October 2024

ForenSeq® DNA Signature Plus Kit Handbook

Interrogate 200 genetic markers using a single streamlined NGS workflow

Sample to Insight

Table of Contents

Kit Contents

ForenSeq DNA Signature Plus Kit Catalog no. No. of reactions			V16000213 96	V16000214 384
Reagent	**Description**	**Cap**	**Quantity**	**Quantity**
Box 1	**Pre-PCR**	–	–	–
FEM	Enzyme Mix	Yellow	2	8
PCR1	PCR1 Reaction Mix	Green	2	8
Box 2	**Post-PCR**	–	–	–
HP3	2 N NaOH	Orange	1	3
HSC	Human Sequencing Control	Pink	1	1
LNA1	Library Normalization Additives 1	Clear	1	4
LNS2	Library Normalization Storage Buffer 2	Clear	1	4
LNW1	Library Normalization Wash 1	Clear	2	8
PCR2	PCR2 Reaction Mix	Purple	2	8
UDI Plate	Unique Dual Index	Clear	1	4
Box 3	**Post-PCR**	–	–	–
LNB1	Library Normalization Beads 1	White	1	4
RSB	Resuspension Buffer	Purple	1	1
SPB2	Sample Purification Beads 2	Red	1	2
Box 4	**Pre-PCR**	–	–	–
NA24385	Control DNA	Black	1	4
DPMA	DNA Primer Mix A	Blue	2	8
DPMB	DNA Primer Mix B	Red	2	8

Shipping and Storage

All reagents in a box are shipped at the same temperature. When a reagent has a different storage temperature than most other reagents in the box, you can initially store the reagent at the same temperature as the other reagents.

After first use, store the reagent at the indicated temperature.

Reagent	Description	Shipping temperature (°C)	Storage temperature (°C)
Box 1	**Pre-PCR**	–	–
FEM	Enzyme Mix	–25 to –15	–25 to –15
PCR1	PCR1 Reaction Mix	–25 to –15	–25 to –15
Box 2	**Post-PCR**	–	–
HP3	2 N NaOH	–25 to –15	–25 to –15
HSC	Human Sequencing Control	–25 to –15	–25 to –15
LNA1	Library Normalization Additives 1	–25 to –15	–25 to –15
LNS2	Library Normalization Storage Buffer 2	–25 to –15	15–25 (room temperature)
LNW1	Library Normalization Wash 1	–25 to –15	2–8
PCR2	PCR2 Reaction Mix	–25 to –15	–25 to –15
UDI Plate	Unique Dual Index	–25 to –15	–25 to –15
Box 3	**Post-PCR**	–	–
LNB1	Library Normalization Beads 1	2–8	2–8
RSB	Resuspension Buffer	2–8	2–8
SPB2	Sample Purification Beads 2	2–8	2–8
Box 4	**Pre-PCR**	–	–
NA24385	Control DNA	–25 to –15	2–8

Reagent	Description	Shipping temperature (°C)	Storage temperature (°C)
DPMA	DNA Primer Mix A	–25 to –15	–25 to –15
DPMB	DNA Primer Mix B	–25 to –15	–25 to –15

When stored correctly, the components are stable until the expiration date printed on the kit box.

Intended Use

The ForenSeq DNA Signature Plus Kit is intended for molecular biology applications in forensic, human identity, and paternity testing. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend that all users of QIAGEN® products adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

Each lot of ForenSeq DNA Signature Plus Kits is tested against predetermined specifications to ensure consistent product quality.

Introduction

The ForenSeq Signature Plus Kit generates dual-indexed libraries for sequencing. Each sample is combined with a primer mix that contains a pair of tagged oligos for each target sequence. PCR cycles link the tags to copies of each target, forming DNA templates consisting of the regions of interest flanked by universal primer binding sequences. The tags are then used to attach index adapters and the resulting library is amplified, purified, and pooled for sequencing. The ForenSeq Signature Plus Kit can be used with the standard PCR1 buffer that is available with the kit or the ForenSeq Enhanced PCR1 Buffer System (ePCR1) that is available as an add-on kit. This handbook summarizes the protocol for using the ForenSeq Signature Plus Kit with the standard PCR1 (PCR1) buffer.

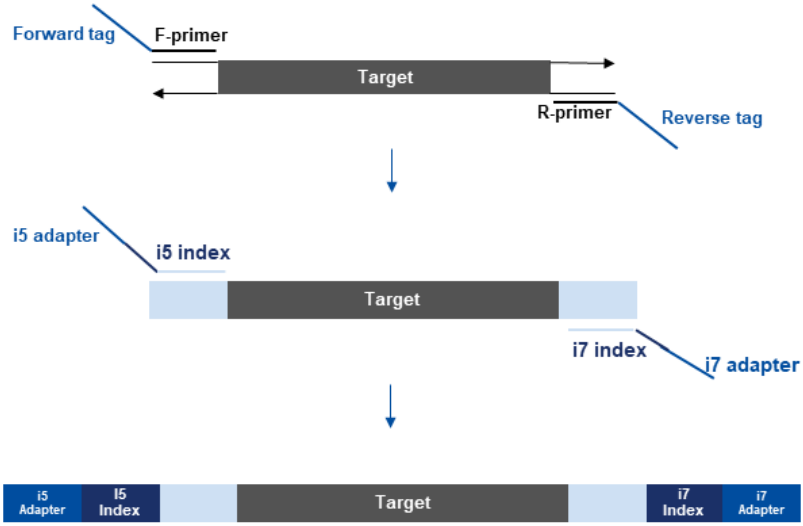

Figure 1. Assay overview.

The ForenSeq Signature Plus Kit offers the following features:

- Simultaneous preparation of up to 96 libraries in one plate. Each library is a collection of amplified DNA fragments from one sample.
- Amplify short tandem repeat (STR) and single-nucleotide polymorphism (SNP) amplicons in one reaction.
- Choose between 2 targeted primer mixes, DNA Primer Mix A (DPMA) or DNA Primer Mix B (DPMB), to prepare samples for databasing or casework.

The ForenSeq Enhanced PCR1 Buffer System offers the following features:

- An optimized buffer system that supports the simultaneous amplification of 96 challenging samples in the presence of high concentrations of inhibitors such as humic acid and hematin.
- Engineered as a minor protocol enhancement, this buffer system can be included into validated workflows without the need for revalidation.

Protocol steps

The following diagram lists the steps to prepare libraries with challenging examples, with hands-on times, total times, and reagents. Safe stopping points are marked between steps.

Step	Hands-on time	Total time	Reagents
Amplify and tag targets*	15 min	3 h 35 min	NA24385, DPMA or DPMB, FEM, PCR1
Enrich targets†	10 min	90 min	UDI Plate, PCR2
Purify libraries†	15 min	30 min	80% EtOH, RSP, SPB2
Safe stopping point	–	–	–
Normalize libraries†	30 min	1 h 20 min	HP3, LNA1, LNB1, LNS2, LNW1

Step	Hands-on time	Total time	Reagents
Safe stopping point	–	–	–
Pool libraries†	10 min	10 min	–
Safe stopping point	–	–	–
Denature and dilute libraries†	10 min	10 min	HP3, HSC, HT1

* Pre-PCR

† Post-PCR

DNA input recommendations

Use 1 ng purified human genomic DNA (gDNA) as input. Before starting the protocol, quantify the input using a fluorometric-based method or qPCR and assess quality.

The kit is compatible with crude lysate from buccal swabs and FTA card stains:

- For crude lysate, use 2 µL input material per sample. See "Consumables" for recommended lysis buffers.
- For FTA paper, use a 1.2 mm FTA card punch for each sample.

Controls

Each preparation must include at least one positive amplification control and at least one negative amplification control. If these controls are not included, troubleshooting support is limited.

The kit includes Control DNA NA24385 for use as the positive amplification control (NA24385), and the negative amplification control is nuclease-free water. The protocol includes instructions to prepare each control.

