage
Lysis Buffer	1	Guanidine hydrochloride in buffered aqueous solution	15°C to 30°C
Wash Buffer I	1	Guanidine hydrochloride and 2-propanol in buffered aqueous solution	15°C to 30°C
Wash Buffer II	2	Buffered aqueous solution-containing salts	15°C to 30°C
Elution Buffer	1	Buffered aqueous solution	15°C to 30°C
Proteinase Buffer	1	Glycerol in buffered aqueous solution	15°C to 30°C
Proteinase K	4	Lyophilized Proteinase K	15°C to 30°C

VeriSeq NIPT Sample Prep Kit, Library Prep Box

Table 3 VeriSeq NIPT Library Prep Box (24) and (48), Part # 20026030 and 15066809

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

VeriSeq NIPT Sample Prep Kit, Accessory Box

Table 5 VeriSeq NIPT Accessory Box, Part # 15066811

Reagent Name on Label	Number of Containers in Kit	Active Ingredients	Storage
DNA Binding Plate	1	Propylene microplate with modified silicone membrane	2°C to 8°C

Reagent Name on Label	Number of Containers in Kit	Active Ingredients	Storage
Resuspension Buffer	1	Buffered aqueous solution	2°C to 8°C
Sample Purification Beads	1	Solid-phase paramagnetic beads in buffered aqueous solution	2°C to 8°C
DNA Quantification Reagent	1	DNA intercalating dye in DMSO	2°C to 8°C
DNA Quantification Standard	1	dsDNA standard, nonspecific DNA, and sodium azide in buffered aqueous solution	2°C to 8°C

VeriSeq NIPT Sample Prep Kit, 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

- Sequencing reagents and consumables required for next-generation sequencing (NGS) system
- Certified DNase/RNase-free water – molecular biology grade
- Ethanol, 100% (200 proof) – molecular biology grade

NOTE Non-molecular biology grade ethanol can potentially negatively impact performance of the assay.

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, refer to the Storage column in the tables under the [Reagents](#) section. 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 seven days at -25°C to -15°C. No additional denaturation is required if stored for this period of time at these conditions.

Equipment and Materials

Equipment and Materials Required, Not Provided

Equipment Required, Not Provided

Equipment	Supplier
<p>A next-generation sequencing (NGS) system with the following capabilities:</p> <ul style="list-style-type: none"> • 2 x 36 bp paired-end sequencing • Compatible with VeriSeq NIPT Sample Prep Kit dual index adapters • Automatic production of BCL files • Two channel chemistry • 400 million paired-end reads per run • Compatible with VeriSeq NIPT Assay Software v2 or a NextSeq 550Dx Sequencing System. 	Instrument supplier or Illumina, part # 20005715
Freezer, -25°C to -15°C	General lab supplier
Microcentrifuge	General lab supplier
Pipette aid	General lab supplier
Refrigerator, 2°C to 8°C	General lab 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
Vortexer	General lab supplier
Centrifuge and rotor assembly for blood collection tubes	
<p>Equivalent:</p> <ul style="list-style-type: none"> • Refrigerated centrifuge capable of 1600 × g with no-brake option • Swinging bucket rotor with buckets • Bucket inserts with 76 mm minimum depth • Insert adapters to support 16 mm x 100 mm blood collection tubes 	General lab supplier

Equipment	Supplier
<p>Recommended:</p> <ul style="list-style-type: none"> Allegra X12R 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 	<p>Beckman Coulter, item # 392304 (120 V or 230 V)</p> <p>Beckman Coulter, item # 369704</p> <p>Beckman Coulter, item # 392805</p> <p>Beckman Coulter, item # 359150</p>
Centrifuge and rotor assembly for microplates	
<p>Equivalent:</p> <ul style="list-style-type: none"> Centrifuge capable of 5600 × g Swinging plate rotor with 96-well plate carriers, 76.5 mm minimum depth. Multifuge X4 Pro-MD 120 V TX-1000BT Sorvall Legend XTR Centrifuge 	<p>General lab supplier</p> <p>Thermo Fisher Scientific # 75016034</p> <p>Thermo Fisher Scientific, catalog # 75004521 (120 V) or catalog # 75004520 (230 V)</p>
<ul style="list-style-type: none"> HIGHPlate 6000 Microplate Rotor Rotor high plate 6000 <p>Support base for microplates</p> <ul style="list-style-type: none"> Recommended: <ul style="list-style-type: none"> MicroAmp 96-Well Support Base 96-Well PCR Plate Carrier 	<p>Thermo Fisher Scientific, catalog # 75003606</p> <p>Thermo Scientific VWR, catalog # 97040-244</p> <p>Thermo Fisher Scientific, catalog # 4379590</p> <p>Thermo Fisher Scientific, catalog # AB-0563/1000</p>
<p>One of the following microplate readers, or equivalent, (fluorometer) with SoftMax Pro v6.2.2–7.1.2:</p> <ul style="list-style-type: none"> Gemini XPS SpectraMax M2, M3, M4, and M5. <ul style="list-style-type: none"> Purple insert is required with microplate reader for use in workflow. 	<p>Molecular Devices, part # XPS</p> <p>Molecular Devices, part # M2, M3, M4, and M5</p>
SpectraMax High-Speed USB, Serial Adapter	Molecular Devices, part # 9000-0938

Equipment	Supplier
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), part # 95475-02 (230 V), or part # 806288 (for Hamilton Company Bonaduz)
VeriSeq Onsite Server v2 or an upgraded VeriSeq Onsite Server	Illumina, part # 20028403 or 20047000 (v2) or 20101927 or # 15076164 or # 20016240 (upgraded)
If using a NextSeq 550Dx Sequencing System: <ul style="list-style-type: none"> • NextSeq 550Dx High Output Reagent Kit v2.5, 75 cycles 	Illumina, part # 20028870

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

Consumable	Supplier
<p>Deep-well reservoir with the following specifications:</p> <ul style="list-style-type: none"> • SLAS 1–2004 microplate format with 96 pyramidal or conical bottom wells and a 240 ml minimum capacity. • Polypropylene with preference for low DNA binding for all sample contact surfaces. • Internal dimensions (liquid level) are compatible with automated aspiration and dispensing steps of VeriSeq NIPT Microlab STAR. • Height dimensions are compatible with automated movements of VeriSeq NIPT Microlab STAR. 	<p>General lab supplier</p> <p>Compatible reservoirs:</p> <ul style="list-style-type: none"> • Corning Axygen, product # RES-SW96-HP-SI • Agilent, product # 201246-100
<p>Reagent tub with the following specifications:</p> <ul style="list-style-type: none"> • Tub that fits securely, but not forced, into the carrier of the VeriSeq NIPT Microlab STAR with tapered bottom and a 20 ml minimum capacity. • Polypropylene that is free from RNase/DNase. • Internal reservoir dimensions (liquid level) generate liquid levels using assay reagent volumes that are compatible with automated aspirating and dispensing steps of VeriSeq NIPT Microlab STAR. • Height dimensions are compatible with automated movements of VeriSeq NIPT Microlab STAR. 	<p>Compatible tubs:</p> <ul style="list-style-type: none"> • Illumina Reagent Tub, part # 20095418
<p>Deep-well plates with the following specifications:</p> <ul style="list-style-type: none"> • SLAS 1–2004, 3–2004, and 4–2004 microplate format with 96 pyramidal or conical bottom wells and a 2 ml minimum well capacity. • Translucent polypropylene, with preference for low DNA binding material for all sample contact surfaces. • Well dimensions generate a liquid level that is compatible with automated aspiration and dispensing steps of VeriSeq NIPT Microlab STAR. • Plate skirt that permits placement of plate barcodes to require position with secure, flat surface adhesion. • Torque-resistant frame able to sustain a minimum of 5600 × g. • Plate height dimensions are compatible with automated movements of VeriSeq NIPT Microlab STAR 	<p>General lab supplier</p> <p>Compatible plates:</p> <ul style="list-style-type: none"> • Eppendorf, part # 0030505301 • Eppendorf, part # 30502302 • USA Scientific, part # 1896-2000

Consumable	Supplier
<p>384-well plate with the following specifications:</p> <ul style="list-style-type: none"> • Microplate with 384 wells, optimized for low-volumes, with a 50 µl minimum well capacity. • Black opaque polystyrene with light-blocking and low DNA binding for all sample contact surfaces. • Well dimensions generate liquid levels that are compatible with automated aspiration and dispensing steps of VeriSeq NIPT Microlab STAR. • Plate height dimensions are compatible with automated movements of VeriSeq NIPT Microlab STAR. • Plate skirt that permits placement of plate barcodes to required position with secure, flat surface adhesion. 	<p>General lab supplier</p> <p>Compatible plates:</p> <ul style="list-style-type: none"> • Corning, product # 3820
<p>96-well plate with the following specifications:</p> <ul style="list-style-type: none"> • Microplate with a torque-resistant frame able to sustain a minimum of 5600 × g and 96 translucent wells with tapered bottoms, raised rims, and a 150 µl minimum well capacity. • Polypropylene that is free from RNase/DNase with low DNA binding for all sample contact surfaces. • Well dimensions generate liquid levels that are compatible with automated aspirating and dispensing steps of VeriSeq NIPT Microlab STAR. • Plate height dimensions are compatible with automated movements of VeriSeq NIPT Microlab STAR. <p>NOTE: Compatible plasticwares with different part numbers, for example, compatible 96 well plates from different manufacturers, may not be directly interchangeable without part-specific calibration of the VeriSeq NIPT Microlab STAR system by Illumina service and support staff. To change between plasticwares, consult your Illumina support team.</p> <ul style="list-style-type: none"> • Plate skirt that permits placement of plate barcodes to required position with secure, flat surface adhesion. • Compatible with thermal cyclers for denaturing. 	<p>General lab supplier</p> <p>Compatible plates:</p> <ul style="list-style-type: none"> • Eppendorf, part # 0030129512 • Eppendorf, part # 30129580 • Eppendorf, part # 30129598 • Eppendorf, part # 30129660 • Eppendorf, part # 30129679 • Bio-Rad, part # HSP9601
<p>One of the following seals:</p> <ul style="list-style-type: none"> • Microseal 'F' Foil • Foil seals 	<p>Bio-Rad, catalog # MSF1001</p> <p>Beckman Coulter, item # 538619</p>
Cell-Free DNA BCT CE	Streck, catalog # 218997
Push Caps	Sarstedt, order # 65.802

Consumable	Supplier
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
Equivalent:	General lab supplier
<ul style="list-style-type: none"> An alcohol-based rapid disinfectant spray A solution of disinfecting detergent 	
Recommended:	
<ul style="list-style-type: none"> Deionized water and 70% ethanol 	

Optional Materials, Not Provided

Consumable	Supplier
Dulbecco's Phosphate-Buffered Saline (DPBS) for no template control (NTC)	General lab supplier
Tube, screw cap, 10 ml (for control samples only)	Sarstedt, order # 60.551
Tube, screw cap, 50 ml	General lab supplier
25 ml serological pipettes	General lab supplier
10 ml serological pipettes	General lab supplier

Specimen Collection, Transport, and Storage



CAUTION

Handle all specimens as if they are potentially infectious agents.

