HiSeq Systems

Denature and Dilute Libraries Guide

Overview

This guide explains how to denature and dilute libraries prepared for clustering on the cBot 2 or cBot and subsequent sequencing on the HiSeq®. Instructions for preparing libraries for on-instrument clustering on the HiSeq 2500 or HiSeq 1500 using a rapid flow cell are also included.

These denature and dilute procedures apply to the following flow cells: HiSeq v4, TruSeq® v3 (HiSeq), HiSeq Rapid v2. They do not apply to HiSeq X® or HiSeq 3000/4000 patterned flow cells.

NOTE

Denaturation and dilution are not necessary for all library types. For example, Nextera XT libraries do not require this step because the Nextera XT protocol results in a ready-to-use normalized concentration of pooled libraries. See the library preparation documentation for the protocol used to prepare libraries.

Cluster Densities

Optimizing cluster densities across every lane of the flow cell is critical to achieving quality data. Optimum cluster density requires accurate quantification of libraries. For recommended quantification methods, see the library prep documentation for your library prep kit.

Cluster Densities for HiSeq Flow Cells

Cluster Kit Version	Cluster Density	When Measured With
HiSeq Cluster Kit v4	950–1050 K/mm²	RTA v1.18, or later
TruSeq Cluster Kit v3	750–850 K/mm²	RTA v1.12, or later
HiSeq Rapid Cluster Kit v2	850–1000 K/mm²	RTA v1.18, or later
HiSeq Rapid Duo cBot Sample Loading Kit	850–1000 K/mm²	RTA v1.18, or later

Consumables

The following consumables are required to denature and dilute libraries. Make sure that you use the appropriate 8-tube strip for your cluster instrument and workflow.

Consumable	Supplier	Purpose
cBot 2 Barcoded Strip Tubes (8 wells)	Illumina, catalog # 20005160	Clustering on the cBot 2 with sample tracking.
0.2 ml 8-tube strip	General lab supplier	Clustering on the cBot, or Clustering on the cBot 2 without sample tracking
Stock 1.0 N NaOH, molecular biology-grade	General lab supplier	Denaturing libraries for cluster generation
HT1 (Hybridization Buffer), thawed and prechilled	Illumina	Diluting denatured libraries and a PhiX control
Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20	General lab supplier	Diluting a PhiX control
[Optional] Illumina PhiX Control	Illumina, catalog # FC-110-3001	Spike-in and control lane options

Best Practices

- Follow the instructions in the order shown, using the specified volumes and incubation parameters.
- A *fresh* dilution of 0.1 N NaOH is required for denaturing libraries and a PhiX control.
  - After denaturing libraries, you can set aside the remaining NaOH to prepare a PhiX control within the next 12 hours.
  - After 12 hours, discard any remaining dilution of NaOH.

Prepare Reagents

The following reagents are required to denature and dilute libraries for sequencing on the HiSeq:

- 0.1 N NaOH
- HT1 (Hybridization Buffer)

Prepare HT1

1 Remove HT1 from -25°C to -15°C storage and thaw at room temperature.

2 Store at 2°C to 8°C until you are ready to dilute denatured libraries.

Prepare a Fresh Dilution of NaOH

1 Combine the following volumes in a microcentrifuge tube:
  - Laboratory-grade water (900 µl)
  - Stock 1 N NaOH (100 µl)

  These volumes result in 1 ml of 0.1 N NaOH.

2 Invert to mix.

Denature and Dilute Libraries for cBot Clustering

Perform the following steps to denature and dilute libraries to a final DNA concentration of 20 pM for cluster generation on the cBot.

NOTE

If your library requires a final concentration > 20 pM, make sure that the NaOH concentration is ≤ 0.05 N in the denaturation solution and ≤ 0.001 N (1 mM) in the final solution diluted with HT1. Higher concentrations inhibit hybridization and decrease cluster density.

Protocol Variations

Follow the appropriate denature and dilute protocol depending on the normalization procedure used during library prep.

- **Standard normalization**—Libraries are normalized using standard library quantification and quality control procedures recommended in the library prep documentation. For these libraries, follow **Protocol A**. See *Protocol A: Standard Normalization Method* on page 5.
- **Bead-based normalization**—Libraries are normalized using a bead-based procedure described in the library prep documentation for methods that support bead-based normalization. For these libraries, follow **Protocol B**. See *Protocol B: Bead-Based Normalization Method* on page 6.

