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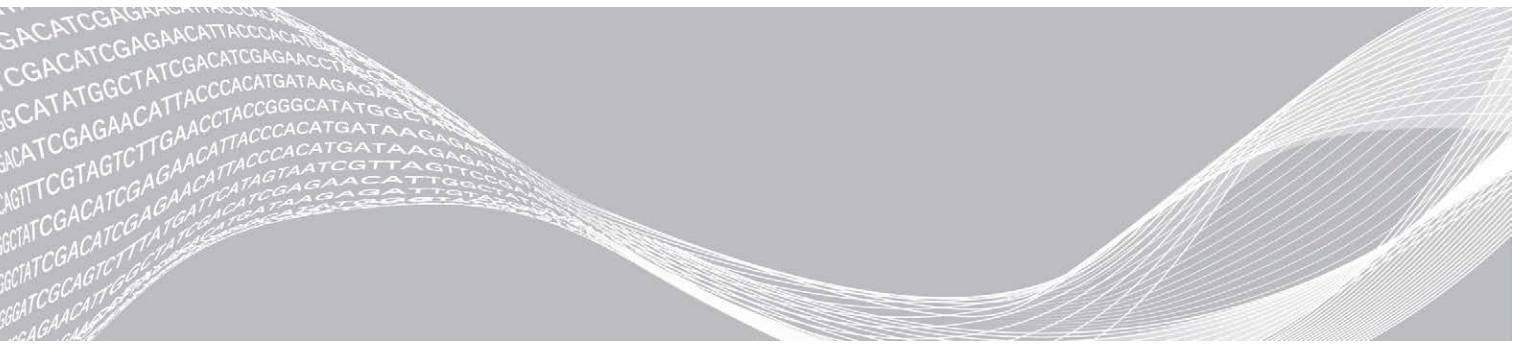
TruSeq Genotype N_e

Reference Guide



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Chapter 1 Overview

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Introduction

Use this protocol to prepare up to 384 uniquely indexed libraries using the Illumina® TruSeq® Genotype N_e kit for targeted genotyping by sequencing. The kit supports genotyping by sequencing up to 5000 targets, including single nucleotide polymorphisms (SNPs) and small indels. This targeted approach allows a wide range of applications, such as parentage assays, purity testing, and molecular breeding.

The Genotype N_e kit offers:

- Reliable identification of SNPs and small indels.
- Streamlined 96-well based workflow with less than 3 hours of hands-on time.
- Bead-based purification, which allows an automation-friendly workflow.
- Custom panels of up to 5000 markers for targeted genotyping of any plant or animal species.
- Compatibility with the MiniSeq™, MiSeq®, and NextSeq® systems for high-quality sequencing results.

DNA Input Recommendations

Quantify the input DNA and assess the DNA quality before beginning library prep.

DNA Type	Supported Amplicon Size	Input
Genomic DNA	150 bp, 175 bp	50 ng

Input DNA Dilution

Quantify and dilute the DNA to the input amount of 50 ng. You can dilute and store more than the required DNA for later use. Dilute DNA in RS1 and SS1 and store as described in *Quantify and Dilute DNA* on page 6.

Input DNA Quantification

Quantify the starting genomic material using a fluorescence-based quantification method, such as a Qubit dsDNA Assay Kit or PicoGreen. Do not use a UV-spectrometer-based method.

Fluorescence-based methods employ a dye specific to double-stranded DNA (dsDNA). They specifically and accurately quantify dsDNA, even when many common contaminants are present. UV spectrometer methods based on 260 OD readings can overestimate DNA concentrations due to the presence of RNA and other contaminants common to gDNA preparations.

Additional Resources

Visit the TruSeq Genotype N_e kit support page on the Illumina website for documentation, software downloads, training resources, and information about compatible Illumina products.

The following documentation is available for download from the Illumina website.

Resource	Description
Custom Protocol Selector	support.illumina.com/custom-protocol-selector.html A wizard for generating customized end-to-end documentation that is tailored to the library prep method, run parameters, and analysis method used for the sequencing run.
TruSeq Genotype Ne Checklist (document # 1000000033140)	Provides a checklist of steps for the experienced user.
TruSeq Genotype Ne Consumables and Equipment List (document # 1000000033141)	Provides an interactive checklist of user-provided consumables and equipment.

For information on the sequencing reagents provided with the TruSeq Genotype Ne kit, see the system guide for your instrument.

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Introduction

This section describes the TruSeq Genotype N_e protocol.

- Before proceeding, confirm the kit contents and make sure that you have the required consumables and equipment.
  - This protocol requires different magnetic stands for pre-PCR and post-PCR procedures.
  - Although the kit includes Amplicon Control Oligo Pool 3 (ACP3), use of a control is not supported so this reagent is not used.
- Follow the protocol in the order described using the specified parameters.
- Prepare up to 384 samples in batches of 96 samples.

Prepare for Pooling

If you plan to pool libraries, record information about your samples before beginning library prep. For more information, see the TruSeq Genotype N_e support page.

Review *Nextera Low Plex Pooling Guidelines (Pub. No. 770-2011-044)* when preparing libraries for Illumina sequencing systems that require balanced index combinations.

Tips and Techniques

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

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Vortexing steps
  - Centrifuge steps
  - Thermal cycling steps

- Apply the adhesive seal to cover the plate, and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' for shaking, centrifuging, and long-term storage.
- Microseal 'A' adhesive film is used for thermal cycling steps to prevent evaporation.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.

Centrifugation

- Centrifuge at any step in the procedure to consolidate liquid or beads in the bottom of the well, and to prevent sample loss.
  - To pellet beads, centrifuge at 280 × g for 1 minute.