- Whole blood specimens of 7–10 ml must be collected in Streck Cell-Free DNA BCT. Do not freeze.
- Transportation of whole blood must comply with all applicable governing regulations for the transport of etiologic agents. Expedited shipping/transportation methods are recommended.
- During transport, store at temperatures between 4°C and 30°C. After samples are received, store at 2°C to 8°C until ready to proceed. The time between blood collection and initial plasma isolation should not exceed 5 days.
- In the event that retesting is required, post-processing samples may be recapped and stored at 4°C for an additional 5 days (up to a total of 10 days after blood collection).

**CAUTION**

Exposure to elevated temperatures above the aforementioned ranges can negatively impact individual sample failure rates and/or sample performance.

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.
- This assay contains dimethyl sulfoxide, a corrosive and combustible liquid. 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 hydrochloride) 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.
- Immediately report any serious incidents related to this product to Illumina and the Competent Authorities of the member states in which the user and the patient are established.
- For environmental, health, and safety information, refer to the safety data sheets (SDS) at support.illumina.com/sds.html.

Procedural Notes

Avoiding Contamination

- Use fresh tips and fresh consumable labware.
- Use aerosol-resistant tips to reduce the risk of carry-over and sample-to-sample cross-contamination.
- Due to the potential for contamination, take extreme care to make sure that well contents remain fully in the well. Do not splash contents. Centrifuge following any vortexing step.
- Follow applicable governing regulations for proper laboratory practice and hygiene when handling blood and blood derivatives.
- Do not use aerosol bleach sprays when performing library preparation. Trace bleach contamination can lead to assay failure.
- When unsealing any plates, take care to place the plate on a firm, flat surface, gripping the plate firmly. Slowly remove the seal ensuring that the seal does not make contact with exposed wells. Take care to not touch exposed wells or disturb contents. Well to well cross-contamination can produce incorrect results.

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.
- Remove all unloadable carriers and clean with an alcohol-based rapid disinfectant spray, De-ionized water, and 70% ethanol, and leave them to dry. If they are heavily soiled, soak them afterward in a solution of disinfecting detergent, rinse with the alcohol-based disinfectant, and leave to dry.
- Open the front cover and wipe the deck with a cloth saturated with De-ionized water and 70% ethanol. The slide blocks in particular must be checked for cleanliness.
- Remove the Basic Vacuum System (BVS) manifold and clean manifold, gasket, and interior compartments of BVS with cloth. Avoid cleaning gasket with ethanol as this can brittle the material.
- 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 De-ionized water and 70% ethanol 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 De-ionized water and 70% ethanol directly onto the surface of the CORE 96-head waste box and chute and wipe it clean.
 - If buildup is difficult to remove from tip wastes, wipe with a cloth wet with DNase/RNase-free water until the buildup is removed. Dispose of the cloth appropriately. Proceed to sterilize with the alcohol-based disinfectant.

- 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 De-ionized water and 70% ethanol. (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 De-ionized water and 70% ethanol.
- Clean the CORE 96-head:
 - Using the same lint-free cloth soaked in De-ionized water and 70% ethanol, 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 De-ionized water and 70% ethanol, ‘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 De-ionized water and 70% ethanol, and wipe dry.
- Clean the Autoload protecting ribbon with a cloth soaked in De-ionized water and 70% ethanol, and wipe without exerting pressure.
- When the deck and components are completely dry, replace the carriers.

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.

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

Do not exceed two NTC samples per batch of 24 or 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

- Take extreme care to avoid cross-contamination – there should be no visible liquid on underside of the seal.
 - Make sure that the exposed underside of the seal does not make contact with exposed wells.
 - Take care not to touch exposed wells.
- Always seal the 96-well plate before the following steps in the protocol:
 - Centrifuge steps
 - Thermal cycling steps
- To seal the plate, apply the foil seal to the plate and then seal. Make sure that pressure is applied across the entire plate and the seal is tight across each individual well.
- Before unsealing the plate, perform the following:
 - Briefly centrifuge the 96-well plate at 1000 × g for 20 seconds.
 - Place the plate on a flat surface, and then remove the seal slowly.

VeriSeq NIPT Microlab STAR

- 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 v2 software interface for prompts and operator instructions.
- Keep the front cover in place during operation.
- Keep the deck clear of all items during operation.
- If presented with the option button **Exclude** during an error handling event, refrain from choosing this option under any circumstances. If the method cannot precede past the error handling event or have limited error handling options, abort the run.
- During plate vacuum steps, if prompted by the VeriSeq NIPT Workflow Manager v2, manually assist forming the seal between the plate and the vacuum manifold.
- Allow the system to dispose of tips from the adapter automatically. Do not manually remove tips unless prompted by the software.
- 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 and reduce the applied vacuum of the system.
- For 24, 48, and 96 sample batches, load a full rack of the individually counted 8-channel tips prior to starting method.

Process Samples

Procedure

1. Complete the following steps for each aliquot:
 - a. Centrifuge barcoded samples at 1600 × g for 10 minutes at 4°C with the brake off.
 - b. Remove the sample tubes when the centrifuge comes to a complete stop.
Begin plasma isolation within 15 minutes after centrifugation. If more than 15 minutes elapse, centrifuge again.
2. Inspect each tube for sample suitability by verifying the following requirements:
 - Sample volume is as expected.
 - Clear separation between the red blood cell and plasma layers of the samples are visible after 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 (ie, 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 and cause samples to overflow into adjacent wells.

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



CAUTION

During an error-handling event, if presented with the Exclude option, do not select it. If the method cannot proceed past the error-handling event and you have limited error-handling options, abort the run.

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.
3. For 24, 48, and 96 sample batches, load a full rack of the individually counted 8-channel tips prior to starting the method.

**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 select **VeriSeq NIPT Method**.
2. Enter a unique Batch ID and user name, and then select **OK**.
Batch ID can contain ≤ 26 characters. You can use numbers, letters, underscores (_), or dashes (-). For example: 2025-10-16_Batch3.
The Batch ID is not case-sensitive. Case-sensitive Batch IDs are not considered unique.
Batch names must be unique and must not differ only in capitalization. For example, batch names Batch01 and batch01 are not unique. This same rule applies for Sample ID naming.
3. Select **New Batch**.
4. After initiation, select **OK** to begin plasma isolation.
5. Select the batch size, and then select **OK**.
6. Select the number of no template controls (NTCs), and then select **OK**.
NTC slots are always the last slots selected. For example, with two NTCs in a 24-sample run, positions 23 and 24 are NTCs.
7. Perform one of the following steps:
 - To load an existing sample sheet, select the sample sheet associated with the batch, and then select **OK**.
 - To proceed without selecting a sample sheet, select **No Sample Sheet**.For information on creating a sample sheet, refer to *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)*.

NOTE Sample type, singleton, or twins, must be accurately recorded for each sample to ensure proper data analysis. If you choose **No Sample Sheet**, make sure you set default sample values in the Workflow Manager Service Tools. Refer to *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)* for more information.

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

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Tip	7–12	1000 µl tips	5
			1000 µl tips (96 batch only)	4, 5
	Tube	15	Prepared blood sample tubes 1–24 (for all batch sizes)	1–24
	Tube	16	Prepared blood sample tubes 25–48 (48 and 96 batch size only)	25–48
	Tube	17	Prepared blood sample tubes 49–72 (96 batch size only)	49–72
	Tube	18	Prepared blood sample tubes 73–96 (96 batch size only)	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] Dulbecco's Phosphate-Buffered Saline (DPBS) - used for no template control (NTC)	5

10. Make sure that the carriers, labware, and reagents are loaded correctly.
11. On the Pre-Spin Deck Verification screen, select **OK**.
12. Observe the ML STAR perform the automated steps.
13. When prompted by the Workflow Manager, make sure the ML STAR loading deck is free of any obstructions to allow the ML STAR to unload the carriers.
14. Select **Unload** to unload the deck.
15. Remove the Intermediate Plasma deep-well plate as follows.
 - a. Inspect the plate for consistent volumes in each well (no pipette errors). The expected volume is 1000 µl.
 - b. Record any inconsistencies when the Plasma Isolation procedure is complete.
 - c. Seal the plate, load with balance, and centrifuge at 5600 × g for 10 minutes with the brake off or on the lowest setting.
16. Select **Yes** to proceed to final Plasma Preparation.

17. Remove the plate seal and reload the plate onto the carrier.

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

18. Select the **Intermediate Plasma plate has been spun** checkbox, and then select **OK**.

19. Observe the ML STAR perform the automated steps.

20. When prompted 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.

21. Select **Unload** to unload the deck.

22. When prompted by the Workflow Manager, empty the carriers and deck.

23. Remove the Final Plasma deep-well plate.

24. Inspect the plate for the following errors:

- Inconsistent volumes in each well. Expected volume is 900 µl.
- Visible cell pellets.
- Excessive hemolysis.

If you observe abnormal visible cell pellets 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, refer to *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)*.

25. When prompted by the Workflow Manager, select **OK**.

26. Enter comments about affected wells, and then select **OK**.

27. Perform one of the following steps.

- To proceed to cfDNA Extraction, select **Yes**.
- To stop, select **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 reservoir tubs and deep-well reservoirs with the name of the reagents.

Reagent	Storage	Instructions
Final Plasma deep-well plate	2°C to 8°C	If previously stored, let stand for 30 minutes to bring to room temperature. Centrifuge at 1000 × g for 20 seconds. Unseal the Final Plasma deep-well plate before use.

3. Slowly add 3.75 ml Proteinase Buffer to each reagent vial of Proteinase K.

- Prepare 3 vials for 24 and 48 samples.
- Prepare 4 vials for 96 samples.