Abbreviations

DNL	Diluted Normalized Libraries
DPMA	Primer Mix A
DPMB	DNA Primer Mix B
gDNA	Genomic DNA
FEM	FEM Enzyme Mix
FSP	ForenSeq Sample Plate
HP3	2 N NaOH
HSC	Human Sequencing Control
HT1	Hybridization Buffer
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNS2	Library Normalization Storage Buffer 2
LNW1	Library Normalization Wash 1
NA24385	Control DNA NA24385
NLP	Normalized Library Plate
NWP	Normalization Working Plate
PBP	Purification Bead Plate
PCR2	PCR2 Reaction Mix
PLP	Purified Library Plate
PNL	Pooled Normalized Libraries
RSB	Resuspension Buffer
SPB2	Sample Purification Beads 2
UDI	Unique dual index plate

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs) available from the product supplier.

Equipment

Equipment	Supplier	Pre-PCR	Post-PCR
20 µL pipettes	General lab supplier	X	X
200 µL pipettes	General lab supplier	X	X
1000 µL pipettes	General lab supplier		X
Benchtop microcentrifuge	General lab supplier	X	X
Magnetic stand-96	Thermo Fisher, part # AM10027		X
Microplate centrifuge	General lab supplier	X	X
One of the following thermoshakers: • BioShake iQ • BioShake XP	QInstruments • item no. 1808-0506 • item no. 1808-0505		X
Rubber roller	General lab supplier	X	X
Thermal cycler, 96-well, with heated lid	See "Thermal cyclers", next section		X
Vortexer	General lab supplier	X	X

Thermal cyclers

The following table lists supported thermal cyclers with recommended settings. If your laboratory has an unlisted thermal cycler, evaluate the thermal cycler before performing the protocol.

Thermal cycler	Temperature mode	Lid temperature
ABI LTI thermal cycler 9700*	9600 emulation	Heated
Bio-Rad	Calculated	Heated, constant at 100°C
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated
Proflex 96-well PCR System†	Not applicable	Heated, constant at 100°C
Veriti™ 96-well thermal cycler†	Standard	Heated, constant at 100°C
Veriti Pro 96-well thermal cycler	Not applicable	105°C
QIAamplifier 96-well thermal cycler	Standard	Heated, constant at 100°C

* Only gold heat blocks are supported.

† Settings were verified after developmental validation of the ForenSeq DNA Signature Prep Kit.

Consumables

- 1.5 mL LoBind microcentrifuge tubes (VWR, catalog # 80077-230)
- 15 mL conical tubes
- 20 µL barrier pipette tips
- 200 µL barrier pipette tips
- 96-well deep well storage plates (midi plates) (Thermo Fisher Scientific, part no. AB-0765, individually sealed)
- 96-well twin.tec® PCR plates, semi-skirted (one of the following suppliers: Eppendorf, cat. no. 951020303; VWR, cat. no. 89136-706)
- Ethyl alcohol, pure (Sigma-Aldrich, cat. no. E7023)
- Microseal 'A' sealing film (Bio-Rad, cat. no. MSA5001)
- Microseal 'B' sealing film, adhesive, optical (Bio-Rad, cat. no. MSB1001)
- One of the following kits:

- MiSeq FGx Reagent Kit (QIAGEN, cat. no. 15066817)
- MiSeq FGx Reagent Micro Kit (QIAGEN, cat. no. 20021681)

- Nuclease-free water
- Multichannel reagent reservoirs, PVC, disposable (VWR, catalog # 730-001)
- RNase/DNase-free 8-tube strips and caps
- (Crude lysate) One of the following kits:
  - QuickExtract DNA Extraction Solution (Epicentre, cat. no. QE09050)
  - SwabSolution Kit (Promega, cat. no. DC8271)
- (FTA Card) 1X TBE Buffer
- (FTA Card) PCR Tube Storage Racks (VWR, cat. no. 80086-074)

Important Notes

Index adapter sequences

The following table lists the 8 bp sequences for the index adapters included in the kit.

Table 1. Sequences for the UDI adapters

Index name	Index 1 bases	Index 2 bases	Plate well location
UDI0001	ATCACGAT	AGCGCTAG	A1
UDI0002	CGATGTAT	GATATCGA	A2
UDI0003	TTAGGCAT	CGCAGACG	A3
UDI0004	TGACCAAT	TATGAGTA	A4
UDI0005	ACAGTGAT	AGGTGCGT	A5
UDI0006	GCCAATAT	GAACATAC	A6
UDI0007	CAGATCAT	ACATAGCG	A7
UDI0008	ACTTGAAT	GTGCGATA	A8
UDI0009	GATCAGAT	CCAACAGA	A9
UDI0010	TAGCTTAT	TTGGTGAG	A10
UDI0011	GGCTACAT	CGCGGTTC	A11
UDI0012	CTTGTAAT	TATAACCT	A12
UDI0013	AGTCAAAT	AAGGATGA	B1
UDI0014	AGTTCCAT	GGAAGCAG	B2
UDI0015	ATGTCAAT	TCGTGACC	B3
UDI0016	CCGTCCAT	CTACAGTT	B4
UDI0017	GTAGAGAT	ATATTCAC	B5

Table 1. Sequences for the UDI adapters (continued)

Index name	Index 1 bases	Index 2 bases	Plate well location
UDI0018	GTCCGCAT	GCGCCTGT	B6
UDI0019	GTGAAAAT	ACTCTATG	B7
UDI0020	GTGGCCAT	GTCTCGCA	B8
UDI0021	GTTTCGAT	AAGACGTC	B9
UDI0022	CGTACGAT	GGAGTACT	B10
UDI0023	GAGTGGAT	ACCGGCCA	B11
UDI0024	GGTAGCAT	GTTAATTG	B12
UDI0025	ACTGATAT	AACCGCGG	C1
UDI0026	ATGAGCAT	GGTTATAA	C2
UDI0027	ATTCCTAT	CCAAGTCC	C3
UDI0028	CAAAAGAT	TTGGACTT	C4
UDI0029	CAACTAAT	CAGTGGAT	C5
UDI0030	CACCGGAT	TGACAAGC	C6
UDI0031	CACGATAT	CTAGCTTG	C7
UDI0032	CACTCAAT	TCGATCCA	C8
UDI0033	CAGGCGAT	CCTGAACT	C9
UDI0034	CATGGCAT	TTCAGGTC	C10
UDI0035	CATTTTAT	AGTAGAGA	C11
UDI0036	CCAACAAT	GACGAGAG	C12
UDI0037	CGGAATAT	AGACTTGG	D1
UDI0038	CTAGCTAT	GAGTCCAA	D2
UDI0039	CTATACAT	CTTAAGCC	D3

Table 1. Sequences for the UDI adapters (continued)

Index name	Index 1 bases	Index 2 bases	Plate well location
UDI0040	CTCAGAAT	TCCGGATT	D4
UDI0041	GACGACAT	CTGTATTA	D5
UDI0042	TAATCGAT	TCACGCCG	D6
UDI0043	TACAGCAT	ACTTACAT	D7
UDI0044	TATAATAT	GTCCGTGC	D8
UDI0045	TCATTCAT	AAGGTACC	D9
UDI0046	TCCCGAAT	GGAACGTT	D10
UDI0047	GTTCCAAT	AATTCTGC	D11
UDI0048	ACCTTGGC	GGCCTCAT	D12
UDI0049	ATATCTCG	ATCTTAGT	E1
UDI0050	GCGCTCTA	GCTCCGAC	E2
UDI0051	AACAGGTT	ATACCAAG	E3
UDI0052	GGTGAACC	GCGTTGGA	E4
UDI0053	CAACAATG	CTTCACGG	E5
UDI0054	TGGTGGCA	TCCTGTAA	E6
UDI0055	AGGCAGAG	AGAATGCC	E7
UDI0056	GAATGAGA	GAGGCATT	E8
UDI0057	TGCGGCGT	CCTCGGTA	E9
UDI0058	CATAATAC	TTCTAACG	E10
UDI0059	GATCTATC	ATGAGGCT	E11
UDI0060	AGCTCGCT	GCAGAATC	E12
UDI0061	CGGAACTG	CACTACGA	F1

Table 1. Sequences for the UDI adapters (continued)