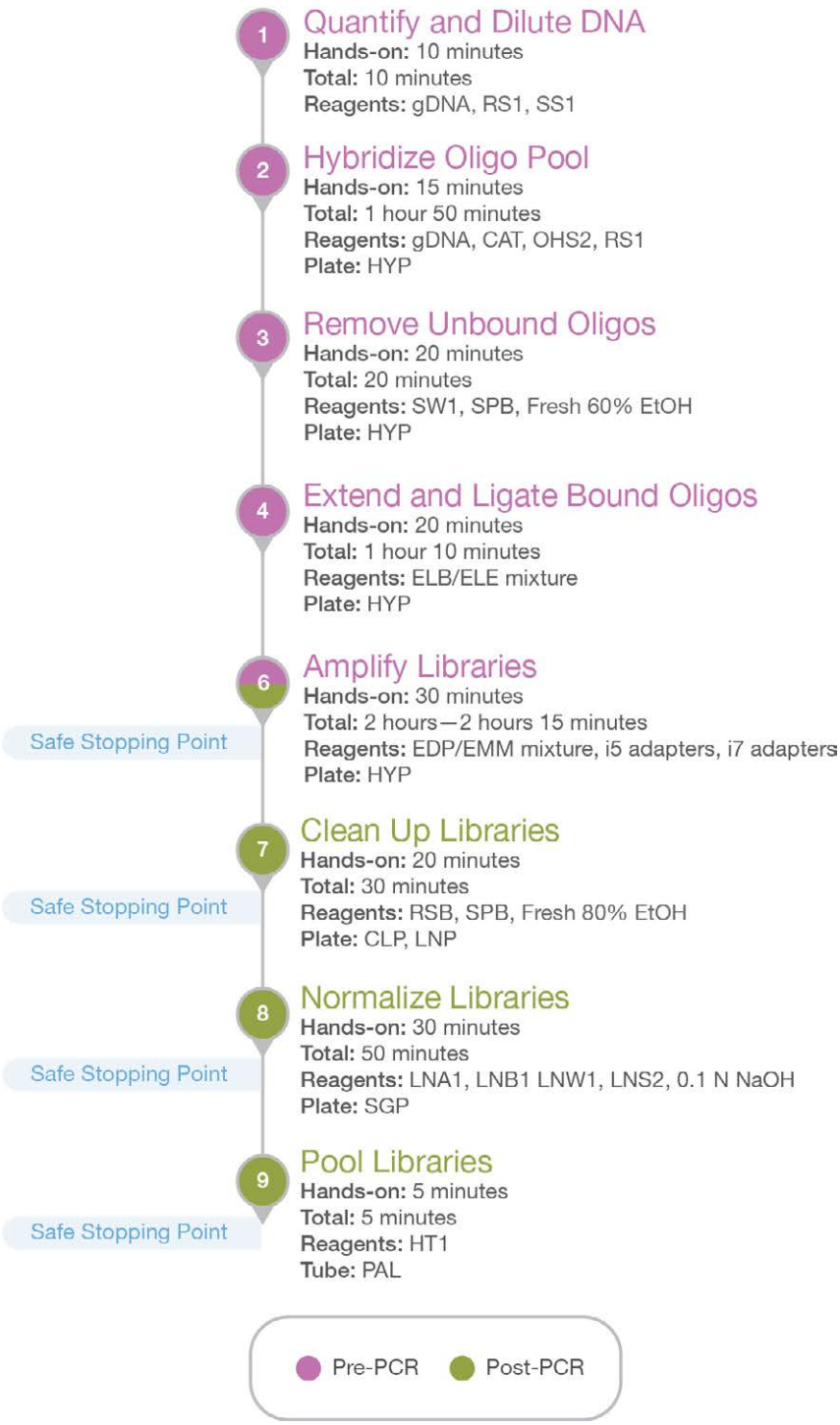
Handling Beads

- Pipette bead suspensions slowly.
- Before, allow the beads to come to room temperature.
- Immediately before use, vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous.
- If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
- When washing beads:
  - Use the appropriate magnetic stand for the plate.
  - Dispense liquid so that beads on the side of the wells are wetted.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand. Do not disturb the bead pellet.

Library Prep Workflow

The following figure illustrates the TruSeq Genotype N_e library prep workflow.

Figure 1 TruSeq Genotype N_e Workflow



Quantify and Dilute DNA

This step quantifies and dilutes input DNA to the appropriate concentration in the required diluent for subsequent steps.

Consumables

- RS1 (Resuspension Solution 1)
- SS1 (Sample Stabilization Solution 1)
- Genomic DNA
- Microcentrifuge tubes

About Reagents

- For later use, you can dilute and store more DNA than is required for the current protocol. Dilute DNA in RS1 and SS1 as described in the procedure.
- Prepare aliquots of diluted DNA in microcentrifuge tubes and store at -25°C to -15°C for up to 4 weeks.
- Store thawed diluted DNA at 2°C to 8°C for up to 2 weeks.
- Avoid repeatedly freezing and thawing diluted DNA.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
DNA	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Flick to mix, and then centrifuge briefly. Do not vortex.
RS1	15°C to 30°C	If stored at 2°C to 8°C, let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge briefly.
SS1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Flick to mix, and then centrifuge briefly.

Procedure

1 Quantify DNA using a fluorometric method, such as Qubit or PicoGreen.

2 Dilute DNA to 25 ng/µl in RS1.

3 Requantify the diluted DNA using the same fluorometric quantification method.

4 In a microcentrifuge tube, further dilute the 25 ng/µl DNA in RS1 to a total volume of 4 µl with a total DNA input of 50 ng.