Protocol A: Standard Normalization Method

Use protocol A to denature and dilute libraries that have been normalized using standard library quantification and quality control procedures recommended in the library prep documentation.

Denature Libraries

1 Combine the following volumes in a microcentrifuge tube:
- 2 nM library (10 µl)
- 0.1 N NaOH (10 µl)

2 Vortex briefly to mix.

3 Centrifuge at 280 × g for 1 minute.

4 Incubate for 5 minutes at room temperature to denature the library into single strands.

5 Add 980 µl prechilled HT1 to 20 µl denatured library to prepare a 20 pM library.

6 Set aside on ice.

Dilute Denatured Libraries

1 Combine the following volumes.

Final Concentration	10 pM	12 pM	15 pM	18 pM	20 pM
20 pM denatured library	500 µl	600 µl	750 µl	900 µl	1000 µl
Prechilled HT1	500 µl	400 µl	250 µl	100 µl	0 µl

2 Invert to mix, and then pulse centrifuge.

3 Set aside on ice.

Prepare an 8-Tube Strip

Use the following instructions to prepare an 8-tube strip and load diluted libraries without a PhiX control. If you plan to use a PhiX control, skip these instructions and proceed to *Denature and Dilute PhiX for cBot Clustering (Optional)* on page 7.

1 Number the tubes of an 8-tube strip:
- **Standard 8-tube strip**—Label the tubes #1 through #8.
- **Barcode labeled 8-tube strip**—From the key end, label the tubes #8 through #1 for a high output flow cell or #2 and #1 for a rapid flow cell.

Figure 1 Barcode Labeled 8-Tube Strip, Numbered

2 Add the appropriate volume of diluted library to the numbered tubes:

Flow Cell	Library	Numbered Tubes
HiSeq v4 flow cell	75 µl	1–8
TruSeq v3 (HS) flow cell	120 µl	1–8

Flow Cell	Library	Numbered Tubes
HiSeq Rapid v2 flow cell	135 µl	1–2
TruSeq v2 (GA) flow cell	120 µl	1–8

3 Set aside on ice.

Protocol B: Bead-Based Normalization Method

Use protocol B to denature and dilute libraries that have been normalized and pooled using a bead-based procedure described in the library prep documentation for methods that support bead-based normalization.

Bead-based normalization procedures can be variable. The actual volume of library varies depending upon library type and experience.

Prepare Incubator

1 Preheat the incubator to 98°C.

Dilute Library to Loading Concentration

1 Using the following table as an example, combine the appropriate volumes of pooled libraries and prechilled HT1 in a microcentrifuge tube. Prepare 120 µl of diluted library for each lane you are loading.

Amplicon Library Pool	Prechilled HT1
6 µl	594 µl
7 µl	593 µl
8 µl	592 µl
9 µl	591 µl
10 µl	590 µl
Nextera XT Library Pool	**Prechilled HT1**
15 µl	585 µl

The total volume is 600 µl.

2 Vortex briefly.

3 Centrifuge at 280 × g for 1 minute.

Denature Diluted Library

1 Place the tube on the preheated incubator for 2 minutes.

2 Immediately cool on ice.

3 Leave on ice for 5 minutes.

4 Set aside on ice.

Prepare an 8-Tube Strip

Use the following instructions to prepare an 8-tube strip and load diluted libraries without a PhiX control. If you plan to use a PhiX control, skip these instructions and proceed to *Denature and Dilute PhiX for cBot Clustering (Optional)* on page 7.

1 Number the tubes of an 8-tube strip:

- **Standard 8-tube strip**—Label the tubes #1 through #8.
- **Barcode labeled 8-tube strip**—From the key end, label the tubes #8 through #1 for a high output flow cell or #2 and #1 for a rapid flow cell.

Figure 2 Barcode Labeled 8-Tube Strip, Numbered

2 Add 120 µl diluted library to the numbered tubes.

3 Set aside on ice.

Denature and Dilute PhiX for cBot Clustering (Optional)

Incorporate a PhiX control by adding PhiX to the flow cell using 1 of 2 methods: PhiX spike-in or PhiX control lane. Make sure that you select a method compatible with the version of control software installed on your sequencing instrument.