Index name	Index 1 bases	Index 2 bases	Plate well location
UDI0062	TAAGGTCA	TGTCGTAG	F2
UDI0063	TTGCCTAG	ACCACTTA	F3
UDI0064	CCATTCGA	GTTGTCCG	F4
UDI0065	ACACTAAG	ATCCATAT	F5
UDI0066	GTGTCGGA	GCTTGCGC	F6
UDI0067	TTCCTGTT	AGTATCTT	F7
UDI0068	CCTTCACC	GACGCTCC	F8
UDI0069	GCCACAGG	CATGCCAT	F9
UDI0070	ATTGTGAA	TGCATTGC	F10
UDI0071	ACTCGTGT	ATTGGAAC	F11
UDI0072	GTCTACAC	GCCAAGGT	F12
UDI0073	CAATTAAC	CGAGATAT	G1
UDI0074	TGGCCGGT	TAGAGCGC	G2
UDI0075	AGTACTCC	AACCTGTT	G3
UDI0076	GACGTCTT	GGTTCACC	G4
UDI0077	TGCGAGAC	CATTGTTG	G5
UDI0078	CATAGAGT	TGCCACCA	G6
UDI0079	ACAGGCGC	CTCTGCCT	G7
UDI0080	GTGAATAT	TCTCATTC	G8
UDI0081	AACTGTAG	ACGCCGCA	G9
UDI0082	GGTCACGA	GTATTATG	G10
UDI0083	CTGCTTCC	GATAGATC	G11

Table 1. Sequences for the UDI adapters (continued)

Index name	Index 1 bases	Index 2 bases	Plate well location
UDI0084	TCATCCTT	AGCGAGCT	G12
UDI0085	AGGTTATA	CAGTTCCG	H1
UDI0086	GAACCGCG	TGACCTTA	H2
UDI0087	CTCACCAA	CTAGGCAA	H3
UDI0088	TCTGTTGG	TCGAATGG	H4
UDI0089	TATCGCAC	CTTAGTGT	H5
UDI0090	CGCTATGT	TCCGACAC	H6
UDI0091	GTATGTTC	AACAGGAA	H7
UDI0092	ACGCACCT	GGTGAAGG	H8
UDI0093	TACTCATA	CCTGTGGC	H9
UDI0094	CGTCTGCG	TTCACAAT	H10
UDI0095	TCGATATC	ACACGAGT	H11
UDI0096	CTAGCGCT	GTGTAGAC	H12

Number of samples

Process at least 8 samples at a time, including positive and negative amplification controls. Preparing master mixes for fewer than 8 samples can introduce pipetting inaccuracies due to small volumes.

Use the following table to determine the maximum number of libraries to pool for a run, depending on primer mix, sample type, and MiSeq FGx® reagent kit. Casework recommendations are intended for samples where DNA mixtures are possible or challenging samples with <1 ng gDNA available and partial degradation.

Table 2. Maximum number of libraries

Primer mix	Sample type	MiSeq FGx Reagent Micro Kit	MiSeq FGx Reagent Kit
DPMA	Database or reference	36	96
	Casework	12	32
DPMB	Database or reference	12	32
	Casework	12	32

Primer mixes

The kit includes 2 primer mixes: DPMA and DPMB. Both primer mixes detect identity informative SNPs (iiSNPs), autosomal STRs (aSTRs), and X- and Y-STRs. In addition to these targets, DPMB detects ancestry-informative SNPs (aiSNPs) and phenotypic-informative SNP (piSNPs).

The ForenSeq Signature Plus Kit supports analysis of these SNPs and STRs from gDNA ranging from high-quality, single-source samples to challenging. One reaction with integrated indexing enables sequencing of up to 96 database samples using DPMA or 32 casework samples using DPMB in one run with the MiSeq FGx Reagent Kit.

Protocol

This chapter describes the ForenSeq Signature Plus protocol with stepwise instructions to prepare libraries for sequencing. For an overview of the protocol with reagents and durations for each step, see "Protocol steps" on page 10.

Before starting, confirm kit contents and make sure that you have the necessary reagents, consumables, and equipment. For a list of items, see "Equipment and Reagents to Be Supplied by User" on page 13.

Important points before starting

Protocol continuity

- Follow the steps in the order indicated using the specified volumes and incubation parameters.
- Unless a safe stopping point is specified, proceed immediately to the next step.

Plate setup

- Create a sample sheet to record the position of each sample, control, and index adapter.
    - Reference the sample sheet throughout the protocol to ensure proper plate setup.

      The *ForenSeq Universal Analysis Software v2.7 Reference Guide* (**qiagen.com/HB-3563**) provides detailed information on sample sheets and input of sample information.

Preventing cross-contamination

- Set up the "Amplify and tag targets", on the previous page, process in a pre-PCR environment. Perform all other processes in a post-PCR environment.
- When adding or transferring samples, change tips between **each sample**.
- When adding adapters or primers, change tips between **each well**.
- When processing smaller sample batches of fewer than 96 libraries at a time, prevent aerosolization by resealing the utilized, pierced UDI wells with Microseal "B" adhesive seal. Trim excess seal with a scissors.
- Do not apply Microseal "B" to unused UDI wells as this will impact the integrity of the single-use pierceable foil.

Sealing the plate

- Apply a microseal to cover the plate and seal with a rubber roller. After each use, discard seals from plates.
- Use Microseal 'A' pressure film for thermal cycling. When using fewer than 96 wells, you can cut the film to size.
- Use Microseal 'B' adhesive film for shaking, centrifuging, and long-term storage. These seals are effective at –40°C to 110°C.

Handling beads

- For optimal performance and yield, confirm that beads are at room temperature before use.
- Aspirate and dispense beads slowly due to viscosity.
- Do not centrifuge plates and tubes containing beads, except when indicated.

- Vortex beads before use and frequently throughout the protocol to resuspend. Resuspended beads are evenly distributed and homogenous in color.
- If beads aspirate into pipette tips during supernatant removal, dispense back to the plate on the magnetic stand and wait until the liquid is clear (approximately 2 min).

Protocol step 1: Amplify and tag targets

This process uses an oligonucleotide primer mix with regions specific to the DNA sequences upstream and downstream of STRs and SNPs to tag and amplify the input gDNA.

Consumables

- NA24385 (Control DNA NA24385)
- DPMA (DNA Primer Mix A) or DPMB (DNA Primer Mix B)
- FEM (Enzyme Mix)
- PCR1 (PCR1 Reaction Mix)
- 1.5 mL LoBind microcentrifuge tubes (2)
- 96-well PCR plate, semiskirted
- Input gDNA
- Microseal 'A' film
- Nuclease-free water
- (FTA card) 1X TBE Buffer (100 µL per FTA card punch)
- (FTA card) Microseal 'B' film
- **Optional**: RNase/DNase-free 8-tube strip and caps

About reagents

- Use PCR1 for standard samples.
- For information on DPMA and DPMB, see "Loci detected with DPMA and DPMB".

Preparation

1. Prepare the following consumables:

Item	Storage temperature (°C)	Instruction
NA24385	2–8	Let stand for 30 min to bring to room temperature. Invert 3 times to mix, and then centrifuge briefly.
1X TBE Buffer*	–25 to –15	Thaw at room temperature. Invert 3 times to mix, and then centrifuge briefly.
DPMA or DPMB	2–8	Let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge.
FEM	–25 to –15	Remove from storage immediately before use, and then return to storage immediately after use. Pipette to mix.
PCR1	–25 to –15	Thaw at room temperature for 30 min. Vortex to mix, and then centrifuge briefly.

* For FTA cards only.

2. Save the following PCR1 program on the thermal cycler in the post-amplification area. (Example program name: Sig Plus PCR1.) See Table 3 on the next page for ramp modes.
   a. Choose the preheat lid option. See Table 3 on the next page for lid temperature
   b. 98°C for 3 min
   c. 8 cycles of:

i. 96°C for 45 s

ii. 80°C for 30 s

iii. 54°C for 2 min with applicable ramp mode

iv. 68°C for 2 min with applicable ramp mode

d. 10 cycles of:

i. 96°C for 30 s

ii. 68°C for 3 min with applicable ramp mode

e. 68°C for 10 min

f. Hold at 10°C

Table 3. Thermal cycler lid temperatures and ramp modes

Thermal cycler	Temperature mode	Lid temperature	Ramp mode
ABI LTI Thermal Cycler 9700	9600 emulation	Heated	8%
Bio-Rad	Calculated	Heated, constant at 100°C	0.2°C/s
Eppendorf Mastercycler Pro S	Gradient S, Simulated Tube	Heated	2%
Proflex 96-well PCR System	Not applicable	Heated, constant at 105°C	0.2°C/s
Veriti 96-well Thermal Cycler	Standard	Heated, constant at 105°C	4%
Veriti Pro 96-well Thermal Cycler	Not applicable	105°C	0.2°C/s
QIAamplifier 96-well Thermal Cycler	Standard	100°C	0.1°C/s

The PCR1 program takes approximately 4.5 h and can be run overnight.