4. Cap the Proteinase K vials and vortex until resuspended.



CAUTION

Do not contaminate the rubber stopper. Getting other substances on the rubber stopper can contaminate future samples.

5. Pool the prepared Proteinase K from all vials into a reagent tub and label it as Proteinase K.

6. Add 100 ml 100% EtOH to each reagent bottle of Wash Buffer II.

- Prepare 1 bottle for 24 and 48 samples.
- Prepare 2 bottles for 96 samples.

7. Invert the Wash Buffer II bottles to mix.

8. Mark the checkboxes on the Wash Buffer II bottles.

9. Label 1 new full-skirt plate Intermediate and apply a plate barcode.

10. Label 1 new full-skirt plate cfDNA Elution and apply a plate barcode.

11. Label 1 new deep-well plate Extraction Intermediate and apply a deep-well plate barcode.

12. Apply a plate barcode to the DNA Binding plate.

13. Apply a foil seal to unused wells for 24 and 48 sample batches.

14. Prepare a 70% EtOH cleaning solution (70% EtOH, 30% DNase/RNase-free water) for cleaning the vacuum system.

15. Prepare the vacuum system as follows.

- Remove the vacuum manifold and clean with 70% EtOH.
Avoid cleaning the gasket with EtOH as this can brittle the material.
- Empty the vacuum waste.
- Make sure that the ML STAR vacuum system is on.

Procedure

1. Select **OK** to start cfDNA Extraction.

2. If the **VeriSeq NIPT Method** is not open:

- a. Open the AppLauncher, and then select **VeriSeq NIPT Method**.
 - b. Enter the Batch ID and user name, and then select **OK**.
3. Load tips onto the tip carriers as follows, and then select **OK**.

**CAUTION**

Before starting method for 24, 48, and 96 sample batches, add full rack of 8-channel tips.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24	Tip	1–6	1000 µl tips	1
		7–12	300 µl tips	1
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

4. Load counted tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Tip	49–54	1000 µl tips	1
			300 µl tips	2
			50 µl tips	3

5. Enter the location of the first and last tips for each tip rack, and then select **OK**.

6. Scan the Extraction Box barcodes.
7. Enter the user name or reagent preparer initials, and then select **OK**.
8. Scan the Accessory Box barcodes.
9. Enter the user name or reagent preparer initials, and then select **OK**.
10. Confirm that barcodes are affixed.
11. Unseal the Final Plasma deep-well plate if necessary.
12. Load plates (barcode-facing right) onto the plate carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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

13. Confirm that the DNA Binding plate is barcoded, then select **OK**.
14. For partial plate batches, apply a trimmed plate seal over the unused wells (columns 4–12 for 24 sample batches and columns 7–12 for 48 sample batches).
15. Load the DNA Binding plate onto the vacuum manifold with the barcode-facing right.
16. Before placing the binding plate on the BVS manifold, visually inspect the wells for any possible obstructions.
This can impede the flow of reagents while under vacuum.
17. If using 24 or 48 sample batches, cover unused wells and seal with foil seal. Select the **Are DNA Binding Plate Columns Sealed?** checkbox, and then select **OK**.
18. Load reagent tubs onto the reagent carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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

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

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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

21. Wait for the automated reagent volume check to complete.
22. Confirm that the vacuum waste is empty (half full recommended), and then select **OK**.
23. Confirm the placement of all carriers, labware, and reagents, and then select **OK** in the Extraction Deck Verification screen.
24. 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.

25. After the final vacuum step, remove the DNA Binding plate and clean the bottom surface with 70% EtOH.
26. Seal any uncovered wells on the DNA Binding plate, and then place the DNA Binding plate on the empty Final Plasma deep-well plate.
27. Centrifuge the DNA Binding plate/Final Plasma plate assembly at $5600 \times g$ for 10 minutes with the brake on.
28. Select **OK**.
29. During DNA Binding plate centrifugation, complete the vacuum cleaning:
- Remove vacuum manifold, and then select **OK**.
 - 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.

- d. Select the **Manifold is on Vacuum** checkbox to initiate the elution plate transfer on vacuum manifold, and then select **OK**.
30. After centrifugation, unseal the wells containing samples on the DNA Binding plate.
31. Place the DNA Binding plate on top of the cfDNA Elution plate that is on the vacuum manifold.
32. Load the DNA Binding plate with the barcode to the right, and then select **OK**.
33. Observe the ML STAR during the automated steps.
34. After the incubation step, select the **Plates are assembled as indicated** checkbox. Confirm that the DNA Binding/cfDNA Elution plate assembly is on a support base (if required by centrifuge).
35. Seal the uncovered wells on the DNA Binding plate.
36. Centrifuge at 5600 × g for 2 minutes with the brake on, and then select **OK**.
37. Visually inspect the cfDNA Elution plate for consistent volumes in each well.
The expected volume is approximately 55 µl.
38. Seal and retain the cfDNA Elution plate for library preparation.
39. When prompted 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.
40. Select **Unload** to unload the deck.
41. Unload all carriers and clean the ML STAR deck, and then select **OK**.
42. Enter comments about affected wells, and then select **OK**.
43. Perform one of the following steps:
 - To continue to Prepare Libraries, select **Yes**.
 - To stop, select **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.

Reagent	Storage	Instructions
A-Tailing Mix	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
cfDNA Elution Plate	-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.
End Repair Mix	-25°C to -15°C	Thaw at room temperature. Vortex to mix.
Hybridization Buffer	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Return to storage after use.
Ligation Mix	-25°C to -15°C	Thaw at room temperature. Vortex to mix, and then centrifuge briefly.
NIPT DNA Adapter Plate	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Centrifuge at 1000 × g for 20 seconds.
Resuspension Buffer	2°C to 8°C	Vortex to mix. Return to storage after use.
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.



CAUTION

When unsealing the NIPT DNA adapter plate take extreme care to avoid well-to-well aerosol cross-contamination, which can produce incorrect results.

3. If the cfDNA Elution plate was stored frozen, prepare it as follows.
 - a. Thaw at room temperature.
 - b. Vortex at 1500 rpm for 1 minute.
 - c. Centrifuge at 1000 × g for 20 seconds.
4. Label one new full-skirt plate Libraries and apply a plate barcode.
5. Prepare 80% EtOH from absolute EtOH. Combine 40 ml 100% EtOH and 10 ml DNase/RNase-free water. Invert to mix.

6. 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 (µl)	Resuspension Buffer (µl)
24, 48	900	1200
96	1800	2400

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

Sample Batch Size	Ligation Mix (µl)	Resuspension Buffer (µl)
24, 48	230	1713
96	440	3278

Procedure

1. Select **OK** to start Library Preparation. If the **VeriSeq NIPT Method** is not already open:
 - a. Open the AppLauncher and select **VeriSeq NIPT Method**.
 - b. Enter the Batch ID and user name, and then select **OK**.
2. Confirm that the following consumables are prepared as indicated in the Reagent Preparation screen:
 - A-Tailing Mix, Ligation Mix, and 80% EtOH
 - Sample Purification Beads, End Repair Mix, and NIPT DNA Adapter Plate
3. Select the checkboxes, and then select **OK**.
4. Scan the Library Prep Box barcodes.
5. Enter the user name or reagent preparer initials, and then select **OK**.
6. Scan the Accessory Box barcodes.
7. Enter the user name or reagent preparer initials, and then select **OK**.

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

Sample Batch Size	Carrier Type	Track	Item	Site Position
24	Tip	1–6	50 µl tips	1
		7–12	300 µl tips	1, 2
48	Tip	1–6	50 µl tips	1, 2
		7–12	300 µl tips	1, 2, 3, 4
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
24, 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 select **OK**.

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

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Multiflex	19–24	cfDNA Elution plate, barcoded	1
			NIPT 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 select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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 select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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. Save the remainder of the 12 ml Hybridization Buffer (HT1) in the container for pooling.
15. Make sure that the carriers, labware, and reagents are loaded as indicated, and then select **OK** in the Library Deck Verification screen.
16. Wait for the automated reagent volume check to complete.
17. Observe the ML STAR during the automated steps.
18. When prompted 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.
19. Select **Unload** to unload the deck.
20. Inspect the Libraries plate for consistent volumes in each well.



CAUTION

If well volumes are inconsistent, samples may fail automated quality control.

21. If storing, seal and retain the Libraries plate.
22. Unload the carriers, clean the deck, and then select **OK**.
23. Enter comments about affected wells, and then select **OK**.
24. Perform one of the following steps:
 - To continue to Quantify Libraries, select **Yes**.
 - To stop, select **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 from date of preparation at -25°C to -15°C.

Quantify Libraries

Preparation

1. Prepare the following reagents:

Reagent	Storage	Instructions
DNA Quantification Reagent	2°C to 8°C	Protect from light. Thaw at room temperature for 30–150 minutes. (Removing reagent at start of the Prepare Libraries procedure is recommended.) Vortex to mix, and then centrifuge briefly.
DNA Quantification Standard	2°C to 8°C	Vortex to mix, and then centrifuge briefly.
Resuspension Buffer	2°C to 8°C	Vortex to mix.

2. If the Libraries plate was stored frozen, prepare it as follows.
 - a. Confirm that the plate was not stored for more than 7 days and thaw at room temperature.
 - b. Vortex to mix
 - c. Centrifuge at 1000 × g for 1 minute.
3. Turn on the fluorometer 10 minutes before use.
4. Apply a plate barcode to a new 384-well plate.
5. Apply a plate barcode to a new full-skirt plate.

Procedure

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

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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

6. Confirm that barcodes are affixed.
7. If needed, unseal the Libraries plate.
8. Load plates (barcode-facing right) onto the Multiflex carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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

9. Load reagent tubes without caps into the tube carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Tube	46	DNA Quantification Standard	1
			DNA Quantification Reagent	2

10. Load reagent tubs onto the reagent carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Reagent	47	New reagent tub (empty)	1
			16 ml Resuspension Buffer	2

11. 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
24, 48, 96	Tip	49–54	1000 µl tips	1
			300 µl tips	2
			50 µl tips	3

12. Enter the location of the first and last tips for each tip rack, and then select **OK**.
13. Make sure that the carriers, labware, and reagents are loaded as indicated, and then select **OK** in the Quant Deck Verification screen.
14. Wait for the automated reagent volume check to complete.
15. Observe the ML STAR during the automated steps.
16. When prompted 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.
17. Select **Unload** to unload the deck.