Control Software	Control Lane Compatibility
HiSeq Control Software (HCS) v2.2, or later	Not compatible
HiSeq Control Software (HCS) v2.0, or earlier	Compatible

A low-concentration PhiX control spike-in at 1% allows direct assessment of error rates for each lane. A PhiX spike-in is important for unbalanced or low-diversity libraries.

Denature a PhiX Library

1 Combine the following volumes to dilute the PhiX library to 2 nM:
- 10 nM PhiX library (2 µl)
- 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 (8 µl)

2 Combine the following volumes in a microcentrifuge tube to prepare a 1 nM PhiX library:
- 2 nM PhiX library (10 µl)
- 0.1 N NaOH (10 µl)

3 Vortex briefly to mix.

4 Centrifuge at 280 × g for 1 minute.

5 Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.

6 Add 980 µl prechilled HT1 to 20 µl denatured PhiX library to prepare a 20 pM PhiX library.

NOTE

The denatured 20 pM PhiX library can be stored up to 3 weeks at -25°C to -15°C. After 3 weeks, cluster numbers can decrease.

Dilute Denatured PhiX Library

1 Dilute the PhiX library as follows.

Flow Cell	Dilution
HiSeq v4 flow cell	Combine the following volumes to dilute to 18 pM: • 20 pM denatured PhiX library (900 µl) • Prechilled HT1 (100 µl)
TruSeq v3 (HiSeq) flow cell	Combine the following volumes to dilute to 12 pM: • 20 pM denatured PhiX library (600 µl) • Prechilled HT1 (400 µl)

2 Set aside on ice.

Combine Library and PhiX Control for PhiX Spike-in

1 Combine the following volumes of denatured PhiX control and library.

Library Type	Volumes
Most libraries (1%)	Prepared library (990 µl) PhiX control (10 µl)
Low-diversity libraries (≥ 10%)	Prepared library (900 µl) PhiX control (100 µl)

2 Set aside on ice.

Prepare an 8-Tube Strip for PhiX Spike-In

1 Number the tubes of an 8-tube strip:
- **Standard 8-tube strip**—Label the tubes #1 through #8.
- **Barcode labeled 8-tube strip**—From the key end, label the tubes #8 through #1 for a high output flow cell or #2 and #1 for a rapid flow cell.

Figure 3 Barcode Labeled 8-Tube Strip, Numbered

2 Add the appropriate volume of library and PhiX solution to the specified tubes:
- For protocol A, use the following volumes.

Flow Cell	Library and PhiX Solution	Numbered Tubes
HiSeq v4 flow cell	75 µl	1–8
TruSeq v3 (HiSeq) flow cell	120 µl	1–8
HiSeq Rapid v2 flow cell	135 µl	1–2

- For protocol B, add 120 µl library and PhiX solution to the 8-tube strip.

3 Set aside on ice.

Prepare an 8-Tube Strip for a PhiX Control Lane

1 Number the tubes of an 8-tube strip:
- **Standard 8-tube strip**—Label the tubes #1 through #8.
- **Barcode labeled 8-tube strip**—Label the tubes #8 through #1 from the key end.

Figure 4 Barcode Labeled 8-Tube Strip, Numbered

2 Add 120 µl PhiX control to tube #4 to designate lane 4 as the control lane.

3 Add 120 µl library to the remaining tubes, #1 through #3 and #5 through #8.

4 Set aside on ice.

Next Steps

After preparing the 8-tube strip, you are ready to set up the clustering run. For instructions, see the *cBot 2 System Guide (document # 15065681)* or the *cBot System Guide (document # 15006165)*.

Denature and Dilute Libraries for HiSeq Clustering

Use the following instructions to denature and dilute libraries for on-instrument clustering on the HiSeq 2500 or HiSeq 1500 using a rapid flow cell.

NOTE

If your library requires a final concentration > 20 pM, make sure that the NaOH concentration is ≤ 0.05 N in the denaturation solution and ≤ 0.001 N (1 mM) in the final solution diluted with HT1. Higher concentrations inhibit hybridization and decrease cluster density.