3. Label a new PCR plate FSP for ForenSeq Sample Plate.
4. Label a new 1.5 mL tube according to your input type.

Input type	Label
Crude lysate	Master Mix
FTA card	FTA Master Mix
Purified DNA	Master Mix

Procedure

Purified DNA

1. Using nuclease-free water, dilute 1 ng purified DNA input to 0.2 ng/µL.
2. In the Master Mix tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.

   Note: Reagent fill volume listed on the tube label represents the volume required for master mix. This volume **does not** take into consideration that the vial is filled with an additional 10% overage.

   - PCR1 (4.7 µL)
   - FEM (0.3 µL)
   - DPMA or DPMB (5 µL)

   For example, for 8 samples, prepare 88 µL master mix: 41.4 µL PCR1, 2.6 µL FEM, and 44 µL DPMA or DPMB.
3. Pipette to mix, and then cap and centrifuge briefly.
4. **Optional**: Evenly distribute the master mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.
5. Add 10 µL Master Mix to each well of the FSP.
6. In a new 1.5 µL tube, combine the following volumes to dilute NA24385 :

- NA24385 (2 µL)
- Nuclease-free water (98 µL)

7. Cap and gently invert three times to mix, and then centrifuge briefly.
8. Add 5 µL diluted NA24385 to at least one well of the FSP as a positive amplification control.
9. Pipette to mix.
10. Add 5 µL nuclease-free water to at least one well of the FSP as a negative amplification control.
11. Pipette to mix.
12. Add 5 µL 0.2 ng/µL DNA to each well of the FSP. Pipette to mix.
13. Seal and centrifuge at 100 × *g* for 30 s.
14. Transport to the post-PCR area.
15. Place on the preprogrammed thermal cycler and run the Sig Plus PCR1 program.
16. Unless you are stopping, proceed to "Enrich targets" on page 32.

Safe stopping point

If you are stopping, seal the plate with Seal "B" and store at 2–8°C for up to 2 days. Alternatively, leave the plate in the thermal cycler overnight.

Crude lysate

1. In the Master Mix tube, combine the following volumes. Multiply each volume by the number of samples and add 10% for overage.

Note: Reagent fill volume listed on the tube label represents the volume required for master mix. This volume **does not** take into consideration that the vial is filled with an additional 10% overage.

- PCR1 (4.7 µL)
- FEM (0.3 µL)
- DPMA or DPMB (5 µL)
- Nuclease-free water (3 µL)

For example, for 8 samples, prepare 114.4 µL master mix: 41.4 µL PCR1, 2.6 µL FEM, 44 µL DPMA or DPMB, and 26.4 µL nuclease-free water.

2. Pipette to mix, and then cap and centrifuge briefly.
3. **Optional**: Evenly distribute the master mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.
4. Add 13 µL master mix to each well of the FSP.
5. In a new 1.5 mL tube, combine the following volumes to dilute NA24385 :
    - NA24385 (2 µL)
    - Nuclease-free water (38 µL)
6. Cap and gently invert 3 times to mix, and then centrifuge briefly.
7. Add 2 µL diluted NA24385 to at least one well of the FSP as a positive template control.
8. Add 2 µL nuclease-free water to at least one well of the FSP as a negative template control.
9. Add 2 µL diluted crude lysate to each remaining well of the FSP.
10. Seal and centrifuge at 100 × *g* for 30 s.
11. Transport to the post-PCR area.
12. Place on the preprogrammed thermal cycler and run the Sig Plus PCR1 program.

13. Unless you are stopping, proceed to “Enrich targets” on page 32.

Safe stopping point

If you are stopping, seal the plate with Seal ”B” and store at 2–8°C for up to 2 days. Alternatively, leave the plate in the thermal cycler overnight.

FTA Card

1. Place a 1.2 mm FTA card punch into each well of the FSP.
2. Add 100 µL 1X TBE Buffer.
3. Place on a PCR tube storage rack.
4. Seal with microseal ‘B’ film and shake at 1800 rpm for 2 min.
5. Centrifuge at 1000 × *g* for 30 s.
6. Remove and discard all supernatant.
7. Add the following volumes to each control well of the FSP:
    - PCR1 (4.7 µL)
    - FEM (0.3 µL)
    - DPMA or DPMB (5 µL)
8. In a 1.5 mL tube, combine the following volumes to dilute NA24385:
    - NA24385 (2 µL)
    - Nuclease-free water (98 µL)
9. Cap and gently invert 3 times to mix, and then centrifuge briefly.
10. Add 5 µL diluted NA24385 to the positive amplification control wells.
11. Pipette to mix.

12. Add 5 µL nuclease-free water to the negative amplification control wells.
13. Pipette to mix.
14. In the FTA Master Mix tube, combine the following volumes. Multiple each volume by the number of samples and add 10% for overage.

    Note: Reagent fill volume listed on the tube label represents the volume required for master mix. This volume **does not** take into consideration that the vial is filled with an additional 10% overage.

    - PCR1 (4.7 µL)
    - FEM (0.3 µL)
    - DPMA or DPMB (5 µL)
    - Nuclease-free water (5 µL)

    For example, for 8 samples, prepare 88 µL master mix: 41.4 µL PCR1, 2.6 µL FEM, and 44 µL DPMA or DPMB.
15. Pipette to mix, and then seal and centrifuge briefly.
16. **Optional**: Evenly distribute FTA Master Mix among each well of an 8-tube strip. Use a multichannel pipette to dispense.
17. Add 15 µL FTA Master Mix to each well of the FSP that contains an FTA card punch.
18. Seal with microseal 'A' film, and then centrifuge at 100 × *g* for 30 s.
19. Transport to the post-PCR area.
20. Place on the preprogrammed thermal cycler and run the Sig Plus PCR1 program.
21. Unless you are stopping, proceed to "Enrich targets" (next section).

Safe stopping point

If you are stopping, seal the plate with Seal "B" and store at 2–8°C for up to 2 days. Alternatively, leave the plate in the thermal cycler overnight.

Protocol step 2: Enrich targets

This process amplifies the DNA and adds the UDI adapters, and the sequences required for cluster generation. The index adapters tag the DNA with a unique combination of sequences that identify each sample for analysis.

Note: When preparing 8 samples, you can perform this process using an 8-tube strip instead of the 96-well PCR plate.

Consumables

- UDI plate
- PCR2 (PCR2 Reaction Mix)
- Microseal 'A' film

About reagents

- Dispense PCR2 slowly to prevent bubbles.

Preparation

1. Prepare the following consumables:

Item	Storage temperature (°C)	Instructions
UDI plate	–25 to –15	Thaw at room temperature. Mix briefly on plate shaker at 1800 rpm, and then centrifuge briefly.
PCR2	–25 to –15	Thaw at room temperature for 20 min, and then invert to mix.

2. Save the following PCR2 program (example program name: Sig Plus PCR2) on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 98°C for 30 s
   - 15 cycles of:
     - 98°C for 20 s
     - 66°C for 30 s
     - 68°C for 90 s
   - 68°C for 10 min
   - Hold at 10°C

   Total program time is approximately 46 min.

Procedure

1. Centrifuge the sealed FSP at 100 × *g* for 30 s.
2. Remove the protective cover from the UDI plate and discard appropriately.
3. Centrifuge at 100 × *g* for 30 s.

4. Using a new pipette tip for each well, pierce the foil covering of the UDI plate and transfer 8 µL UDI adapter to each sample well in the FSP. See Table 4 on the facing page Table 4 for the UDI Adapter Plate layout. When finished with UDI plate, cover processed UDI wells with microseal “B” adhesive seal. Trim excess with scissors.

   Reseal the pierced wells of the UDI plate.