18. Unload the Libraries plate.
 - a. Inspect the plate for consistent volumes in each well.
 - b. Seal the Libraries plate and store at room temperature until the fluorometric data analysis is complete.
19. Unload the remaining 96-well plates and check for consistent volumes in each well.

Gross errors in volume can indicate an issue with pipetting steps.
20. Unload the 384-well plate and check for liquid in the appropriate wells.
21. Seal the plate with a foil seal.
22. Centrifuge at 1000 × g for 20 seconds.
23. Incubate at room temperature for 10 minutes, protected from light.
24. Unload all carriers.
25. Clean the ML STAR deck, and then select **OK**.

**CAUTION**

Do not discard quantification reagents until data is obtained. You require the reagents if you need to perform requantification.

26. After incubation, remove the foil seal and load the 384-well plate onto the microplate reader. Make sure to use the purple adapter plate (Part Number: 0310-4336) provided by Molecular Devices or equivalent if applicable by instrument used.
 - Make sure that A1 is in the top-left corner when loading.
27. Double select the VeriSeq NIPT template to open it in SoftMax Pro.
28. Select **New Experiment** in the Home tab.
29. Select **Read**.
30. Export the data as XML as follows.
 - a. Right select **Plate**, and then select **Rename**.
 - b. Scan the barcode of the Quantification plate, and then select **OK**.
 - c. In the upper-left corner of the screen, select the plate icon, and then select **Export** from the menu.
 - d. Select the **Expt name** checkbox, set the plate date option to raw, set the output format to XML, and then select **OK**.
 - e. Set the output file path and name, and then select **Save**.

The Hamilton computer must be able to access the file location. Do not use spaces in the file name or file path.

Analysis

1. On the ML STAR, in the Scanner Information screen, enter the fluorometer ID.
2. Enter comments about the fluorometer run, and then select **OK**.
3. Navigate to the *.xml quantification file that contains the fluorometric data, and then select **OK**.

4. Review the standards curve and sample concentration analysis results, and then select **OK**.
5. If you must rescan the plate, select **Rescan**.
Samples are time and light sensitive. When necessary, perform the rescan immediately.
6. Enter comments about affected wells, and then select **OK**.
7. Assess the results and proceed as follows.
 - If the results pass specification, proceed to [Pool Libraries on page 36](#). For specifications, refer to the quantitation QC metrics and boundaries table in the *VeriSeq NIPT Solution v2 Software Guide* (document # 1000000067940).
 - If the results fail specification, the system aborts the method. Repeat the quantification procedures beginning with [Preparation on page 32](#).
8. Perform one of the following steps:
 - To proceed to [Pool Libraries on page 36](#), select **Yes**.
 - To stop, select **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:

Reagent	Storage	Instructions
Hybridization Buffer	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Return to storage after use.

2. If the Libraries plate was stored frozen, prepare it as follows.
 - a. Confirm that the plate was not stored for more than 7 days and thaw at room temperature.
 - b. Vortex at 1500 rpm for 1 minute.
 - c. Centrifuge at 1000 × g for 20 seconds.
 - d. Pipette to mix.
3. Label an empty pooling tube Pool A. For 96 samples, label a second empty pooling tube Pool B.

4. Save the following denature program on the thermal cycler with a heated lid.
 - a. Choose the preheated lid option and set to 102°C.
 - b. Set the reaction volume to 50 µl.
 - c. Set the ramp rate to maximum ($\geq 2^\circ\text{C}$ per second).
 - d. Incubate at 96°C for 10 minutes, and then 4°C for 5 seconds.
 - e. Hold at 4°C.

Procedure

1. Place the Libraries plate on the preprogrammed thermal cycler and run the denature program. Do not denature Libraries plate before the quantification has passed QC metrics, as you may want to perform requantification.
2. Centrifuge the Libraries plate at 1000 × g for 20 seconds.
3. Select **OK** to start pool libraries.
4. If the VeriSeq NIPT Method is not open:
 - a. Open the AppLauncher, and select **VeriSeq NIPT Method**.
 - b. Enter the Batch ID and user name, and then select **OK**.
5. Select the pool concentration, and then select **OK**.
The target cluster density is 220–260 K/mm².

NOTE Pooling concentrations and/or pooling volumes may need to be increased for 24 sample batches to maintain similar cluster densities obtained with 48/96 sample batches.

6. If prompted by the Workflow Manager, perform one of the following steps:
 - To load a sample sheet, select the sample sheet associated with the batch, and then select **Load**.
 - To use system default values for remaining sample types, sex reporting, or screen type, select **Use Default** for each setting.
For information about creating a sample sheet, refer to the *VeriSeq NIPT Solution v2 Software Guide* (document # 1000000067940).
7. Select **Start** to begin timer for denaturing plate.
8. Load tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Tip	7–12	50 µl filter tips	1

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

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

10. Load pooling tubes onto the tube carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 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

11. Load reagent tubs onto the reagent carrier as follows, and then select **OK**.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Reagent	47	3 ml Hybridization Buffer	1

12. Load tips onto the tip carriers as follows.

Sample Batch Size	Carrier Type	Track	Item	Site Position
24, 48, 96	Tip	49–54	1000 µl filter tips	1
			300 µl filter tips	2
			50 µl filter tips	3

13. Enter the location of the first and last tips for each tip rack, and then select **OK**.
14. Make sure that the carriers, labware, and reagents are loaded as indicated.
15. In the Pooling Deck Verification screen, select **OK**.
16. Observe the ML STAR during the automated steps.
17. Enter comments about the affected wells, and then select **OK**.
18. When prompted 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.
19. Select **Unload** to unload the deck.
20. Unload the tube carrier.
21. Cap each pooling tube, vortex, and then centrifuge briefly.
22. Select **OK**.
23. Sequence libraries as soon as possible after pooling. Seal the Libraries plate and store at -25°C to -15°C for up to 7 days to allow repooling.

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 Pooled Libraries for Sequencing

Preparation

1. Prepare the following reagents:

Reagent	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 by completing the following fields in the Local Run Manager VeriSeq NIPT Module module:
 - a. Run Name
 - b. **[Optional]** Run Description
 - c. Pool Barcode

**CAUTION**

The Pool Barcode entered in the Local Run Manager module must match the Pool Barcode entered in the Workflow Manager. Incorrect run configurations are rejected by the analysis software and require resequencing.

For more information on using the Local Run Manager VeriSeq NIPT Module, refer to the *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)*.

Procedure

1. Combine the following volumes to the reagent cartridge, and then pipette to mix.
 - Hybridization Buffer (900 µl)
 - 450 µl Pool A (450 µl)
2. Proceed with sequencing using the reference guide for your next-generation sequencing instrument. For a NextSeq 550Dx, refer to the *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)* (or refer to the applicable package insert as listed on the Illumina Support page www.support.illumina.com).
3. Confirm correct run configuration when prompted.
4. If necessary, repeat this procedure for Pool B.
 - To achieve target cluster density range, the library plate can be repooled using a different pooling concentration on the Hamilton. Repooling invalidates the original pool.
 - Alternatively, the ratio of pool to HT1 (450 µl + 900 µl) can be modified to achieve target cluster density range.

Next Generation Sequencing

The VeriSeq NIPT Solution v2 can be used with a next-generation sequencing system with the following specifications:

- Capable of 2x36 paired-end reads
- Compatible with index adapters in the VeriSeq NIPT Sample Prep Kit
- Two channel chemistry
- Automatic production of BCL (*.bcl) files (raw data from sequencing instrument)
- 400 million paired-end reads per run
- Compatible with VeriSeq NIPT Assay Software v2

The NextSeq 550Dx is compatible with the VeriSeq NIPT Solution v2

Sequence Data Analysis

After sequencing is complete, sequencing data is automatically sent to the VeriSeq NIPT Assay Software v2 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, refer to the *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)*.

Interpretation of Results

The VeriSeq NIPT Solution v2 algorithm employs a sophisticated statistical model that combines several different types of information from the collection of paired-end sequenced library fragments. This model is used to detect regions of the genome that are under or overrepresented in the library of each sample. Importantly, this model accounts for whether the degree of under or overrepresentation is quantitatively consistent with an aneuploid event in the fetal genome at the level of fetal fraction estimated for the library.

For all chromosomes, paired-end sequencing data are aligned with the reference genome (HG19). Unique nonduplicated aligned reads are aggregated into 100 kb bins. The corresponding bin counts are adjusted for GC bias and according to previously established region-specific genomic coverage. Using such normalized bin counts, statistical scores are derived for each autosome by comparing the coverage regions that can be affected by aneuploidy with the rest of the autosomes. A log likelihood ratio (LLR) is computed for each sample by taking into account these coverage-based scores and the estimated fetal fraction. The LLR is the probability of a sample being affected given the observed coverage and fetal fraction versus the probability of a sample being unaffected given the same observed coverage. The calculation of this ratio also takes into account the estimated uncertainty in fetal fraction. For subsequent calculations, the natural logarithm of the ratio is used. The Assay Software assesses the LLR for each target chromosome and each sample to provide a determination of aneuploidy.

During batch creation, you must define the type of a sample (singleton or twin), the screening type (basic or genomewide), and the sex chromosome reporting (Yes, No, and SCA) desired for each sample. Together, these options determine the information reported for each sample.

For all sample types, the screening type determines which autosomal anomalies are reported. For the basic screening type, only whole chromosome trisomy events involving chromosomes 13, 18 and 21 are reported. For the genomewide screening type, whole or partial chromosome deletion or duplication of any autosomal chromosome are reported. The length of the smallest reportable partial chromosome deletion or duplication is 7 Mb.

For singleton samples, you can disable sex chromosome reporting. You can also configure to report sex chromosome aneuploidies either with or without reporting the sex of euploid samples.

For twin samples, if Yes is selected for sex chromosome reporting, the result is limited to reporting the presence or absence of a Y chromosome in the library. Sex chromosome aneuploidy cannot be reported for twin samples.

NOTE When all samples in a batch have the same reported sex, an email/WebUI error notification will alert the user with a sample admixture/contamination warning. The batch will be invalidated, and no report will be produced. (Applicable for VeriSeq NIPT Solution v2 server software v2.2 and above.)

A result of ANOMALY DETECTED indicates the sample screens positive for one or more anomalies consistent with the selected screen type and sex chromosome reporting option. When an anomaly is detected, the report provides a description of the anomaly in cytogenetic notation.