Protocol Variations

Follow the appropriate denature and dilute protocol depending on the normalization procedure used during library prep.

- **Standard normalization**—Libraries are normalized using standard library quantification and quality control procedures recommended in the library prep documentation. For these libraries, follow **Protocol A**. See *Protocol A: Standard Normalization Method* on page 9.
- **Bead-based normalization**—Libraries are normalized using a bead-based procedure described in the library prep documentation for methods that support bead-based normalization. For these libraries, follow **Protocol B**. See *Protocol B: Bead-Based Normalization Method* on page 10. (For TruSight® Tumor 170 libraries, follow *Protocol C: TruSight Tumor 170 Library Denaturation and Dilution Method* on page 11.)
- **TruSight Tumor 170 library denaturation and dilution method**—For TruSight Tumor 170 libraries, follow **Protocol C**. See *Protocol C: TruSight Tumor 170 Library Denaturation and Dilution Method* on page 11.

Protocol A: Standard Normalization Method

Use protocol A to denature and dilute libraries that have been normalized using standard library quantification and quality control procedures recommended in the library prep documentation.

Denature Libraries

1 Combine the following volumes in a microcentrifuge tube:
   - 2 nM library (10 µl)
   - 0.1 N NaOH (10 µl)

2 Vortex briefly to mix.

3 Centrifuge at 280 × g for 1 minute.

4 Incubate for 5 minutes at room temperature to denature the library into single strands.

5 Add 980 µl prechilled HT1 to 20 µl denatured library to prepare a 20 pM library.

6 Set aside on ice.

Dilute Denatured Libraries

1 Combine the following volumes in a 1.5 ml or 1.7 ml microcentrifuge tube.

Final Concentration	2 pM	6 pM	10 pM	12 pM	16 pM	20 pM
20 pM denatured library	42 µl	126 µl	210 µl	252 µl	336 µl	420 µl
Prechilled HT1	378 µl	294 µl	210 µl	168 µl	84 µl	0 µl

2 Invert to mix, and then pulse centrifuge.

3 Set aside on ice.
The microcentrifuge tube is loaded onto the HiSeq during run setup.

Protocol B: Bead-Based Normalization Method

Use protocol B to denature and dilute libraries that have been normalized and pooled using a bead-based procedure described in the library prep documentation for methods that support bead-based normalization. (For TruSight Tumor 170 libraries, follow *Protocol C: TruSight Tumor 170 Library Denaturation and Dilution Method* on page 11.)

Bead-based normalization procedures can be variable. The actual volume of library varies depending upon library type and experience.

Prepare Incubator

1 Preheat the incubator to 98°C.

Dilute Library to Loading Concentration

1 Using the following table as an example, combine the appropriate volumes of pooled libraries and prechilled HT1 in a 1.5 ml or 1.7 ml microcentrifuge tube. Prepare 120 µl of diluted library for each lane you are loading.

Amplicon Library Pool	Prechilled HT1
6 µl	594 µl
7 µl	593 µl
8 µl	592 µl
9 µl	591 µl
10 µl	590 µl

Nextera XT Library Pool	Prechilled HT1
15 µl	585 µl

The total volume is 600 µl.

2 Vortex briefly.

3 Centrifuge at 280 × g for 1 minute.

Denature Diluted Library

1 Place the tube on the preheated incubator for 2 minutes.

2 Immediately cool on ice.

3 Leave on ice for 5 minutes.

4 To add a PhiX control, proceed to *Denature and Dilute PhiX for HiSeq Clustering Using Protocols A–B (Optional)* on page 12. Otherwise, see *Next Steps* on page 13.

Protocol C: TruSight Tumor 170 Library Denaturation and Dilution Method

Use protocol C to denature and dilute TruSight Tumor 170 libraries.

Use the following procedures to achieve optimal cluster density:

- Sequence 12 libraries (6 DNA and 6 RNA) per run to achieve maximum coverage for each library.
- If you are sequencing DNA libraries only, you can sample up to 6 libraries.
- If you are sequencing RNA libraries only, you can sample up to12 libraries.
- Contact Illumina Technical Support if you are sequencing other combinations of DNA and RNA libraries.