Table 4. UDI plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	UDI0001	UDI0002	UDI0003	UDI0004	UDI0005	UDI0006	UDI0007	UDI0008	UDI0009	UDI0010	UDI0011	UDI0012
B	UDI0013	UDI0014	UDI0015	UDI0016	UDI0017	UDI0018	UDI0019	UDI0020	UDI0021	UDI0022	UDI0023	UDI0024
C	UDI0025	UDI0026	UDI0027	UDI0028	UDI0029	UDI0030	UDI0031	UDI0032	UDI0033	UDI0034	UDI0035	UDI0036
D	UDI0037	UDI0038	UDI0039	UDI0040	UDI0041	UDI0042	UDI0043	UDI0044	UDI0045	UDI0046	UDI0047	UDI0048
E	UDI0049	UDI0050	UDI0051	UDI0052	UDI0053	UDI0054	UDI0055	UDI0056	UDI0057	UDI0058	UDI0059	UDI0060
F	UDI0061	UDI0062	UDI0063	UDI0064	UDI0065	UDI0066	UDI0067	UDI0068	UDI0069	UDI0070	UDI0071	UDI0072
G	UDI0073	UDI0074	UDI0075	UDI0076	UDI0077	UDI0078	UDI0079	UDI0080	UDI0081	UDI0082	UDI0083	UDI0084
H	UDI0085	UDI0086	UDI0087	UDI0088	UDI0089	UDI0090	UDI0091	UDI0092	UDI0093	UDI0094	UDI0095	UDI0096

5. Briefly centrifuge PCR2, and then pipette to mix.
6. **Optional**: Evenly distribute PCR2 among each tube of an 8-tube strip. Use a multichannel pipette to dispense.
7. Add 27 µL PCR2 to each well. Pipette to mix.
8. Seal and centrifuge at 100 × *g* for 30 s.
9. Place on the preprogrammed thermal cycler and run the Sig Plus PCR2 program.

Safe stopping point

If you are stopping, seal the plate with Seal "B" and store at 2–8°C for up to 7 days. Alternatively, leave the plate in the thermal cycler overnight.

Protocol step 3: Purify libraries

This process uses purification beads to purify the amplified libraries from other reaction components.

Consumables

- RSB (Resuspension Buffer)
- SPB2 (Sample Purification Beads 2)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plate
- 96-well PCR plate, skirted or semiskirted
- PVC reagent reservoir
- Microseal 'B' film

Preparation

1. Prepare the following consumables:

Item	Storage temperature (°C)	Instructions
RSB	2–8	Let stand for 30 min to bring to room temperature. Vortex and invert to mix.
SPB2	2–8	Let stand for 30 min to bring to room temperature. Vortex for at least 1 min and invert to mix.

2. Label plates as follows.

Plate type	Label
Midi	PBP (Purification Bead Plate)
PCR	PLP (Purified Library Plate)

3. Add the calculated volume to the applicable vessel:

Number of libraries	Vessel
<16	1.5 mL tube
16–96	Reagent reservoir or each well in a column of a new midi plate
>96	Reagent reservoir

Procedure

1. Add 45 µL SPB2 to each well of the PBP.
2. Centrifuge the sealed FSP at 100 × *g* for 30 s.
3. Transfer 45 µL reaction from each well of the FSP to the corresponding well of the PBP.
4. Discard the FSP plate.
5. Seal the PBP and shake at 1800 rpm for 2 min.
6. Incubate at room temperature for 5 min.
7. Place on the magnetic stand and wait until the liquid is clear (approximately 5 min).
8. Remove and discard all supernatant.
9. Keep on the magnetic stand and wash as follows.
    a. Add 200 µL fresh 80% EtOH to each well.
    b. Incubate for 30 s.
    c. Remove and discard all supernatant.

10. Wash a **second** time.
11. Seal and centrifuge at 100 × *g* for 30 s.
12. Place on the magnetic stand.
13. With a 20 µL pipette, remove residual EtOH from each well.
14. Remove from the magnetic stand.
15. Add 52.5 µL RSB to each well.
16. Seal and shake at 1800 rpm for 2 min.
17. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 2 min.
18. Incubate at room temperature for 2 min.
19. Place on the magnetic stand and wait until the liquid is clear (approximately 5 min).
20. Transfer 50 µL supernatant from each well of the PBP to the corresponding well of the PLP.
21. Seal and centrifuge at 100 × *g* for 30 s.

Safe stopping point

If you are stopping, seal the plate with seal 'B' and store at –25°C to –15°C for up to 1 year.

Protocol step 4: Normalize libraries

This process normalizes the concentration of each library for even representation without post-PCR quantification and individual normalization. Samples of varying types and input amounts achieve consistent cluster density, optimizing the resolution of each library in the pool.

Consumables

- HP3 (2 N NaOH)
- LNA1 (Library Normalization Additives 1)

- LNB1 (Library Normalization Beads 1)
- LNS2 (Library Normalization Storage Buffer 2)
- LNW1 (Library Normalization Wash 1)
- Nuclease-free water
- One of the following tubes:
    - 1.5 mL microcentrifuge tube
    - 15 mL conical tube
- 96-well midi plate
- 96-well PCR plate, skirted or semiskirted
- PVC reagent reservoir
- Microseal 'B' film

About reagents

The volumes combined in the LNA1/LNB1 Master Mix tube and the 0.1 N HP3 tube include overage, so calculating additional overage is not necessary.

Important: This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For complete environmental, health, and safety information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety** where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Preparation

1. Prepare the following consumables:

Item	Storage temperature (°C)	Instructions
HP3	–25 to –15	Thaw at room temperature for ≥30 min. Vortex to mix, and then centrifuge briefly.
LNA1	–25 to –15	Thaw at room temperature for ≥30 min. Vortex with intermittent inversion.
LNB1	2–8	Let stand for 30 min to bring to room temperature. Vortex for at least 1 min, inverting 5 times every 15 s. Pipette to mix until the bead pellet at the bottom is resuspended.
LNW1	2–8	Let stand for 30 min to bring to room temperature.
LNS2	15–30	Remove from storage.

2. Label vessels as follows.

Vessel type	Label
1.5 mL tube or 15 mL conical tube	LNA1/LNB1 Master Mix
1.5 mL tube or 15 mL conical tube	0.1 N HP3
Midi plate	NWP for Normalization Working Plate
PCR plate	NLP for Normalization Library Plate

3. Dedicate separate hazardous waste disposal containers for liquids and solids.

Procedure

1. In the LNA1/LNB1 Master Mix tube, combine the following volumes. Multiply each volume by the number of samples, but do not add overage.

- LNA1 (46.8 µL)
- LNB1 (8.5 µL)

  For example, for 8 samples, combine 374.4 µL LNA1 and 68 µL LNB1.

2. Vortex, and then invert several times to mix.
3. Transfer the entire volume to a reagent reservoir.
4. Add 45 µL LNA1/LNB1 Master Mix to each sample well of the NWP.
5. To clear any beads that might have aspirated, place the PLP on the magnetic stand and wait until the liquid is clear (approximately 2 min).
6. Transfer 20 µL supernatant from each well of the PLP to the corresponding well of the NWP.
7. Seal the NWP and shake at 1800 rpm for 30 min.
8. While the plate is shaking, perform steps 9–11 to save time later in the process.
9. In the 0.1 N HP3 tube, combine the following volumes. Multiply each volume by the number of samples, but do not add overage.
   - Nuclease-free water (33.3 µL)
   - HP3 (1.8 µL)

   For example, 8 samples require 266.4 µL nuclease-free water and 14.4 µL HP3.
10. Invert several times to mix, and then set aside.
11. Add 30 µL LNS2 to each sample well of the NLP.
12. Immediately after shaking, place the NWP on the magnetic stand and wait until the liquid is clear (approximately 2 min).
13. Remove and discard all supernatant.
14. Remove from the magnetic stand.

15. Wash as follows.
    a. Add 45 µL LNW1 to each well.
    b. Seal and shake at 1800 rpm for 5 min.
    c. Place on the magnetic stand and wait until the liquid is clear (approximately 2 min).
    d. Remove and discard all supernatant.
    e. Remove from the magnetic stand.
16. Wash a **second** time.
17. Seal and centrifuge at 100 × *g* for 30 s.
18. Place on the magnetic stand and wait until the liquid is clear (approximately 2 min).
19. With a 20 µL pipette, remove residual LNW1 from each well.
20. Remove from the magnetic stand.
21. Add 32 µL freshly prepared 0.1 N HP3 to each well.
22. Seal and shake at 1800 rpm for 5 min.
23. If the beads are not fully resuspended, pipette to mix or reshake at 1800 rpm for 5 min.
24. Place on the magnetic stand and wait until the liquid is clear (approximately 2 min).
25. Transfer 30 µL supernatant from the NWP to the corresponding well of the NLP.
26. Pipette to mix.
27. Seal and centrifuge at 100 × *g* for 30 s.