The VeriSeq NIPT Assay Software v2 uses statistics generated during sequencing to provide a fetal fraction estimation (FFE) for each sample. The FFE is the estimated fetal cfDNA component that 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 v2 uses 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. Negative calls are reported only if the 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.

In addition to iFACT, the VeriSeq NIPT Assay Software v2 assesses several other QC metrics during analysis. The additional metrics include assessments of coverage uniformity on reference genomic regions and the distribution of cfDNA fragment lengths. 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, the sample can be reprocessed provided sufficient plasma volume is in the blood collection tube.

The VeriSeq NIPT Solution v2 generates data for use in a final report. It does not generate a final report for the patient. Customers are responsible for the design and contents of the final report to be delivered to the point of care physician. Illumina is not responsible for the accuracy of wording in the final report for customers.



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 a NextSeq 500/550 sequencing system or a NextSeq 550Dx sequencing system with the following configurations:

	NextSeq 500/550	NextSeq 550Dx
On-instrument Software	NextSeq Control Software 4.0	NextSeq Operating Software 1.3
Reagent Kit Version	NextSeq 500/550 High Output v2.5 (75 cycle) Reagent Kit	NextSeq 550Dx High Output v2.5 (75 cycle) Reagent Kit
Sequencing Method	2x36 paired-end sequencing run in high output mode	2x36 paired-end sequencing run in high output mode

Clinical Study

The clinical accuracy of the VeriSeq NIPT Solution v2 was demonstrated by evaluating plasma samples from pregnant women with singleton and twin pregnancies. Samples were obtained from deidentified banked plasma samples that were previously processed from peripheral whole blood specimens. Over 45,000 samples were considered for study inclusion. These samples underwent previous prenatal screening for fetal chromosome aneuploidies and partial deletions and duplications of 7 Mb or greater. All samples from affected pregnancies and a subset of consecutive samples from unaffected pregnancies were eligible for testing if clinical outcomes were available and sample criteria were met. A total of 2,335 samples were in the testing analysis set. From this set, 2,328 samples were from singleton pregnancies and seven samples were from twin pregnancies.

Of these samples, 28 (1.2%, 28/2335) samples failed assay QC on first pass during the analysis of the completed sequencing data:

- 27 iFACT failures (one XO, 26 unaffected)
- One failure for data outside of expected range

Demographics and Pregnancy Characteristics

Maternal age, gestational age, and trimester of pregnancy are summarized in [Table 7](#) for the samples in the genomewide screen, including known mosaic samples. The majority (98%) of testing samples represent first trimester pregnancy.

The demographics were assessed between the basic and genomewide cohorts and showed no statistical difference. Demographics and pregnancy characteristics were similar whether known mosaics were included or excluded.

Table 7 Demographics and Pregnancy Characteristics

Summary Statistic	Genomewide (including known mosaics)
Number of samples	2307*
Maternal age – years	
Mean	35.08
Standard Deviation	4.04
Median	34.95
25th percentile, 75th percentile	32.31, 37.79
Minimum, maximum	20.22, 53.02
Gestational age at blood draw - weeks	
Mean	10.93
Standard Deviation	1.20
Median	10.57
25th percentile, 75th percentile	10.29, 11.14
Minimum, maximum	10.00, 27.86
Trimester of pregnancy – n (%)	
< First (<14 weeks)	2,252 (98%)
Second	54 (2%)
Third (≥ 27 weeks)	1 (0%)

* Final samples presented contained 7 twins.

Clinical Performance

Results as called by the VeriSeq NIPT Solution v2 were compared to the clinical reference standard outcomes. All study samples had clinical reference standard outcomes (clinical truth) related to fetal chromosomal aneuploidy status and partial deletions and duplications of 7 Mb or greater. The clinical reference standard outcome for samples included in this study depended on results of chromosome analysis or a newborn physical exam with an NGS-based NIPT negative screen. Trained study staff performed classification of clinical reference standard data in accordance with the Medical Coding document from the sponsor.

Chromosome analysis methods included karyotyping, fluorescence in situ hybridization (FISH), or comparative genomic hybridization chromosome microarray (CMA). Chromosome analysis was performed on neonatal or infant peripheral blood or saliva, products of conception (POC) samples, amniocytes, chorionic villi, placental tissues, or postnatal umbilical cord blood.

Mosaicism is defined as the presence of two or more cell lines of different chromosomal composition in an individual. The cell lines originate from the same zygote. The type and level of mosaicism varies and is dependent on the timing of mosaic events during embryogenesis and fetal development. Different types of mosaicism appear in prenatal diagnoses depending on the distribution of abnormal versus normal cell lines over cytotrophoblast, mesenchyme, or the fetus.¹⁰ Although mosaicism can be seen with any chromosomal anomaly, the prevalence of mosaicism in rare trisomies is higher than in the trisomies of chromosomes 21, 18, and 13 (T21, T18, and T13).¹¹ In the performance evaluation, mosaic cases were included in genomewide analysis, as the purpose of this screen type for this assay is to detect rare autosomal aneuploidies (RAAs).

Basic Screen Performance

For the basic screen, anomalies include T21, T18, and T13. A total of 2,243 singleton and twin samples were included in the analysis. All seven twin pregnancies were correctly detected as T21 and are not reported in the following table.

Table 8 Sensitivity and Specificity of the VeriSeq NIPT Solution v2 for Detecting Trisomies 21, 18, & 13 in a Basic Screen for Singleton Pregnancies (Excluding Known Mosaics)

	T21	T18	T13
Sensitivity	> 99.9% (130/130)	> 99.9% (41/41)	> 99.9% (26/26)
2-sided 95% CI	97.1%, 100%	91.4%, 100%	87.1%, 100%
Specificity	99.90% (1982/1984)	99.90% (1995/1997)	99.90% (2000/2002)
2-sided 95% CI	99.63%, 99.97%	99.64%, 99.97%	99.64%, 99.97%

The assay performance in the basic screen as shown in [Table 8](#) is calculated excluding a subset of 64 samples affected by RAAs, autosomal partial deletions or duplications, or known mosaicism. These 64 samples included eight T21 and three T18 mosaics. Five of these 11 samples were identified as affected with the anomaly detected by the VeriSeq NIPT Assay Software v2.

Genomewide Screen Performance

For the genomewide screen, any anomaly includes trisomies, monosomies, and partial deletions or duplications of 7 Mb or greater. Samples for the genomewide screen contained 36 samples with known mosaicism. A total of 2,307 singleton and twin samples were tested. All seven twin pregnancies were correctly detected as having a chromosome 21 anomaly and are not reported in the following tables.

Genomewide Screen Performance for Any Anomaly

Table 9 Sensitivity and Specificity of the VeriSeq NIPT Solution v2 for Detecting Any Anomaly in the Genomewide Screen (Including Known Mosaics)

	Sensitivity	Specificity
Estimate % (n/N)	95.5% (318/333)	99.34% (1954/1967)
2-sided 95% CI	92.7%, 97.3%	98.87%, 99.61%

Genomewide Screen Performance for Rare Autosomal Aneuploidy

Table 10 Sensitivity and Specificity of the VeriSeq NIPT Solution v2 for Rare Autosomal Aneuploidy (RAA) in the Genomewide Screen (Including Known Mosaics)

	Sensitivity	Specificity
Estimate % (n/N)	96.4% (27/28)	99.80% (2001/2005)
2-sided 95% CI	82.3%, 99.4%	99.49%, 99.92%

Genomewide Screen Performance for Partial Deletions and Duplications

Table 11 Sensitivity and Specificity of the VeriSeq NIPT Solution v2 for Partial Deletions and Duplications of 7 Mb or More in the Genomewide Screen (Including Known Mosaics)

	Sensitivity	Specificity
Estimate % (n/N)	74.1% (20/27)	99.80% (2000/2004)
2-sided 95% CI	55.3%, 86.8%	99.49%, 99.92%

Differences in Performance Between the Basic and Genomewide Screens

The scoring methodology for common trisomies and sex chromosome aneuploidies is the same for both the basic and genomewide screens. The basic screen only applies the algorithm to T21, T18, and T13. However, the genomewide screen expands upon this methodology to assess for all trisomies and RAAs and partial duplications and deletions.

There are two differences between the performance reporting described between basic and genomewide screens. First, for the genomewide screen, samples with known mosaicism both for common trisomies and for RAAs and partial deletions and duplications were included for performance metrics. Second, the genomewide screen can preferentially report the detection of a partial duplication or deletion over a full trisomy. The presence of a full trisomy in addition to a partial duplication or deletion can be seen by referencing the LLR score provided in the supplementary report.

Inclusion of Mosaics in Genomewide Screen

Mosaicism is listed as a limitation of this assay. When mosaicism is present, the fetal signal of an anomaly is reduced and therefore may be more challenging to detect without compromising the overall specificity of the assay. However, because mosaicism is more relevant for expanded content, samples with mosaicism were included in the genomewide screen.

Of the 64 samples included in the genomewide screen but not the basic screen, 36 samples were identified as having mosaicism by the clinical reference standard. Of these 36 samples, 23 calls matched the clinical reference standard.

Partial Deletion or Duplication versus Whole Chromosome Aneuploidy Detection

The VeriSeq NIPT Solution v2 features menu options for both a basic screen and a genomewide screen. In the basic screen, an ANOMALY DETECTED result is only reported when a full aneuploidy is detected on chromosomes 21, 18, or 13 and if all quality control metrics are met. In the genomewide screen, the system detects aneuploidy across all autosomes and partial deletion and duplication events of at least 7 Mb.

While using the genomewide screen, in cases in which both a whole chromosome event as well as a CNV event within the same chromosome exceeds the LLR threshold the system gives reporting precedence to a partial deletion or duplication event over the whole chromosome call if the size of the partial deletion or duplication covers approximately 75% or less of the chromosome on which the event is detected. If the partial deletion and duplication region detected is larger than 75% of the size of the chromosome, the event is reported as a full trisomy or monosomy of the entire chromosome if simultaneously the LLR threshold for the whole chromosome is also exceeded. Due to this, substantially large deletions and duplications that are less than or equal to 75% of the size of the chromosome can be indicative of a whole chromosome aneuploidy.

In all samples, the LLR score for the whole chromosome classification is available in the supplementary report. The LLR score should be reviewed with respect to the specified cutoff in [95% Detection Probabilities for Average Regions by Size for the VeriSeq NIPT Solution v2 on page 57](#) prior to interpreting the result. For example, a CNV call where the Chromosome-level LLR scores exceeding the cutoff provide further support for an interpretation consistent with an aneuploidy of the whole chromosome, refer to [Table 12](#) as an example.