5 Add 1 µl SS1 to the 4 µl of 50 ng DNA.
These volumes result in 5 µl of 50 ng input DNA.

Table 1 Example for 50 ng DNA Input

Reagent	Stock Concentration	Volume
DNA	25 ng/µl	2 µl
RS1	--	2 µl
SS1	5x	1 µl

Hybridize Oligo Pool

This step hybridizes a custom oligo pool that contains upstream and downstream oligos specific to your targeted regions of interest. Perform replicates to increase confidence in somatic variant calls.

Consumables

- ELB (Extension-Ligation Buffer)
- ELE (Extension-Ligation Enzyme)
- CAT (TruSeq Genotype N_e Oligos)
- OHS2 (Oligo Hybridization for Sequencing 2)
- RS1 (Resuspension Solution 1)
- HYP (Hybridization Plate) barcode label
- Diluted DNA
- 96-well PCR plate
- Microseal 'B' adhesive film
- Microcentrifuge tubes
- RNase/DNase-free 8-tube strips and caps
- Prepare for a subsequent step:
  - SPB (Sample Purification Beads)
  - SW1 (Stringent Wash 1)

WARNING

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 additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

About Reagents

- CAT
  - You can dilute and store more than the required amount of CAT for later use. Dilute CAT in RS1 as described in the procedure.
  - Use a multichannel pipette to dispense diluted CAT from a PCR 8-tube strip that contains 70 µl in each tube.
  - Prepare aliquots of diluted CAT and store at -25°C to -15°C for up to 12 months.
- OHS2
  - Aspirate and dispense slowly due to the viscosity of the reagent.
  - Before each use, vortex thoroughly and then centrifuge briefly. Make sure that all precipitates have dissolved.
  - When mixing, mix thoroughly.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
DNA	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Flick to mix, and then centrifuge briefly. Do not vortex.
CAT	-25°C to -15°C	Thaw at room temperature for 30 minutes. Vortex to mix, and then centrifuge briefly.
OHS2	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex thoroughly to mix, and then centrifuge briefly.
RS1	15°C to 30°C	If stored at 2°C to 8°C, let stand for 30 minutes to bring to room temperature. Vortex to mix, and then centrifuge briefly.
SPB	2°C to 8°C	Let stand to bring to room temperature in preparation for the procedure to remove unbound oligos. Do not exceed 25°C.
SW1	2°C to 8°C	Let stand to bring to room temperature in preparation for the procedure to remove unbound oligos.
ELB	-25°C to -15°C	Thaw at room temperature for 20 minutes, and then place on ice.
ELE	-25°C to -15°C	Place on ice.

2 Save the following HYB program for 25 µl reaction volume on a Bio-Rad thermal cycler:
- Choose the preheat lid option and set to 100°C.
- Step 1: 95°C for 3 minutes.
- Step 2: From 90°C, decrease by 0.5°C, hold for 30 seconds, ramp at 0.1°C per second.
- Step 3: Go to step 2 for 59x.
- Step 4: From 60°C, decrease by 0.5°C, hold for 1 minute, ramp at 0.1°C per second.
- Step 5: Go to step 4 for 19x.
- Step 6: From 50°C, decrease by 1°C, hold for 2 minutes, ramp at 0.1°C per second.
- Step 7: Go to step 6 for 9x.
- Step 8: From 40°C, hold for 10 minutes, ramp at 0.1°C per second.

3 Label a new 96-well PCR plate HYP.

Procedure

1 Dilute 2.5 µl CAT with 2.5 µl RS1 per sample well in a microcentrifuge tube.

2 Pulse vortex to mix, and then centrifuge briefly.

3 Add 5 µl RS1 to one well as a no template control.

4 Add 5 µl diluted DNA to the remaining wells.

5 Add 5 µl diluted CAT to all wells.

6 Add 15 µl OHS2 to each well. Using a P20 pipette, pipette slowly to mix.

7 If bubbles form, centrifuge the plate at 100 × g for 20 seconds.

8 Place on the preprogrammed thermal cycler and run the HYB program.

9 For 96 samples, combine ELE and ELB as follows. Scale accordingly for up to 384 samples.

a Transfer 137 µl ELE to the contents of the ELB tube.
b Flick and invert to mix. Do not vortex.

NOTE

Prepare the full amount of ELE/ELB mixture for the kit. If necessary, store aliquots at -25°C to -15°C for up to 3 months. Do not allow more than six freeze-thaw cycles.

10 Place the ELB/ELE mixture on ice for use during the procedure to extend and ligate bound oligos.

Remove Unbound Oligos

This step uses SPB to remove unbound oligos from gDNA. Three wash steps with SW1 and one wash step with 60% ethanol ensure the complete removal of unbound oligos.

Consumables and Equipment

- SW1 (Stringent Wash 1)
- SPB (Sample Purification Beads)
- Freshly prepared 60% ethanol (EtOH)
- Magnetic Stand
  - DynaMag-96 Side Skirted Magnet (use with 96-well full-skirted PCR plates)
  - DynaMag-96 Side Magnet (use with Eppendorf 96-well twin.tec PCR plates)

WARNING

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. Use SW1 in a hood. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

About Reagents

- Rinse SW1 over any beads on the side of the well when pipetting.
- SPB
  - Make sure that beads are at room temperature.
  - Vortex SPB vigorously before each use.
  - When mixing, mix thoroughly.