Prepare Incubator

1 Preheat the incubator to 96°C.

Denature Libraries

1 Incubate each pooled library tube in a heat block at 96°C for 2 minutes.

2 Invert each tube two times to mix.

3 Centrifuge briefly, and then place on ice for 5 minutes.

4 [Optional] The denatured RNA and DNA libraries can be stored at -25°C to -15°C for up to 30 days. If you are using denatured RNA and DNA libraries that have been stored frozen, repeat steps 1–3 to redenature, mix, and cool tubes before proceeding to the next step.

Dilute Libraries

Choose one of the following dilution procedures to produce a denatured library solution. If you are sequencing the same number of DNA and RNA libraries, pool at a 4:1 ratio of DNA to RNA. If you are sequencing an unequal number of libraries (for example, 7 DNA + 3 RNA), contact Illumina Technical Support.

Sequence cDNA and DNA Libraries Simultaneously

1 Transfer 20 µl of denatured DNA library to a new, screw-top microcentrifuge tube.

2 Add 5 µl of denatured RNA library to the tube.

3 Add 475 µl HT1 buffer to the tube to make a 1:20 dilution.

4 Vortex to mix.

5 Centrifuge briefly.

Sequence DNA Libraries

1 Transfer 10 µl of denatured DNA library to a new, screw-top microcentrifuge tube.
2 Add 190 µl HT1 buffer to the tube to make a 1:20 dilution.
3 Vortex to mix.
4 Centrifuge briefly.

Sequence cDNA Libraries

1 Transfer 10 µl of denatured RNA library to a new, screw-top microcentrifuge tube.
2 Add 190 µl HT1 buffer to the tube to make a 1:20 dilution.
3 Vortex to mix.
4 Centrifuge briefly.

Dilute Denatured Libraries to Final Loading Concentration

1 Transfer 130 µl of denatured library solution into a new, snap-cap microcentrifuge tube.
2 Add 1170 µl HT1 buffer to the tube.
3 Vortex to mix.
4 Centrifuge briefly.
5 To add a PhiX control, proceed to *Denature and Dilute PhiX for HiSeq Clustering Using Protocol C (Optional)* on page 13. Otherwise, see *Next Steps* on page 14.

Denature and Dilute PhiX for HiSeq Clustering Using Protocols A–B (Optional)

A low-concentration PhiX control spike-in at 1% allows direct assessment of error rates for each lane. A PhiX spike-in is important for unbalanced or low-diversity libraries.

Denature a PhiX Library

1 Combine the following volumes to dilute the PhiX library to 2 nM:
   - 10 nM PhiX library (2 µl)
   - 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 (8 µl)
2 Combine the following volumes in a microcentrifuge tube to prepare a 1 nM PhiX library:
   - 2 nM PhiX library (10 µl)
   - 0.1 N NaOH (10 µl)
3 Vortex briefly to mix.
4 Centrifuge at 280 × g for 1 minute.
5 Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.
6 Add 980 µl prechilled HT1 to 20 µl denatured PhiX library to prepare a 20 pM PhiX library.

NOTE

The denatured 20 pM PhiX library can be stored up to 3 weeks at -25°C to -15°C. After 3 weeks, cluster numbers can decrease.

Dilute Denatured PhiX Library

1 Combine the following volumes to dilute the PhiX library to 12 pM:
- 20 pM denatured PhiX library (600 µl)
- Prechilled HT1 (400 µl)

2 Set aside on ice.

Combine Library and PhiX Control for PhiX Spike-In

1 Combine the following volumes of diluted PhiX control and library.

Library Type	Volumes
Most libraries (1%)	Prepared library (416 µl) PhiX control (4 µl)
Low-diversity libraries (≥ 10%)	Prepared library (378 µl) PhiX control (42 µl)

2 Set aside on ice until you are ready to load the HiSeq during run setup.

Next Steps

After loading libraries into the microcentrifuge tube, you are ready to set up the sequencing run. For instructions, see the *HiSeq 2500 System Guide (document # 15035786)* or the *HiSeq 1500 System Guide (document # 15035788)*.