In the clinical study, there were two singleton pregnancy samples with substantially large duplications (one on chromosome 21 and one on chromosome 18) that were less than 75% of the relative size of the chromosome (refer to [Table 12](#)). Both events were reported as a partial duplications rather than a full trisomy for that chromosome. The LLR scores for these events were above the cutoff consistent with an affected outcome for a full trisomy. For either a partial duplication or a full trisomy call, the follow-up management for a positive NIPT call is offering the patient confirmation testing via prenatal diagnosis.

Table 12 Examples of Large Duplication Events Identified in the Genomewide Screen

	Clinical Truth	Genomewide System Output	Size of Anomaly (Mb)	% of Chromosome	LLR Scores
Sample 1	Trisomy 21 singleton	Partial duplication on 21	22.50	48.9	19.43
Sample 2	Trisomy 18 singleton	Partial duplication on 18	47.00	60.2	12.99

Refer to the *VeriSeq NIPT Solution v2 Software Guide (document # 1000000067940)* for additional information on Quality Control metrics used to report aneuploidy results.

Sex Chromosomes

The VeriSeq NIPT Solution v2 sex chromosome results were compared to the clinical reference standard outcome and are summarized in the following table. The percent concordance was calculated for each sex chromosome within each clinical reference standard outcome. Percent concordance was calculated as the number of samples in which the VeriSeq NIPT Solution v2 sex chromosome call matched the clinical reference standard classification, divided by the total number of samples with the same clinical reference standard classification.

Table 13 Percent Concordance for Fetal Sex Classification*

Fetal Sex Classification		Phenotype from the Newborn Physical Exam		Cytogenetic Results								
		Female	Male	XX	XY	XO	XXX	XXY	XYY	Other**	Missing	
Detected	Karyotype											
Anomaly Not Detected	XX	997	0	21	0	2	0	0	0	0	0	0
Anomaly Not Detected	XY	0	966	0	15	0	0	0	0	0	0	1
Anomaly Detected	XO	0	0	0	0	19	0	0	1	0	0	0
Anomaly Detected	XXX	0	0	0	0	0	17	0	0	1	0	0
Anomaly Detected	XXY	0	0	0	0	0	0	23	0	1	0	0
Anomaly Detected	XYY	0	0	0	0	0	0	0	11	0	0	0
Total		997	966	21	15	21	17	23	12	2	1	
Percent Concordant		100	100	100	100	90.5	100	100	91.7	Not Applicable	Not Applicable	

* Five twin pregnancies were correctly classified as presence of Y. Two pregnancies were correctly classified as no presence of Y.

** Other cytogenetic results were XXXXX and XXYY.

Positive Predictive Value and Negative Predictive Value of the VeriSeq NIPT Solution v2

Positive predictive value (PPV) and negative predictive value (NPV) of the test provide information regarding the ability of the test to inform clinical decisions based on test sensitivity, specificity, and pretest probability that a fetus is trisomy affected (prevalence). Because PPV and NPV depend on prevalence and the prevalence for these aneuploidies can vary across different subject populations, PPV and NPV were calculated for a range of plausible prevalence values based on the sensitivity and specificity values observed in the basic screen (without known mosaics) of the clinical accuracy study. [Table 17](#) is based on the genomewide screen (with known mosaics).

Table 14 Trisomy 21 Prevalence, PPV, and NPV in Basic Screen (Excluding Known Mosaics)

Prevalence (%)	PPV (%)	NPV (%)
0.05	33.17	> 99.99
0.10	49.82	> 99.99
0.20	66.53	> 99.99
0.50	83.29	> 99.99
1.00	90.93	> 99.99
1.50	93.79	> 99.99
2.00	95.29	> 99.99

Table 15 Trisomy 18 Prevalence, PPV, and NPV in Basic Screen (Excluding Known Mosaics)

Prevalence (%)	PPV (%)	NPV (%)
0.03	23.06	> 99.99
0.05	33.31	> 99.99
0.10	49.99	> 99.99
0.20	66.68	> 99.99
0.30	75.03	> 99.99
0.40	80.04	> 99.99
0.50	83.38	> 99.99

Table 16 Trisomy 13 Prevalence, PPV, and NPV in Basic Screen (Excluding Known Mosaics)

Prevalence (%)	PPV (%)	NPV (%)
0.01	9.10	> 99.99
0.02	16.68	> 99.99
0.05	33.37	> 99.99

Prevalence (%)	PPV (%)	NPV (%)
0.10	50.05	> 99.99
0.20	66.73	> 99.99

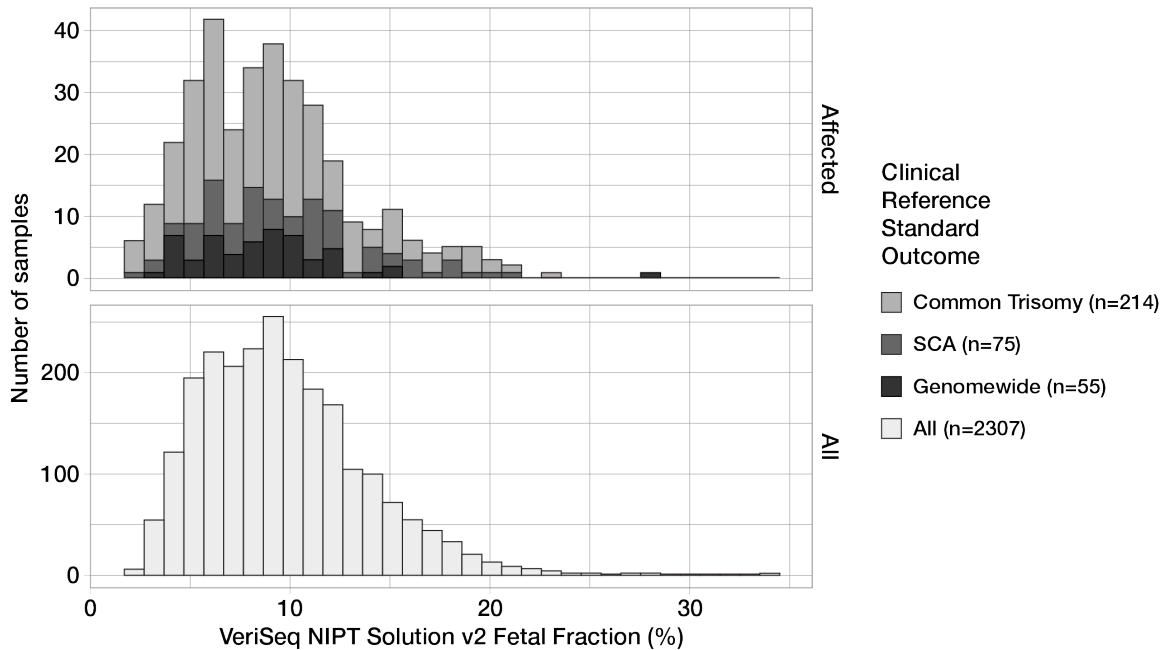
Table 17 Any Anomaly Prevalence, PPV, and NPV in Genomewide Screen (Including Known Mosaics)

Prevalence (%)	PPV (%)	NPV (%)
0.01	1.42	> 99.99
0.02	2.81	> 99.99
0.05	6.74	> 99.99
0.10	12.64	> 99.99
0.20	22.45	99.99
0.50	42.07	99.98
1.00	59.34	99.95
1.50	68.75	99.93
2.00	74.68	99.91

Fetal Fraction Distribution

The distribution of VeriSeq NIPT Solution v2 Fetal Fraction (FF) estimates from the genomewide screen with mosaics are shown by Clinical Reference Standard outcome category in [Figure 1](#).

Figure 1 Fetal Fraction Distribution



5 samples had anomalies across multiple categories.
 Common trisomy includes samples with trisomy 21, 18, and/or 13.
 Genomewide includes samples with RAA or partial deletions and/or duplications.

FF estimates ranged from 2% to 34% overall with a median of 9% and interquartile (IQ) range of 6% to 12%. The median FF estimate for common trisomies and events detected by the genomewide screen is 8% and for SCAs is 9%. The range in FF estimates was consistent for all the outcomes. There is no apparent shift in the distribution of FF among common trisomies, SCAs, events detected by the genomewide screen, or all samples in the genomewide analysis.

Performance in Twin Pregnancies

Estimating Trisomy 13, 18, and 21 and Chromosome Y Performance in Twin Pregnancies

Due to the low prevalence of trisomy 21, 18, and 13 in twin pregnancies, only a small number of affected twin samples were available for the clinical study. To estimate the performance of the VeriSeq NIPT Solution v2 in twin pregnancies, *in silico* models based on observations from clinical samples were used to simulate populations of twin pregnancies. This simulation was 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 (1,044 trisomy 21, 307 trisomy 18, and 192 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. The reduction was 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 v2 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 18). 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.

To estimate chromosome Y sensitivity in twin samples, sets of XY/XY and XX/XY twins were simulated. A weighted average representing their prevalence in the intended use population was taken (1 XY/XY: 1 XX/XY). To estimate chromosome Y specificity in twins, a set of XX/XX twins was simulated. The overall fetal fraction values were simulated according to the known distribution of fetal fraction in clinical twin samples.

For XY/XY and XX/XY twins, corresponding chromosome Y scores were estimated using the known relationship between fetal fraction and chromosome Y scores in clinical singleton samples classified as male. For XX/XY twins only, affected (ie, male) fetal fraction values were simulated using the known distribution of fetal fraction ratios observed between twins from the same pregnancy, as determined from sex discordant clinical twin samples. A conservative approach was taken whereby the affected fraction was selected such that it corresponded to the smaller of the two twins. For each simulated XX/XY sample, the chromosome Y score was multiplied by the affected fraction.

For XX/XX twins, chromosome Y scores were sampled from those scores observed in clinical singleton samples classified as female. The chromosome Y score and the overall fetal fraction were then used to classify each simulated sample as chromosome Y present or chromosome Y absent using the standard VeriSeq NIPT Solution v2 algorithm.

Sensitivity was calculated by determining how often the simulated XY/XY or XX/XY twins were correctly classified as chromosome Y present. Specificity was calculated by determining how often the simulated XX/XX twins were correctly classified as chromosome Y absent. 95% confidence intervals were estimated based on the number of real clinical twin samples in the original data set that were classified as either chromosome Y present or chromosome Y absent.