Preparation

1 Transfer 3 ml SPB to a tube for pre-PCR use.

2 Transfer 6 ml SPB to a tube for post-PCR use.

3 Prepare 200 µl per well of fresh 60% ethanol from 100% ethanol.

Procedure

1 Add 25 µl SPB to each well of the HYB plate. Pipette slowly to mix.

2 Incubate at room temperature for 5 minutes.

3 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

4 Remove and discard all supernatant from each well.

5 Keeping the plate on the magnetic stand, wash beads three times as follows.

   a Add 80 µl SW1 to each well.
   b Incubate at room temperature for 30 seconds.
   c Remove and discard all supernatant from each well.

6 Use a 20 µl pipette to remove residual SW1 from each well.

7 Add 80 µl of 60% EtOH to each well.

8 Incubate at room temperature for 30 seconds.

9 Remove and discard all supernatant from each well.

10 Use a 20 µl pipette to remove residual EtOH from each well.

11 Air-dry for up to 5 minutes. Proceed ***immediately*** to the next step.

Extend and Ligate Bound Oligos

This step connects the hybridized upstream and downstream oligos. A DNA polymerase extends from the upstream oligo through the targeted region, followed by ligation to the 5′ end of the downstream oligo using a DNA ligase. The result is the formation of products containing the targeted regions of interest flanked by the sequences required for amplification.

Consumables

- EDP (Enhanced DNA Polymerase)
- ELB/ELE mixture
- EMM (Enhanced Master Mix)
- Microseal 'B' adhesive seal
- Microcentrifuge tube (1)
- Prepare for a subsequent step:
  - Index 1 (i7) adapters (N7XX)
  - Index 2 (i5) adapters (S5XX)

About Reagents

- Do not allow more than six freeze-thaw cycles of EMM.
- ELB/ELE mixture
  - Invert and flick to mix, and then centrifuge briefly. Do not vortex.
  - Prepare the full amount of the mixture.
  - If needed, store aliquots at -25°C to -15°C for up to 3 months.
  - Do not allow more than six freeze-thaw cycles.

Preparation

1 Save the following EXT_LIG program for a 22 µl reaction volume on a thermal cycler:
- Choose the preheat lid option and set to 100°C
- 37°C for 45 minutes
- 70°C for 20 minutes
- Hold at 4°C

2 Prepare the following consumables.

Reagent	Storage	Instructions
ELB/ELE mixture	-25°C to -15°C	If frozen, thaw at room temperature and then place on ice. Invert and flick to mix, and then centrifuge briefly. Do not vortex.
EDP	-25°C to -15°C	Place on ice. Flick to mix, and then centrifuge briefly.
EMM	-25°C to -15°C	Thaw at room temperature for 20 minutes. Vortex to mix.
Index adapters (i7 and i5)	-25°C to -15°C	Only prepare adapters being used. Thaw at room temperature for 20 minutes in preparation for the procedure to amplify libraries. Invert each tube to mix. Centrifuge briefly.

Procedure

1 Remove the plate from the magnetic stand.

2 Using a P100 or P200 pipette, add 22 µl ELB/ELE mixture to each well.

3 Using a pipette set to 20 µl or a P20 pipette, pipette to mix. Make sure that no beads remain in the pipette.

4 If bubbles form, centrifuge at 100 × g for 20 seconds.

5 Place on the thermal cycler and run the EXT_LIG program.

6 Combine EDP and EMM in a microcentrifuge tube as follows.

Number of Samples	EDP	EMM
1	1.1 µl	21 µl
96	106 µl	2006 µl

Volumes include an additional 10%.

7 Pipette the EDP/EMM mixture to mix, and then centrifuge briefly.

8 Place the EDP/EMM mixture on ice for use during the procedure to amplify libraries.

Amplify Libraries

This step amplifies the extension-ligation products and adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for cluster formation.

Consumables

- EDP/EMM mixture
- Index 1 adapters (N7XX)

- Index 2 adapters (S5XX)
- Microseal 'B' adhesive seal

Preparation

1 Save the following PCR program on a thermal cycler using the appropriate number of PCR cycles, which are listed in the following table.
   - 95°C for 3 minutes
   - X cycles of:
     - 98°C for 20 seconds
     - 68°C for 20 seconds
     - 72°C for 30 seconds
   - 72°C for 1 minute
   - Hold at 10°C

Amplicon Plexity	Number of PCR Cycles (X)[1]
< 96 amplicons	28
97–384 amplicons	26
385–999 amplicons	25
1000–1999 amplicons	24
2000–5000 amplicons[2]	23

[1] To achieve desired library yield and specificity, optimize the PCR cycle number for your oligo pool.

[2] Alignment specificity might be reduced for high plexity panels designed with larger amplicon sizes.

NOTE

PCR cycles are based on 50 ng input DNA and the number of amplicons in the CAT.

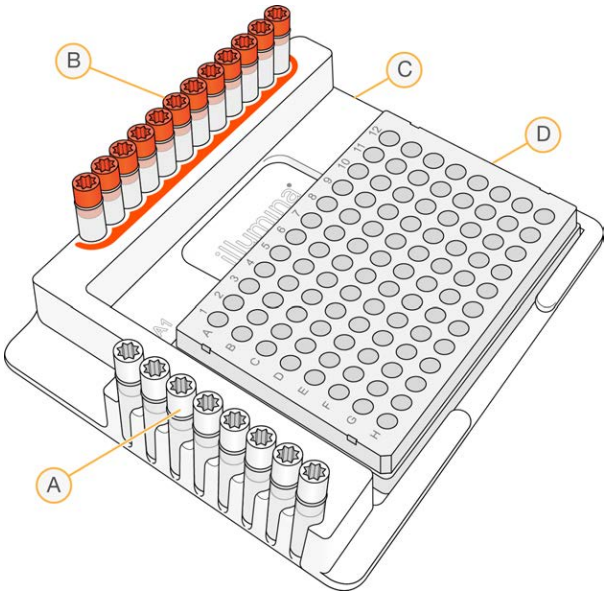
2 If using an iceless cooler, equilibrate the temperature to 2°C to 8°C.