Safe stopping point

If you are stopping, seal the plate with seal 'B' and store at –25°C to –15°C for up to 30 days.

Protocol step 5: Pool libraries

This process combines equal volumes of each normalized library to create a pool of libraries that are sequenced together on the same flow cell.

Consumables

- 1.5 mL microcentrifuge tube
- RNase/DNase-free 8-tube strip and caps
- Microseal 'B' film

Preparation

1. Select libraries to pool for sequencing. For recommendations, see "Number of samples" on page 21.
2. Label the 1.5 mL tube PNL for Pooled Normalized Libraries.

Procedure

1. Using a multichannel pipette, transfer 5 µL of each library to a new 8-tube strip.
2. Seal the NLP and store in the post-PCR area at –25°C to –15°C for ≤30 days.
3. Transfer libraries from each well of the 8-tube strip to the PNL tube.
4. Cap and vortex to mix, and then centrifuge briefly.

Safe stopping point

If you are stopping, cap the tube and store at –25°C to –15°C for up to 30 days.

Protocol step 6: Denature and dilute libraries

This process dilutes libraries to the loading concentration, adds a sequencing control, and uses a heat-based method to denature the libraries for sequencing.

Note: Start this process when you are ready to prepare sequencing reagents and set up the run. Delays can impact template loading.

Consumables

- HP3 (2 N NaOH)
- HSC (Human Sequencing Control)
- MiSeq FGx Reagent Kit contents:
    - HT1 (Hybridization Buffer)
    - Reagent cartridge
- Nuclease-free water
- Pooled libraries
- 1.5 mL microcentrifuge tubes (2)

Preparation

1. Prepare the following consumables:

Item	Storage temperature (°C)	Instructions
HP3	–25 to –15	Thaw at room temperature for ≥30 min, and then centrifuge briefly.
HSC	–25 to –15	Let stand for 30 min to bring to room temperature. Invert to mix, and then centrifuge.
HT1	–25 to –15	Thaw at room temperature, and then vortex to mix.
Reagent cartridge	–25 to –15	Thaw in a water bath at room temperature.

2. Preheat the microheating system to 96°C.
3. Label 2 new 1.5 mL tubes:
    - Denatured HSC
    - DNL for Denatured Normalized Libraries

Procedure

1. In the Denatured HSC tube, combine the following volumes:
    - HSC (2 µL)
    - HP3 (2 µL)
    - Nuclease-free water (36 µL)
2. Pipette gently to mix. Cap and centrifuge briefly to mix.
3. Incubate at room temperature for 5 min.
4. Add 591 µL HT1 to the DNL tube.

5. Place the PNL tube in the preheated microheating system and incubate for 2 min.
6. Immediately transfer 7 µL library from the PNL tube to the DNL tube.
7. Pipette to mix.
8. Cap the PNL tube and store at –25°C to –15°C for ≤30 days. Exceeding 30 days significantly reduces cluster density.
9. Add 4 µL denatured HSC to the DNL tube.
10. Pipette to mix. You can store the denatured HSC at room temperature for ≤1 day.
11. Cap and vortex to mix, and then centrifuge briefly.
12. Immediately transfer the entire volume to the reagent cartridge per instructions in the *MiSeq FGx Sequencing System Reference Guide* (document # VD2018006).

Appendix: Amplicon Information

Loci detected with DPMA and DPMB

The following tables list loci detected with DPMA or DPMB. **Loci in the piSNPs and aiSNPs are exclusive** to DPMB. All other loci are detected with both primer mixes.

- Amplicon lengths exclude 120 bp for adapter sequences.
- Amplicon start and end positions are the one-base endpoints of the entire amplicon, including the sequence that matches primers on the hg19 human reference genome.
- Amelogenin is a genetic marker that confirms the gender of the biological sample donor. The size range is 106–112 bp and the control DNA is male.

Autosomal STRs

Locus	Repeats	Amplicon length (bp)	Chromosome	NA24385 Control Alleles
D1S1656	7–21.3	133–192	1	13,14
TPOX	4–16	61–109	2	8,8
D2S441	7–17	137–177	2	11,15
D2S1338	10–33.1	110–203	3	22,24
D3S1358	8–22	138–194	3	15,16
D4S2408	8–13	98–118	4	8,9
FGA	12.2–53	150–312	4	20, 23
D5S818	4–20	98–162	5	11,12
CSF1PO	5–17	72–120	5	10,12
D6S1043	8–26	154–226	6	11,14

Locus	Repeats	Amplicon length (bp)	Chromosome	NA24385 Control Alleles
D7S820	5–21.1	118–183	7	11,12
D8S1179	6–20	82–138	8	13,16
D9S1122	8–15	104–132	9	12,12
D10S1248	7–20	124–176	10	14,16
TH01	3–14	96–140	11	9,9.3
vWA	11–26	135–195	12	16,18
D12S391	13–28	229–289	12	22,22
D13S317	5–17	138–186	13	11,13
PentaE	5–28.4	362–481	15	10,18
D16S539	4–17	132–184	16	11,11
D17S1301	9–15	130–154	17	11,11
D18S51	6–40	136–272	18	13,16
D19S433	4–27	148–240	19	14,16.2
D20S482	9–17	125–157	20	14,14
D21S11	12–41.2	147–265	21	30,31.2
PentaD	1.1–19	209–298	21	9,12
D22S1045*	8–19	201–245	22	16,16

* Interpret with caution. See “Interpreting locus D22S1045” on page 58 for more information.

X-STRs

Locus	Repeats	Amplicon length (bp)	Chromosome	NA24385 Control Alleles
DXS10074	7–22	184–244	X	17
DXS10103	14–21	157–185	X	18

Locus	Repeats	Amplicon length (bp)	Chromosome	NA24385 Control Alleles
DXS10135	15.3–34	239–312	X	19
DXS7132	11–20	175–211	X	13
DXS7423	10–18	188–220	X	15
DXS8378	8–14	434–458	X	12
HPRTB	8–17	193–229	X	13

Y-STRs

Locus	Repeats	Amplicon length (bp)	Chromosome	NA24385 Control Alleles
DYF387S1	30–44	207–263	Y	38,40
DYS19	9–19	269–309	Y	14
DYS385a-b	7–28	232–316	Y	16,18
DYS389I	9–17	236–268	Y	13
DYS389II	24–34	283–323	Y	29
DYS390	17–28	290–334	Y	25
DYS391	5–16	119–163	Y	10
DYS392*	6–17	318–362	Y	13
DYS437	10–18	194–226	Y	14
DYS438	6–16	129–179	Y	11
DYS439	6–17	167–211	Y	10
DYS448	14–26	330–402	Y	20
DYS460	7–14	348–376	Y	11
DYS481	17–32	129–174	Y	26
DYS505	9–15	162–186	Y	13

Locus	Repeats	Amplicon length (bp)	Chromosome	NA24385 Control Alleles
DYS522	8–17	298–334	Y	12
DYS533	7–17	186–226	Y	12
DYS549	10–14	210–226	Y	12
DYS570	10–26	142–206	Y	18
DYS576	10–25	163–223	Y	18
DYS612	26–33	275–296	Y	37
DYS635	15–30	242–302	Y	21
DYS643	7–15	141–181	Y	9
Y-GATA-H4	8–15	159–187	Y	10

* Interpret with caution. See "Interpreting locus DYS392" on page 61 for more information.