Denature and Dilute PhiX for HiSeq Clustering Using Protocol C (Optional)

A low-concentration PhiX control spike-in at 1% allows direct assessment of error rates for each lane. A PhiX spike-in is important for unbalanced or low-diversity libraries.

Prepare Reagents

Prepare HP3

1 Remove HP3 from 2°C to 8°C storage, and bring to room temperature.

Prepare a Fresh Dilution of NaOH

1 Combine the following volumes in a microcentrifuge tube:
- RNase/DNase-free water (950 µl)
- HP3 (50 µl)

The result is 1 ml of 0.1 N NaOH.

2 Invert the tube several times to mix.

CAUTION

Use the fresh dilution within **1 hour**.

Prepare PhiX Control

Dilute PhiX to 2 nM

1 Thaw a tube of 10 nM PhiX stock (10 µl/tube).

2 Combine the following volumes in a microcentrifuge tube.
- 10 nM PhiX (2 µl)
- RSB (8 µl)

The total volume is 10 µl at 2 nM.

3 Pipette up and down five times to mix.

Denature PhiX

1 Combine the following volumes in a microcentrifuge tube.
- 2 nM PhiX (10 µl)
- 0.1 N NaOH, freshly diluted (10 µl)

2 Vortex to mix.

3 Centrifuge briefly.

4 Incubate at room temperature for 5 minutes.

Dilute Denatured PhiX to Loading Concentration

1 Add 980 µl of prechilled HT1 to the tube of denatured PhiX.
The total volume is 1 ml at 20 pM.

2 Invert to mix, and then centrifuge at 280 × g for 1 minute.

NOTE

[Optional] Store the denatured 20 pM PhiX at -25°C to -15°C for up to 3 weeks as single-use 50 µl aliquots.

Combine Library and PhiX Control

1 Combine the following volumes of denatured PhiX control and denatured library.
- Denatured 20 pM PhiX control (2.5 µl)
- Denatured library (total volume of the denatured library solution)

2 Vortex to mix.

3 Centrifuge briefly.

4 Set aside on ice until you are ready to load the HiSeq 2500 during run setup.

Next Steps

After denaturing and diluting your libraries and preparing the optional PhiX control, you are ready to set up the sequencing run. For instructions, see the *HiSeq 2500 System Guide (document # 15035786)* .

Revision History

Document	Date	Description of Change
Document # 15050107 v06	January 2019	Corrected the concentration from 4 nM to 2 nM in the first dilution step for denaturing and diluting PhiX for TruSight Tumor 170 libraries.
Document # 15050107 v05	January 2019	Added Protocol C for denaturing and diluting TruSight Tumor 170 libraries to the HiSeq clustering section. Added a new procedure for denaturing and diluting PhiX for TruSight Tumor 170 libraries.
Document # 15050107 v04	July 2018	Removed references to GAIIx.
Document # 15050107 v03	November 2016	Corrected Illumina catalog # for cBot 2 Barcoded Strip tubes to 20005160.
Document # 15050107 v02	March 2016	Added procedures for denaturing and diluting libraries that have been normalized using a bead-based procedure. Organized procedures as Protocol A and Protocol B.
Document # 15050107 v01	January 2016	Included cBot 2 as a compatible cluster instrument. Instructions for denaturing and diluting libraries for cBot clustering apply to cBot 2 and cBot. Updated instructions for labeling the tubes of an 8-tube strip. Added Illumina catalog # for 8-tube strips with barcode labels. Removed information about library storage, which varies by library prep kit and is available in the library prep guides. Noted that the procedures described in this guide do not apply to sequencing on a HiSeq 3000/4000 flow cell.
Part # 15050107 Rev. C	November 2014	Added cluster densities for HiSeq Rapid Duo cBot Sample Loading Kit, HiSeq Rapid Cluster Kit v2, and TruSeq Rapid Cluster Kit. Added volumes of diluted libraries, with and without PhiX control, for HiSeq Rapid v2 flow cell.
Part # 15050107 Rev. B	June 2014	Added PhiX spike-in instructions for use with rapid run on-instrument clustering on the HiSeq 2500 or HiSeq 1500. Updated URL for Safety Data Sheets (SDS).
Part # 15050107 Rev. A	April 2014	Initial release.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

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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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