Procedure

1 Arrange the Index 1 (i7) adapters in columns 1–12 of the TruSeq Index Plate Fixture.

2 Arrange the Index 2 (i5) adapters in rows A–H of the TruSeq Index Plate Fixture.

Figure 2 TruSeq Index Plate Fixture



A Rows A–H: Index 2 (i5) adapters (white caps)
B Columns 1–12: Index 1 (i7) adapters (orange caps)
C TruSeq Index Plate Fixture
D HYP plate

3 Place the HYP plate containing beads on a TruSeq Index Plate Fixture.

4 Add 4 µl of each Index 1 (i7) adapter down each column. Replace the cap on each i7 adapter tube with a new orange cap.

5 Add 4 µl of each Index 2 (i5) adapter across each row. Replace the cap on each i5 adapter tube with a new white cap.

6 Place the plate on ice or iceless cooler.

7 Add 20 µl EDP/EMM mixture to each well. Pipette to mix.

8 Centrifuge at 280 × g for 1 minute.

9 Place the plate on ice or iceless cooler.

10 Immediately transfer to the post-PCR area.

11 Place on the preprogrammed thermal cycler and run the PCR program for the appropriate number of cycles.
The beads remain in the wells during PCR.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up Libraries

This step uses SPB (Sample Purification Beads) to purify the PCR products from other reaction components.

Consumables and Equipment

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Barcode labels
  - CLP (Cleanup Plate)
  - LNP (Library Normalization Plate)
- 96-well midi plates (2)
- Microseal 'B' adhesive seals
- Freshly prepared 80% ethanol (EtOH)
- Magnetic stand-96 (use with midi 96-well storage plates)

About Reagents

- Vortex SPB vigorously before use.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Do not exceed 25°C.
RSB	15°C to 30°C	If frozen, thaw at room temperature for 20 minutes. Vortex to mix.

2 Prepare 400 µl fresh 80% ethanol per well from 100% ethanol.

3 Label a new midi plate CLP.

4 Label a new midi plate LNP.

Procedure

1 Centrifuge the HYP plate at 280 × g for 1 minute.

2 Add 36 µl SPB to each well of the CLP plate.

3 Place the HYP plate on a magnetic stand and wait until the liquid is clear (~1 minute).

4 Transfer 45 µl clear supernatant from each well of the HYP plate to the corresponding well of the CLP plate. Transfer as few beads as possible.

5 Shake the plate at 1800 rpm for 2 minutes.

6 Incubate at room temperature for 5 minutes.

7 Centrifuge at 280 × g for 1 minute.

8 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

9 Remove and discard all supernatant from each well.

10 Wash two times as follows.

   a Add 200 µl freshly prepared 80% EtOH to each sample well.

b Incubate on the magnetic stand for 30 seconds.
c Remove and discard all supernatant from each well.

11 Using a 20 µl pipette, remove residual EtOH from each well.

12 Remove from the magnetic stand and air-dry for up to 5 minutes.

13 Add 25 µl RSB to each well.

14 Shake the plate at 1800 rpm for 2 minutes.

15 If beads are not resuspended, pipette to mix and then shake at 1800 rpm for 2 minutes.

16 Incubate at room temperature for 2 minutes.

17 Centrifuge at 280 × g for 1 minute.

18 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

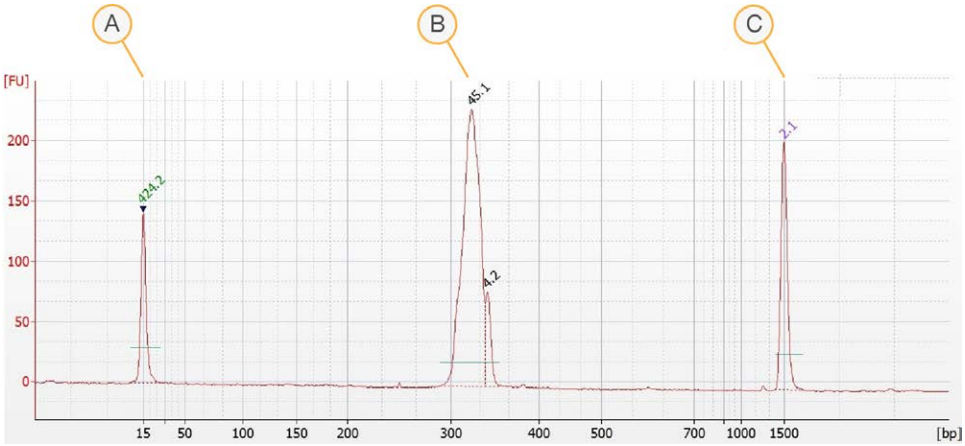
19 Transfer 20 µl purified library from each well of the CLP plate to the corresponding well of the LNP plate.

20 From the remaining liquid in the CLP plate, run an aliquot of the samples and control using any of the following methods:
- 5 µl on a 4% agarose gel.
- For up to six samples, 1 µl on an Agilent Bioanalyzer using a DNA 1000 chip.
- For up to 96 samples, 1 µl on an Agilent 2200 TapeStation using the D1000 ScreenTape assay.
- For up to 288 samples, 2 µl on an Advanced Analytical Fragment Analyzer using the Standard Sensitivity NGS Fragment Analysis Kit.