Identity-informative SNPs

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs10495407	109	1	238439234	238439342	GA
rs1294331	85	1	233448359	233448443	CT
rs1413212	64	1	242806767	242806830	TC
rs1490413	98	1	4367256	4367353	AA
rs560681	90	1	160786641	160786730	AG
rs891700	115	1	239881850	239881964	GG
rs1109037	118	2	10085691	10085808	GA
rs12997453	100	2	182413195	182413294	AG
rs876724	119	2	114945	115063	CT
rs907100	115	2	239563542	239563656	GG

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs993934	120	2	124109120	124109239	AG
rs1355366	119	3	190806041	190806159	CC
rs1357617	120	3	961696	961815	AT
rs2399332	157	3	110300999	110301155	TT
rs4364205	98	3	32417576	32417673	GG
rs6444724	120	3	193207306	193207425	TC
rs1979255	102	4	190318007	190318108	GG
rs2046361	120	4	10968994	10969113	TT
rs279844	167	4	46329584	46329750	AA
rs6811238	120	4	169663541	169663660	GG
rs13182883	169	5	136633252	136633420	AG
rs159606	104	5	17374845	17374948	GG
rs251934	97	5	174778619	174778715	TT
rs338882	157	5	178690599	178690755	GA
rs717302	110	5	2879333	2879442	GA
rs13218440	170	6	12059928	12060097	GG
rs1336071	120	6	94537182	94537301	CC
rs214955	120	6	152697629	152697748	CC
rs727811	115	6	165045254	165045368	GG
rs321198	165	7	137029715	137029879	CC
rs6955448	120	7	4310285	4310404	CT
rs737681	120	7	155990742	155990861	TC

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs917118	109	7	4456953	4457061	CC
rs10092491	116	8	28411037	28411152	CT
rs2056277	104	8	139399038	139399141	CC
rs4606077	151	8	144656710	144656860	CC
rs763869	85	8	1375576	1375660	GG
rs1015250	117	9	1823702	1823818	GC
rs10776839	103	9	137417271	137417373	GG
rs1360288	119	9	128967994	128968112	CC
rs1463729	99	9	126881396	126881494	CT
rs7041158	115	9	27985907	27986021	TT
rs3780962	94	10	17193284	17193377	AG
rs735155	170	10	3374133	3374302	CC
rs740598	120	10	118506839	118506958	AG
rs826472	153	10	2406511	2406663	CC
rs964681	105	10	132698394	132698498	TT
rs10488710	118	11	115207134	115207251	CG
rs1498553	111	11	5708981	5709091	CT
rs2076848	118	11	134667502	134667619	TT
rs901398	90	11	11096173	11096262	TT
rs10773760	99	12	130761623	130761721	AA
rs2107612	103	12	888262	888364	AA
rs2111980	94	12	106328186	106328279	CC

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs2269355	65	12	6945881	6945945	CC
rs2920816	157	12	40862976	40863132	GG
rs1058083	76	13	100038193	100038268	AG
rs1335873	109	13	20901665	20901773	AA
rs1886510	116	13	22374646	22374761	GA
rs354439	170	13	106938320	106938489	TT
rs1454361	118	14	25850765	25850882	TT
rs4530059	170	14	104769099	104769268	GA
rs722290	101	14	53216686	53216786	GC
rs873196	114	14	98845506	98845619	CT
rs1528460	115	15	55210664	55210778	CT
rs1821380	118	15	39313343	39313460	CG
rs8037429	63	15	53616876	53616938	TT
rs1382387	89	16	80106318	80106406	AA
rs2342747	104	16	5868645	5868748	AG
rs430046	119	16	78016980	78017098	CT
rs729172	104	16	5606153	5606256	GG
rs740910	113	17	5706552	5706664	AA
rs8078417	143	17	80461847	80461989	CC
rs938283	98	17	77468433	77468530	TT
rs9905977	170	17	2919324	2919493	GG
rs1024116	98	18	75432317	75432414	TT

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs1493232	75	18	1127945	1128019	AA
rs1736442	153	18	55225698	55225850	TC
rs9951171	119	18	9749789	9749907	AA
rs576261	76	19	39559780	39559855	CC
rs719366	170	19	28463281	28463450	GA
rs1005533	158	20	39487066	39487223	GA
rs1031825	126	20	4447416	4447541	CC
rs1523537	117	20	51296076	51296192	CC
rs445251	119	20	15124865	15124983	GG
rs221956	97	21	43606933	43607029	TC
rs2830795	114	21	28608089	28608202	AG
rs2831700	79	21	29679639	29679717	GG
rs722098	101	21	16685561	16685661	AA
rs914165	156	21	42415865	42416020	GG
rs1028528	78	22	48362256	48362333	AG
rs2040411	68	22	47836378	47836445	GA
rs733164	120	22	27816711	27816830	AG
rs987640	120	22	33559450	33559569	TT

Phenotypic-informative SNPs

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs28777	92	5	33958916	33959007	AA
rs12203592	110	6	396273	396382	CC
rs4959270	161	6	457655	457815	AC
rs683	120	9	12709246	12709365	AA
rs1042602	113	11	88911659	88911771	AC
rs1393350	99	11	89010977	89011075	GG
rs12821256	119	12	89328278	89328396	TT
rs12896399	73	14	92773627	92773699	GT
rs2402130	120	14	92801169	92801288	AA
rs1800407	119	15	28230246	28230364	CC
N29insA	112	16	89985688	89985799	CC
rs1110400	173	16	89986044	89986216	TT
rs11547464	173	16	89986044	89986216	GG
rs1805005	213	16	89985774	89985986	GG
rs1805006	213	16	89985774	89985986	CC
rs1805007	173	16	89986044	89986216	CC
rs1805008	173	16	89986044	89986216	CC
rs1805009	227	16	89986484	89986710	GG
rs201326893_ Y152OCH	173	16	89986044	89986216	CC
rs2228479	213	16	89985774	89985986	AA
rs885479	173	16	89986044	89986216	AA
rs2378249	118	20	33218028	33218145	AA

Ancestry-informative SNPs

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs2814778	120	1	159174650	159174769	TT
rs3737576	98	1	101709521	101709618	TT
rs7554936	106	1	151122413	151122518	TT
rs10497191	101	2	158667153	158667253	CC
rs1834619	84	2	17901444	17901527	GG
rs1876482	120	2	17362526	17362645	GG
rs260690	115	2	109579681	109579795	AA
rs3827760	108	2	109513546	109513653	AA
rs6754311	98	2	136707920	136708017	CC
rs798443	84	2	7968221	7968304	AA
rs12498138	119	3	121459545	121459663	GG
rs1919550	117	3	121364112	121364228	AA
rs1229984	120	4	100239288	100239407	CC
rs3811801	114	4	100244261	100244374	GG
rs4833103	95	4	38815462	38815556	CC
rs7657799	116	4	105375396	105375511	TT
rs7722456	114	5	170202901	170203014	TT
rs870347	119	5	6844995	6845113	AA
rs16891982*	108	5	33951621	33951728	GG
rs192655	70	6	90518235	90518304	AA
rs3823159	119	6	136482701	136482819	AA

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs917115	71	7	28172543	28172613	TC
rs1462906	84	8	31896545	31896628	CC
rs1871534	71	8	145639652	145639722	GG
rs2196051	120	8	122124216	122124335	AG
rs6990312	111	8	110602270	110602380	GT
rs3814134	104	9	127267664	127267767	AA
rs4918664	168	10	94920962	94921129	AA
rs1079597	167	11	113296227	113296393	CC
rs174570	120	11	61597179	61597298	CC
rs2238151	113	12	112211753	112211865	CT
rs671	136	12	112241658	112241793	GG
rs1572018	116	13	41715225	41715340	TC
rs2166624	71	13	42579949	42580019	GG
rs7326934	96	13	49070482	49070577	GG
rs7997709	85	13	34847693	34847777	TT
rs9522149	119	13	111827125	111827243	CC
rs200354	165	14	99375246	99375410	GG
rs12439433	100	15	36219979	36220078	AG
rs1426654	92	15	48426457	48426548	AA
rs1800414	116	15	28196969	28197084	TT
rs735480	108	15	45152321	45152428	TT
rs12913832*	119	15	28365523	28365641	GG

Locus	Amplicon length (bp)	Chromosome	Amplicon start position	Amplicon end position	NA24385 Control Alleles
rs459920	78	16	89730800	89730877	TC
rs11652805	119	17	62987113	62987231	TT
rs17642714	118	17	48726060	48726177	AT
rs2593595	102	17	41056210	41056311	AA
rs4411548	158	17	40658440	40658597	CT
rs4471745	67	17	53568849	53568915	GG
rs2042762	83	18	35277568	35277650	TT
rs3916235	120	18	67578894	67579013	TC
rs4891825	106	18	67867615	67867720	GA
rs7226659	149	18	40488180	40488328	GG
rs7251928	200	19	4077044	4077243	AC
rs310644	89	20	62159472	62159560	TC
rs2024566	88	22	41697312	41697399	AA

* Also used for phenotype prediction.