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

	Trisomy 21	Trisomy 18	Trisomy 13	Presence of Y
Sensitivity	96.4%	95.7%	93.6%	> 99.9%
2-Sided 95% CI	(86.4%, 98.9%)	(68.3%, 99.4%)	(64.1%, 98.9%)	(99.9%, > 99.9%)
Specificity	99.9%	> 99.9%	> 99.9%	> 99.9%
2-Sided 95% CI	(99.8%, > 99.9%)	(99.9%, > 99.9%)	(99.9%, > 99.9%)	(99.7%, > 99.9%)

Table 18 provides point estimates and estimated 95% confidence intervals for the sensitivity and specificity of VeriSeq NIPT Solution v2 to detect trisomy 21, 18, 13, and the presence of Y in 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 18 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 to estimate accuracy of aneuploidy detection.

Analytical Performance

Precision

To assess and quantify assay precision, a reanalysis of data using the VeriSeq NIPT Solution v2 analysis pipeline software from two previous studies from the VeriSeq NIPT Solution was conducted:

- Multisite Reproducibility study that comprised three runs by three operators across three sites using a single lot of reagent for a total of nine runs.
- Within-Lab Precision study that comprised 12 runs at a single site using two ML STARs, two sequencing instrument systems, and three sequencing reagent lots.

The objective of the precision study was to quantify the precision of the assay with regards to trisomy 21 (T21) and Chromosome Y and estimate variability among different instruments, library preparation kits, and sequencing reagent lots. Reproducibility for conditions not described above was not assessed as part of the studies.

A 5% fetal fraction T21 pool was created by combining cfDNA extracted from maternal plasma from pregnant women (with a T21 affected fetus) and cfDNA extracted from plasma from nonpregnant women. A 10% fetal fraction maternal-male (XY fetus) cfDNA pool was also created. The sample panel for each study for each run included 4 replicates of the 5% fetal fraction T21 affected sample pool and 20 replicates of the 10% fetal fraction maternal-male cfDNA pool. Testing was performed over 10 days for a total of 21 runs for the two studies combined.

T21 and the presence of chromosome Y were chosen for evaluation based on representativeness of clinical conditions and complexity of anomaly detection. As the smallest human autosome, the size of chromosome 21 has a direct impact on the sensitivity of T21 detection, particularly at low fetal fraction values such as those used in this study. Chromosome Y, as present in maternal plasma, is exclusively fetal in origin and therefore easier for the assay to detect.

The observed mean and standard deviations for the Chromosome 21 LLR Score and the Chromosome Y normalized chromosomal values (NCV) showed that replicate standard deviation (SD) was the largest source of variability. Variation between sites, instruments, and reagent lots added an insignificant amount of variability, as evidenced by the difference between Total SD and Replicate SD in [Table 19](#) and [Table 20](#).

Table 19 Summary of Multisite (Reproducibility) Sequencing Response Standard Deviation (SD)

Response	N	Mean	Replicate SD	Total Reproducibility SD*
Chromosome 21 LLR Score	36	34.43	11.36	11.36
Chromosome Y NCV	180	190.56	7.96	10.20

* Total includes variability due to site, operator, run, day, and replicate.

Table 20 Summary of Within Lab Sequencing Response Precision

Response	N	Mean	Replicate SD	Total Within Lab SD*
Chromosome 21 LLR Score	48	36.01	9.07	10.25
Chromosome Y NCV	240	198.68	7.63	7.82

* Total includes variability due to sequencing instrument, reagent lot, operator, run, day, and replicate.

An additional study was performed to compare VeriSeq NIPT Solution v2 sequencing precision (total standard deviation) using the version 2.0 of a flow cell versus version 2.5. The study included two types of flow cells (v2.0 and v2.5), three sequencing kit lots, four instrument systems, and two sequencing runs per combination for a total of 48 runs at a single site. One sequencing pool was prepared from cfDNA plates that were manually prepared. The sample panel included 4 replicates of the 5% fetal fraction T21 affected sample pool and 20 replicates of the 10% fetal fraction maternal-male (XY fetus) cfDNA pool. Results from the study are presented in [Table 21](#) and support that there is no difference in sequencing precision when using flow cell v2.0 versus flow cell v2.5.

Table 21 Summary of Flow Cell v2.0 versus Flow Cell v2.5 Sequencing Response Precision

Response	Number of Observations per Version	v2.0 Total SD*	v2.5 Total SD*	Statistical Result**
Chromosome 21 LLR Score	96	9.56	8.44	Statistically Equivalent (p-value=0.25)
Chromosome Y NCV	480	7.74	7.38	Statistically Equivalent (p-value=0.38)

* Total includes variability due to sequencing instrument, reagent lot, run, day, replicate

**Based on F-test for equality of variances (standard deviations squared)

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, for a 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

The impact of potentially interfering substances was assessed in the VeriSeq NIPT Solution by evaluating the performance of the assay in the presence of such substances.

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

Table 22 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 can 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 storage¹²) 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 four 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 23 Potentially Interfering Substances (exogenous)

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

Limit of Detection

The Limit of Detection (LOD) is defined as the level of fetal fraction that corresponds to the 95% detection probability of a condition of interest, such as T21. To assess the LOD of VeriSeq NIPT Solution v2 for various common conditions, studies and statistical analyses were conducted.

The probability of detection of a condition of interest in an affected sample processed by VeriSeq NIPT Solution v2 primarily depends on three factors:

- Fetal fraction
- Sequencing depth
- Size and complexity of the genomic region of interest

Assuming constant sequencing depth, a given aberration is easier to detect in a sample with a higher fetal fraction percentage than in a sample with a lower fetal fraction percentage. Conversely, assuming constant fetal fraction, a given aberration is easier to detect in a sample with a higher sequencing depth than in a sample with a lower sequencing depth. Lastly, aberrations in smaller or more complex genomic regions are harder to detect than aberrations in larger or less complex genomic regions, assuming constant fetal fraction and sequencing depth.

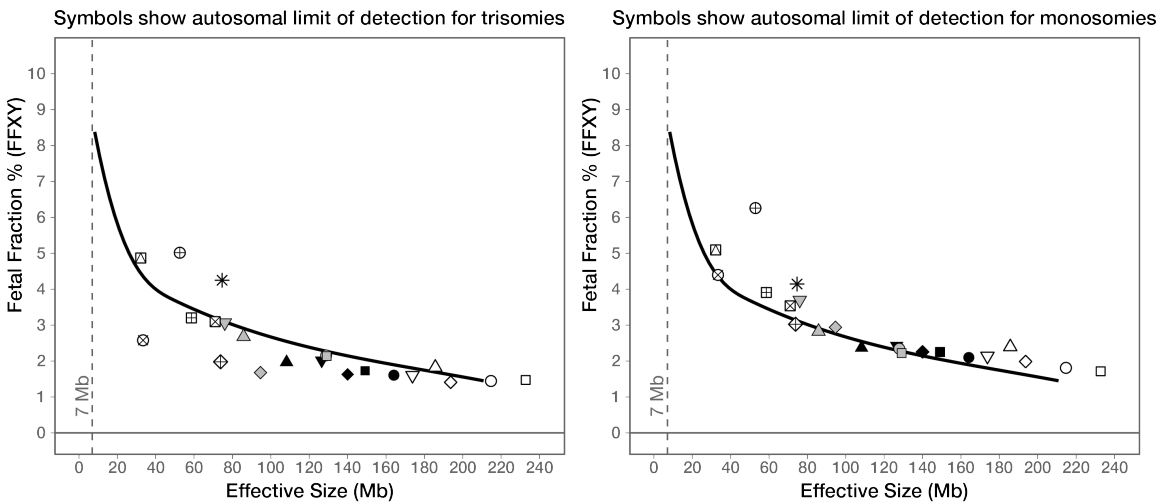
To determine the LOD for T21 detection, samples comprising mixtures of pooled T21 samples and pooled unaffected samples were analyzed. The two types of analyte were mixed over a titration series to create a set of seven fetal fraction levels (0, 2, 3, 4, 5, 6, and 10%). Each level was represented by a total of 10 replicates.

To further increase the resolution of the fetal fraction grid for the LOD analysis, the data from this study was augmented with data obtained from an in silico dilution. The effects of experimental dilution and titration were simulated by the controlled mixing of sequencing data. The data from this in silico titration covered a set of 14 fetal fraction levels (1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 4.25, and 4.50%) with 32 replicates for each level. A probit analysis was applied to the resulting data to determine the LOD for T21.

Independently, a statistical model using fetal fraction, sequencing depth, and genomic size/complexity was developed to predict the detection probability of any aberration in any sample. This model was established from the data corresponding to a set of 1,405 XY samples. The LOD for T21, as predicted by this model, was determined to be concordant with the probit-based estimate described above. This statistical model was used to estimate LOD values for aneuploidies on all autosomes and for partial deletions and duplications.

Figure 2 shows the 95% detection probability for average regions by size and the autosomal limits of detection for all trisomies and all monosomies. CNV LLR Cutoff 15.1.

Figure 2 95% Detection Probabilities for Average Regions by Size for the VeriSeq NIPT Solution v2



Chr	Symbol	Trisomy		Monosomy	
		LLR Cutoff	LoD (%)	LLR Cutoff	LoD (%)
1	○	7	1.44	13.2	1.80
2	□	9	1.47	13.6	1.71
3	◇	5	1.41	13.8	1.99
4	△	7	1.82	15.2	2.39
5	▽	7.6	1.60	17	2.14
6	●	7.3	1.60	15.4	2.09
7	■	6.6	1.73	14	2.25
8	◆	5.8	1.63	14.8	2.25
9	▲	8	1.97	13.6	2.37
10	▼	8.8	2.01	14.7	2.42
11	○	12.2	2.14	15.7	2.35

Chr	Symbol	Trisomy		Monosomy	
		LLR Cutoff	LoD (%)	LLR Cutoff	LoD (%)
12	■	11.6	2.14	12.8	2.22
13	◇	3	1.68	16.5	2.94
14	△	12.7	2.68	14.7	2.82
15	▽	9.8	3.07	16.4	3.69
16	⊠	10.7	3.10	15.3	3.54
17	*	16.8	4.25	15.7	4.14
18	⊕	3	1.98	11.3	3.02
19	⊕	15.5	5.01	27.5	6.26
20	⊠	10.6	3.20	18.2	3.91
21	⊗	2.5	2.58	13.2	4.40
22	⊠	13.5	4.87	15.3	5.09

Troubleshooting

VeriSeq NIPT Solution v2 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	Blood is not separated 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 (hemolysis of sample).	Redraw sample.	Frozen samples will not separate. Improper transport or storage conditions might lead to hemolysis of samples.