Table 2 Expected PCR Product Sizes

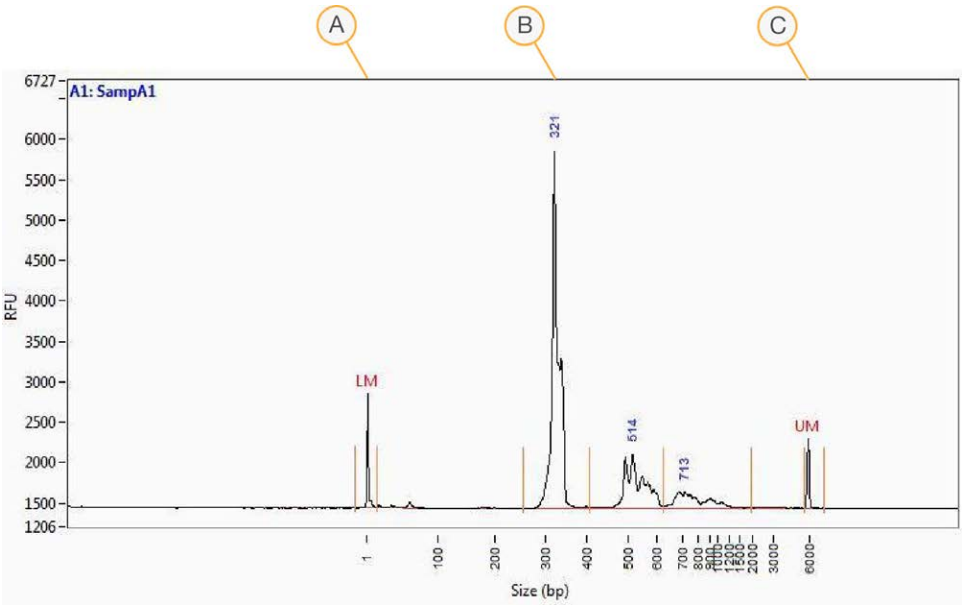
Amplicon Size	PCR Product Size
150 bp	~280 bp
175 bp	~310 bp

Figure 3 Bioanalyzer Trace Example



A Lower Marker
B Expected PCR Product for 175 bp Amplicons (~320 bp)
C Upper Marker

Figure 4 Fragment Analyzer Example



A Lower Marker
B Expected PCR Product for 175 bp Amplicons (~320 bp)
C Upper Marker

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 6 months.

Normalize Libraries

This step normalizes the quantity of each library for balanced representation in pooled libraries. Only samples containing DNA require processing through the subsequent steps.

Consumables and Equipment

- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- LNW1 (Library Normalization Wash 1)
- LNS2 (Library Normalization Storage buffer 2)
- SGP (Storage Plate) barcode label
- 0.1 N NaOH (freshly prepared)
- 96-well PCR plate, skirted
- 15 ml conical tube
- Microseal 'B' adhesive seals
- Magnetic stand-96 (use with midi 96-well storage plates)

WARNING

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 additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

WARNING

This set of reagents contains ß-mercaptoethanol. Perform the following procedure in a hood or well-ventilated area.

About Reagents

- When mixing, mix thoroughly.
- Mix only the amounts of LNA1 and LNB1 required for the current experiment.
- Use a P1000 pipette to transfer LNB1 to LNA1.
- Store remaining LNA1 and LNB1 separately at their respective temperatures.
- Vortex LNB1 thoroughly before use, and make sure that it is resuspended. Homogeneous resuspension is essential for consistent cluster density on the flow cell.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
LNA1	-25°C to -15°C	Thaw at room temperature. Let stand for 30 minutes to bring to room temperature. Vortex to mix. Inspect in front of a light. Make sure that all precipitate has dissolved.
LNB1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vortex for at least 1 minute. Invert intermittently to resuspend. Make sure that the bottom of the tube is free of pellets.
LNW1	2°C to 8°C	Thaw at room temperature. Let stand for 30 minutes to bring to room temperature.
LNS2	15°C to 30°C	Vortex to mix.

2 Prepare fresh 0.1 N NaOH.

3 Label a new midi plate SGP.

Procedure

1 Add 44 µl LNA1 per library to a new 15 ml conical tube.

2 Use a P1000 pipette to resuspend LNB1.

3 Transfer 8 µl LNB1 per library to the 15 ml conical tube of LNA1. Invert to mix.

4 Add 45 µl LNA1/LNB1 to each well of the LNP plate.
Each well contains 20 µl library.

5 Shake at 1800 rpm for 30 minutes.
Shorter durations can affect library representation and cluster density.

6 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

7 Remove and discard all supernatant.

8 Remove from the magnetic stand.

9 Wash two times as follows.

   a Add 45 µl LNW1 to each library well.
   b Shake at 1800 rpm for 5 minutes.
   c Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
   d Remove and discard all supernatant.

10 Use a 20 µl pipette to remove residual LNW1 from each well.

11 Remove from the magnetic stand.

12 Add 30 µl fresh 0.1 N NaOH to each well.

13 Shake at 1800 rpm for 5 minutes.

14 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

15 Add 30 µl LNS2 to each well of the SGP plate.

16 Transfer 30 µl supernatant from each well of the LNP plate to the corresponding well of the SGP plate.

17 Centrifuge the SGP plate at 1000 × g for 1 minute.

SAFE STOPPING POINT

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

Pool Libraries

This step pools libraries by combining equal volumes of normalized libraries in one tube.

Consumables

- PAL (Pooled Amplicon Library) barcode label
- Microcentrifuge tube
- RNase/DNase-free 8-tube strips and caps

Preparation

1 If the SGP plate was stored frozen, prepare as follows.

   a Thaw at room temperature.
   b Centrifuge at 1000 × g for 1 minute.
   c Pipette to mix.