Interpreting locus D22S1045

The following sections provide example interpretation methods to help interpret the aSTR locus D22S1045. Determine actual values and methods based on application and internal validation data.

Locus D22S1045 might indicate elevated *n* – 1 repeat stutter, particularly with decreased marker coverage. Heterozygote imbalance might occur regardless of marker coverage. When determining the presence of a DNA mixture, consider multilocus genotype.

Data trends for D22S1045

Elevated *n* – 1 stutter can occur in low coverage situations, particularly for stutter in STR positions and lengths ≥15. Stutter percentages increase as coverage decreases, and in extreme cases can approach or surpass the read depth of the parent allele.

Heterozygote imbalance can occur at low or high locus coverage. Imbalance increases with a larger spread between allele lengths (for example, 11,18). The following figure shows progressively decreasing intralocus balance (allele count ratio [ACR]) as the allele number spread increases.

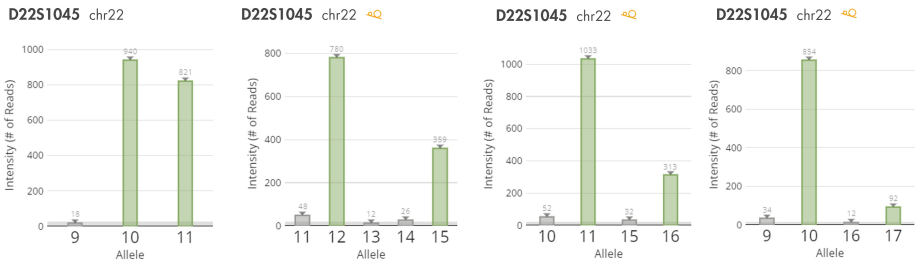

Figure 2. Decreasing intralocus balance.

Genotype determination at D22S1045

The following flowcharts illustrate example methods of genotype determination for locus D22S1045. The read values in each flowchart are intended as conservative examples that demonstrate an interpretation method using specific read level guidelines. Base actual values and methods on laboratory application and internal validation data.

One typed allele

In this hypothetical example allele H, where H is the true call, and INC is an inconclusive result. An inconclusive result is a conservative conclusion that eliminates inadvertently typing

stutter position. The asterisk (*) accounts for potential drop-out due to imbalance.

Figure 3. Flowchart for one typed allele.

Two typed alleles

In the following figure, H is the allele with highest number of reads and L is the allele with lower number of reads. An inconclusive result (INC) is a conservative conclusion that eliminates the chance of accidentally typing stutter position when two potential alleles are present with <150 available reads.

H,L indicates a true allele and L < 50 reads outside the *n* – 1 position = obligate sister. H,* indicates a true allele, and L <50 reads or L in the *n* – 1 position might be elevated stutter.

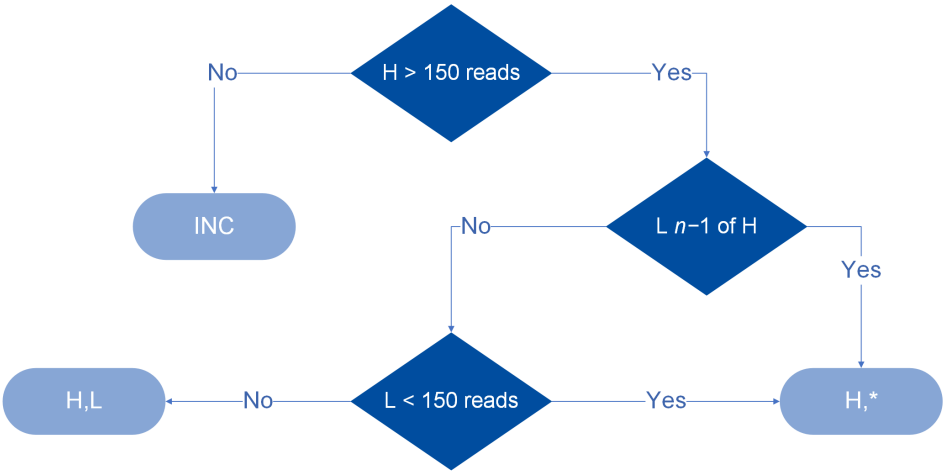

Figure 4. Flowchart for two typed alleles.

Interpreting locus DYS392

The following sections provide example interpretation methods to help interpret the Y-STR locus DYS392. Determine actual values and methods based on application and internal validation data.

Locus DYS392 might indicate elevated *n* – 1 repeat stutter, particularly with decreased marker coverage. Consider multilocus genotype when determining the presence of a DNA mixture.

Data trends for DYS392

Elevated *n* – 1 stutter can occur when locus coverage is low. Stutter increases as coverage decreases, and in extreme cases can approach or surpass the read depth of the parent allele.

Genotype determination at DYS392

The following flowcharts illustrate example methods of genotype determination for locus DYS392. The read values in each flowchart are intended as conservative examples that demonstrate an interpretation method using specific read level guidelines. Base actual values and methods on laboratory application and internal validation data.

One typed allele

In the following figure, H is the example allele, H is a true allele, and INC indicates an inconclusive result. An inconclusive result is a conservative conclusion that eliminates inadvertently typing stutter position.

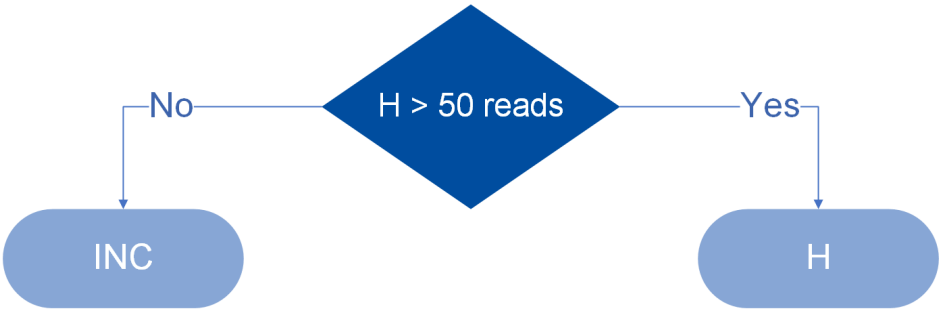

Figure 5. Flowchart for one typed allele.

Stutter position typing

In the following figure, H is the allele with the highest number of reads and L is the allele with the lowest number of reads. INC is an inconclusive result and conservative conclusion to eliminate accidentally typing stutter position. A conclusion of H or L indicates the potential for either to be elevated stutter. A conclusion of H is a true allele, even with L at a high *n* – 1 stutter percent.

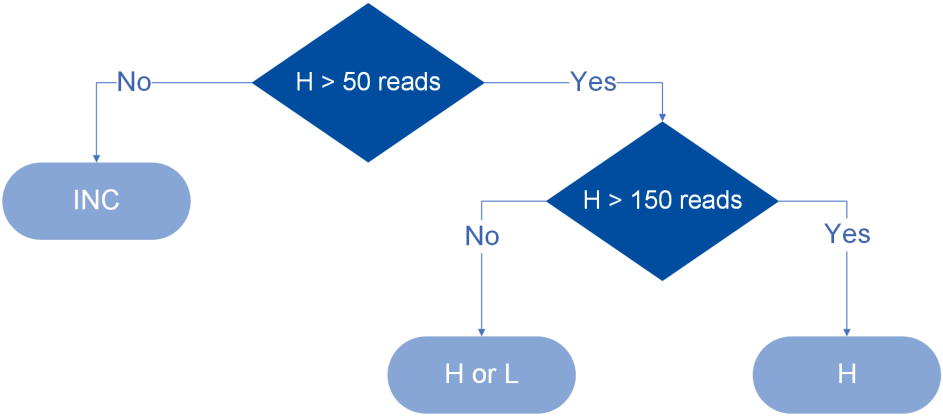

Figure 6. Flowchart for *n* – 1 stutter position typing with parent allele.

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Document Revision History

Date	Changes
10/2024	Initial release

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