Failure Mode	Possible Result	Interpretation	Recommended Action	Comments
Sample clog or slow flow	Plasma contamination	Individual samples can 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 that samples were transported or stored improperly before processing. 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	Possible causes are as follows: <ul style="list-style-type: none"> • Insufficient genetic input • Mistransfer during sample handling • Sequencing reagent failure 	Check Sample Annotation. Check for similar performance on previous samples in relative plate position. Retest sample.	Indicates either an insufficient 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 overdilution of the sample for sequencing.
	Low FF or non-excluded sites (NES) count	Insufficient data generated to accurately report.	Retest from plasma.	
Quantification QC failure	Failed quantification run. Batch median below minimum	Insufficient process yield.	Repeat quantification. If repeat fails, contact Illumina Technical Support.	Non-passing standard curve metrics either indicate issues with library preparation (ie, use of non-biological grade ethanol) or issues with the quantification process.
	Failed quantification run	Standard curve failure.	Repeat quantification. If repeat 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 v2 only accepts numbers, letters, underscores, and dashes for all data fields.	Rename batch using a name that does not contain special characters.
Batch Creation	EM0051	The Batch ID is greater than 36 characters in length.	The VeriSeq NIPT Solution v2 limits the length of batch names to 36 characters or less.	Rename batch using a name that is fewer than 36 characters.
Batch Creation	EM0076	Unable to connect to VeriSeq Onsite Server v2	The VeriSeq Onsite Server v2 is not responding to data requests from the Workflow Manager.	<ol style="list-style-type: none"> 1. Make sure that the ML STAR is connected to the network. 2. Make sure that the VeriSeq Onsite Server v2 is on. 3. Check that the ML STAR can connect to the VeriSeq Onsite Server v2 (via ping request). 4. If previous steps do not resolve the issue, contact Illumina Technical Support.
Batch Creation	EM0118	This batch has been failed and cannot be further processed.	The specified batch has already failed and cannot be processed any further.	The batch record on the VeriSeq Onsite Server v2 indicates that the selected batch has failed. No further processing is allowed. Create another batch with the required samples.

Process Step	Error Code	Error Dialog	Description	User Resolution
Batch Creation	Not applicable	This batch has already completed processing. Would you like to repool?	The indicated batch has processed through pooling. The only allowable processing is repool.	Repool as follows. <ul style="list-style-type: none"> • Select Re-Pool. • Abort method and ensure batch name is correct before repooling.
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 the duplicates and relabel or replace them. 3. Reload the 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. Do one of the following options: <ul style="list-style-type: none"> • Add missing samples to the batch and reload samples. • Abort method, modify the sample sheet as needed. Restart method.
Plate Load	Not applicable	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 does 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, and then fully close the release line clips. 3. Make sure that the vacuum controller and pump are turned on. 4. Check the vacuum waste bottle. If the waste bottle is more than half full, empty the waste bottle. 5. If the problem persists, contact Illumina Technical Support.
cfDNA Extraction	WE0153	Pressure in the vacuum chamber is too high.	If the measured vacuum pressure is too high before the pressure control starts, the system might be malfunctioning.	On the back of the controller, make sure that all vacuum connections and lines are secure.

Process Step	Error Code	Error Dialog	Description	User Resolution
cfDNA Extraction	WE0996	Vacuum failed to seal.	The seal failure must be resolved before continuing.	<p>Verify seal failure is resolved prior to selecting OK.</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. Listen for the vacuum hum and observe the flow of water through the binding plate. 3. Open the trace view on the Workflow Manager. After the actual pressure reading reaches at least 50 pressure units less than the ambient reading, select OK to continue with cfDNA Extraction. 4. If the required pressure reading is not reached during the allotted time, select OK to continue with the first lysate load. 5. Pause the method after the lysate is dispensed onto the binding plate. Reseat and forcibly press down on the binding plate. 6. If the lysate fails to flow through the plate, contact Illumina Technical Support.
cfDNA Extraction	WM0219	If Vacuum is on, manually rest the pump.	The vacuum might 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.

Process Step	Error Code	Error Dialog	Description	User Resolution
cfDNA Extraction	EE0477	An error has occurred while moving a plate. (iSWAP error)	If an iSWAP error is encountered (plate drop, failed to pick up, etc), the system prompts you 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 the plate cannot be recovered, abort the run. • If the plate can be recovered, follow the displayed instructions to complete the plate transfer manually.
cfDNA Extraction	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 v2 is not responding to data requests from the Workflow Manager.	<ol style="list-style-type: none"> 1. Make sure that the ML STAR is connected to the network. 2. Make sure that the VeriSeq Onsite Server v2 is on. 3. Check that the ML STAR can connect to the VeriSeq Onsite Server v2 (via ping request).
	EA0774	Connection Error. The API server connection failed to validate.	The VeriSeq Onsite Server v2 has stopped responding to data requests from the Workflow Manager.	Make sure that: <ol style="list-style-type: none"> 1. Make sure that the ML STAR is connected to the network. 2. Check that the ML STAR can connect to the VeriSeq Onsite Server v2 (via ping request). 3. Make sure that the VeriSeq Onsite Server v2 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 # 1000000078751 v09	April 2024	<p>Removed</p> <ul style="list-style-type: none"> • Obsolete part #20030577. • Maximum tube capacity requirement for blood collection tube centrifuge. <p>Added</p> <ul style="list-style-type: none"> • New part #20101927 to the VeriSeq Onsite Server v2. • Dimension unit for the 10 ml blood collection tubes. • Clarification on the compatible versions of SoftMax Pro. • Clarification note to state that only compatible plasticware should be used to ensure interchangeability with the VeriSeq NIPT Microlab STAR. • Note regarding sample admixture contamination warning to the Interpretation of Results section. • Caution statement do not freeze whole blood specimen collected in Streck Cell-Free DNA BCT. • Caution statement to avoid sample exposure to elevated temperatures. • Clarification regarding assay limitations and reproducibility conditions. • Clarification for CNV LLR Cutoff to Figure 2 in Limit of Detection section. <p>Updated</p> <ul style="list-style-type: none"> • Compatible reagent tub reference from Roche Reagent Tub to Illumina Reagent Tub and added new part number. • Thermo Fisher Multifuge X4 Pro-MD catalog part number to # 75016034. • Caution statement that inconsistent well volumes may cause samples to fail automated QC. • Reference to instrument package inserts.
Document # 1000000078751 v08	August 2022	<p>Update the workflow part number</p> <p>Removed instruction to pipette to mix if library plate was frozen.</p>

Document	Date	Description of Change
Document # 1000000078751 v07	May 2022	<p>Split Limitations of the Procedure into VeriSeq NIPT Solution v2 Reporting and include first two bullets. Remaining text in a new header Limitations of the Assay.</p> <p>Removed</p> <ul style="list-style-type: none"> • VeriSeq from all reagent labeling. • Apply a plate barcode to VeriSeq NIPT Adapter Plate in Prepare Libraries preparation. <p>Added</p> <ul style="list-style-type: none"> • The word certified to DNase/RNase-free water. • One of the following microplate readers, or equivalent and SpectraMax M2, M3, M4, M5, and note. • To the VeriSeq NIPT Microlab STAR section to explain what to do during an error handling event. • A note to visually inspect wells. • Instructions for 24 and 48-sample batches throughout protocol sections. • Steps for when to use the purple adapter plate or equivalent. • Verbiage to Demographics and Pregnancy Characteristics section to include first trimester pregnancy results. • A bullet to the Deep Well plate specifications to include torque-resistant. <p>Updated</p> <ul style="list-style-type: none"> • Verbiage for unique batch names for clarity and include an example. • Symbols and formatting for Notes, Cautions, and Warnings. • Results of the test subbullets. • Guanidine thiocyanate to guanidine hydrochloride. • CVS to BVS (Basic Vacuum System) • Verbiage for using the genomewide screen and the LLR score. • Specs: Reagent tub specs, deep-well plates, 384 well plates, 96 well plates
Document # 1000000078751 v06	August 2021	Updated EU Authorized Representative address.

Document	Date	Description of Change
Document # 1000000078751 v05	December 2020	<p>Updated Principles of Procedure, Warnings and Precautions, and Product Labeling sections with additional clarifications to fulfill regulatory requests.</p> <p>Minor updates to content in protocol to match current Illumina style and organization.</p> <p>Corrected description of chromosome 21 as "second smallest human autosome" to "smallest human autosome" in Precision section of Analytic Performance.</p> <p>Added caution statements to address improper use of reservoirs and risks of sample amalgamation to Isolate Plasma Preparation and Interpretation of Results sections.</p> <p>Added new server and software part numbers for the release of new server model and software part number updates.</p> <p>Added cautions to protocol and troubleshooting information for addressing and preventing sample overflows.</p> <p>Updated active ingredients in the reagent DNA Quantification Standard in the Accessory Box to align with the Safety Data Sheet.</p> <p>Updated naming conventions of the Local Run Manager VeriSeq NIPT module for consistency with other documentation.</p> <p>Added revision history.</p>
Document # 1000000078751 v04	October 2020	Minor corrections.
Document # 1000000078751 v03	September 2020	Updated materials list to present specifications of labware along with known compatible options.
Document # 1000000078751 v02	February 2020	<p>Updated presentation of Clinical Performance information to better convey the differences between basic and genomewide screen types.</p> <p>Added new Differences in Performance Between the Basic and Genomewide Screens section.</p> <p>Removed contradictory information about the optionality of the supplementary report from the Principles of Procedure section.</p> <p>Updated naming convention of the VeriSeq NIPT Workflow Manager v2 software throughout the document for stylistic consistency.</p> <p>Updated labeling of Australia and Illumina Netherlands addresses to reflect recent changes.</p>

Document	Date	Description of Change
Document # 1000000078751 v01	August 2019	Removed duplicate step in Extract cfDNA caused by publishing software error.
Document # 1000000078751 v00	May 2019	Initial release.

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A Summary of Safety and Performance (SSP) is located at <https://ec.europa.eu/tools/eudamed>, after launch of the European Database on Medical Devices (Eudamed). It is linked to the Basic UDI-DI (0081627002NIPTRP).