2 Label a new Eppendorf tube PAL.

Procedure

1 Centrifuge the SGP plate at 1000 × g for 1 minute.

2 Transfer 5 µl of each library to an 8-tube strip, column by column.

3 Seal the plate and store at -25°C to -15°C.

4 Transfer the contents of the 8-tube strip to the PAL tube. Pipette to mix.

5 Denature and dilute the library pool to the appropriate loading concentration for the sequencing run. For instructions, see the denature and dilute libraries guide for your instrument.

SAFE STOPPING POINT

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

Supporting Information

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Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed kit contents, and obtained all required consumables and equipment.

Acronyms

Acronym	Definition
ACP3	Amplicon Control Oligo Pool 3
CLP	Cleanup Plate
EDP	Enhanced DNA Polymerase
ELB	Extension-Ligation Buffer
ELE	Extension-Ligation Enzyme
EMM	Enhanced Master Mix
CAT	TruSeq Genotype Ne Oligos
HT1	Hybridization Buffer
HYP	Hybridization Plate
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNP	Library Normalization Plate
LNS2	Library Normalization Storage Buffer 2
LNW1	Library Normalization Wash 1
OHS2	Oligo Hybridization for Sequencing Reagent 2
PAL	Pooled Amplicon Library
RS1	Resuspension Solution 1
RSB	Resuspension Buffer
SGP	Storage Plate
SNP	Single Nucleotide Polymorphism
SPB	Sample Purification Beads
SS1	Sample Stabilization Solution 1
SW1	Stringent Wash 1

Kit Contents

Make sure that you have all the kit components identified in this section before starting the library prep procedures. The TruSeq Genotype N_e kit is provided in a custom configuration that includes library prep reagents, index adapters, and sequencing reagents.

Kit Name	Catalog #
TruSeq Genotype N_e (96 Samples)	20018978

Library Prep Reagents

Reagents for preparing TruSeq Genotype N_e libraries are provided in three DNA Amplicon Assay v2 boxes and one oligo box.

Box 1 – DNA Amplicon Assay v2 (16 samples), Store at -25°C to -15°C in the Pre-PCR Area

Quantity	Reagent	Description
1	ACP3	Amplicon Control Oligo Pool 3
1	EDP	Enhanced DNA Polymerase
1	ELB	Extension-Ligation Buffer
1	ELE	Extension-Ligation Enzyme
1	EMM	Enhanced Master Mix

Box 1 also contains the HYP barcode label.

Box 2 – DNA Amplicon Assay v2 (16 samples), Store in the Pre-PCR Area

Quantity	Reagent	Description	Storage Temperature
1	LNB1	Library Normalization Beads 1	2°C to 8°C
1	OHS2	Oligo Hybridization for Sequencing Reagent 2	2°C to 8°C
1	RS1	Resuspension Solution 1	15°C to 30°C
1	SPB	Sample Purification Beads	2°C to 8°C
1	SS1	Sample Stabilization Solution 1	2°C to 8°C
1	SW1	Stringent Wash 1	2°C to 8°C

Box 3 – DNA Amplicon Assay v2, Store in the Post-PCR Area

Quantity	Reagent	Description	Storage Temperature
1	HT1	Hybridization Buffer	-25°C to -15°C
1	LNA1	Library Normalization Additives 1	-25°C to -15°C
1	LNS2	Library Normalization Storage Buffer 2	15°C to 30°C
2	LNW1	Library Normalization Wash 1	2°C to 8°C
2	RSB	Resuspension Buffer	15°C to 30°C

Box 3 also contains the CLP, LNP, SGP, PAL, and DAL barcode labels.

TruSeq Genotype N_e Oligo Box, Store at -25°C to -15°C

Quantity	Reagent	Description
1	CAT	TruSeq Genotype N_e Oligos

Index Adapters

Index adapters for TruSeq Genotype N_e libraries are provided in the Nextera® XT Index Kit v2 Sets A–D. Combining all four sets achieves 384 index combinations.

Nextera XT Index Kit v2 Set A (96 indexes, 192 samples), Store at -25°C to -15°C in the Pre-PCR Area

Quantity	Reagent Name
8	Index Adapters S502, S503, S505–S508, S510, and S511
12	Index Adapters N701–N707, N710–N712, N714, and N715

Nextera XT Index Kit v2 Set B (96 indexes, 192 samples), Store at -25°C to -15°C in the Pre-PCR Area

Quantity	Reagent Name
8	Index Adapters S502, S503, S505–S508, S510, and S511
12	Index Adapters N716, N718–N724, and N726–N729

Nextera XT Index Kit v2 Set C (96 indexes, 192 samples), Store at -25°C to -15°C in the Pre-PCR Area

Quantity	Reagent Name
8	Index Adapters S513, S515–S518, and S520–S522
12	Index Adapters N701–N707, N710–N712, N714, and N715

Nextera XT Index Kit v2 Set D (96 indexes, 192 samples), Store at -25°C to -15°C in the Pre-PCR Area

Quantity	Reagent Name
8	Index Adapters S513, S515–S518, and S520–S522
12	Index Adapters N716, N718–N724, and N726–N729

Index Adapter Replacement Caps, Store at 15°C to 30°C in the Pre-PCR Area

Quantity	Description
1	i7 Index Tube Caps, Orange
1	i5 Index Tube Caps, White

Sequencing Reagents

A TruSeq Genotype Ne kit includes the reagents and flow cell required for a sequencing run on the MiniSeq, NextSeq, or MiSeq system.

MiniSeq Reagents

Quantity	Description	Storage Temperature
1	MiniSeq Reagent Cartridge (150 cycles)	-25°C to -15°C
1	MiniSeq Flow Cell	2°C to 8°C
1	Hybridization Buffer (HT1)	-25°C to -15°C

NextSeq Reagents

Quantity	Description	Storage Temperature
1	NextSeq 500 High Output Reagent Cartridge v2 (150 cycles)	-25°C to -15°C
1	NextSeq 500 High Output Flow Cell v2	2°C to 8°C
1	NextSeq 500/550 Buffer Cartridge v2	15°C to 30°C
1	NextSeq Accessory Box v2	-25°C to -15°C

MiSeq Reagent Kit v3

Table 3 Box 1 of 2

Quantity	Description	Storage Temperature
1	MiSeq v3 Reagent Tray (150 Cycles, PE)	-25°C to -15°C
1	Hybridization Buffer (HT1)	-25°C to -15°C

Table 4 Box 2 of 2

Quantity	Description	Storage Temperature
1	PE MiSeq Flow Cell	2°C to 8°C
1	Incorporation Buffer (PR2)	2°C to 8°C

Consumables and Equipment

Make sure that you have the required user-supplied consumables and equipment before starting the protocol.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Use separate sets of consumables and equipment for pre-PCR and post-PCR procedures. Different types of magnetic stands are needed for pre-PCR and post-PCR procedures.

Consumables

Consumable	Supplier
10 N NaOH, molecular biology grade[1]	General lab supplier
20 µl barrier pipette tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
200 µl barrier pipette tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
1000 µl barrier pipette tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
One of the following plate types: • Hard-shell 96-well skirted PCR plates, low-profile, skirted • Eppendorf 96-well twin.tec PCR plates, semiskirted	One of the following suppliers, depending on plate type: • Bio-Rad, catalog # HSP-9601 • Fisher Scientific, catalog # E9-510-20303
96-well storage plates, 0.8 ml (midi plate)	Fisher Scientific, catalog # AB-0859 or AB-0765
Adhesive seal roller	General lab supplier
Conical tubes, 15 ml	General lab supplier
DNA molecular weight markers	General lab supplier
Ethanol, 100% for molecular biology	General lab supplier
Ice bucket	General lab supplier
Microcentrifuge tubes	General lab supplier
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
PCR grade water	General lab supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
One of the following library quality assessment methods: • 4% Agarose gel • Standard Sensitivity NGS Fragment Analysis Kit (1–6000 bp) • DNA 1000 Kit	One of the following suppliers, depending on method: • General lab supplier • Advanced Analytical Technologies, part # DNF-473 • Agilent Technologies, catalog # 5067–1504
[Optional] TruSeq Index Plate Fixture Kit[2]	Illumina, catalog # FC-130-1005

[1] Prepare from tablets or use a standard solution.

[2] A reusable part for setting up index adapters.

Equipment

Pre-PCR Equipment

Equipment	Supplier
Iceless cooler for 96-well plates	General lab supplier
96-well thermal cycler (with heated lid) See *Thermal Cyclers*.	General lab supplier

Equipment	Supplier
One of the following magnetic stands, depending on the type of PCR plate: • DynaMag-96 Side Skirted Magnet (use with 96-well full-skirted PCR plates) • DynaMag-96 Side Magnet (use with Eppendorf 96-well twin.tec PCR plates)	Life Technologies: • Catalog # 12027 • Catalog # 12331D
Microplate centrifuge	General lab supplier

Post-PCR Equipment

Equipment	Supplier
Magnetic stand-96 (use with midi 96-well storage plates)	Life Technologies, catalog # AM10027
One of the following: • BioShake iQ high-speed thermal mixer • BioShake XP high-speed lab shaker	Q.Instruments: • Order # 1808-0506 • Order # 1808-0505
Microplate centrifuge	General lab supplier
One of the following library quality assessment methods: • Fragment Analyzer Automated CE System • 2100 Bioanalyzer Desktop System • Gel electrophoresis supplies and apparatus • Agilent 2200 TapeStation	One of the following suppliers, depending on method: • Advanced Analytical Technologies, part # FSv2-CE2 or FSv2-CE10 • Agilent Technologies, catalog # G2940CA • General lab supplier • Agilent Technologies, catalog # G2964AA
Heat block for 1.5 ml centrifuge tubes	General lab supplier

Thermal Cyclers

Use the following recommended settings for selected thermal cycler models. Before performing library prep, validate any thermal cyclers not listed.

NOTE

The Bio-Rad thermal cyclers might provide superior specificity.

Thermal Cycler	Block Type	Ramp Rate	Lid Temp	Block Rate	Vessel Type
Bio-Rad S1000	Standard	0.1°C	Heated, Constant at 100°C	--	Bio-Rad Hard-Shell 96-Well Skirted PCR Plates, low-profile, skirted
Bio-Rad C1000	Standard	0.1°C	Heated, Constant at 100°C	--	Bio-Rad Hard-Shell 96-Well Skirted PCR Plates, low-profile, skirted
Bio-Rad T100	Standard	0.1°C	Heated, Constant at 100°C	--	Eppendorf twin.tec PCR Plate 96, semiskirted
Applied Biosystems GeneAmp PCR System 9700	Gold	1%	Heated, Constant at 100°C	9600 for HYB and EXT_LIG programs or MAX for PCR program	Eppendorf twin.tec PCR Plate 96, semiskirted
Applied Biosystems Veriti 96-Well	Alloy	2% to 2.1%	Heated, Constant at 100°C	--	Eppendorf twin.tec PCR Plate 96, semiskirted

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

Region	Toll Free	Regional
North America	+1.800.809.4566	
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Sweden	+46 850619671	+46 200883979
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Other countries	+44.1799.534000	

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

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