USER GUIDE

ion torrent
by life technologies™

Ion Amplicon Library Preparation (Fusion Method)

for use with: Ion Torrent Personal Genome Machine™ System

Publication Part Number 4468326 Rev. B
Revision Date 4 November 2011

life technologies™

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WEB SITE

www.iontorrent.com

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Part Number 4468326 Rev. B
11/2011

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Description

IMPORTANT! Before performing this procedure, read and understand the information the "Safety" section in this document.

This user guide describes how to generate amplicon libraries, by a fusion PCR method, for templated bead preparation and sequencing on the Personal Genome Machine™ (PGM™) System. Amplicon libraries are composed of multiple genomic target sequences that have been amplified by PCR.

Visit the Ion Community **http://ioncommunity.iontorrent.com** and **www.appliedbiosystems.com/iontorrent** for information about other methods and kits for preparation of Ion libraries, including the following options:

- Fragment libraries from mechanically or enzymatically sheared genomic DNA
- Libraries from short (<150 bp) or long (>400 bp) amplicons
- Libraries from total or small RNA
- Barcoded genomic DNA or amplicon libraries
- Automated library preparation

Method

The fusion PCR method uses fusion primers to attach the Ion A and truncated P1 (trP1) Adapters to the amplicons as they are generated in PCR. The fusion primers contain the A and trP1 sequences at their 5′-ends adjacent to the target-specific portions of the primers (see **Fusion PCR primer design** below).

Guidelines for designing amplicon libraries and primers

Library and primer design are critical to the success of the fusion PCR method. Use the following guidelines when planning your library generation strategy:

- The target region is the portion of the genome that will be sequenced in the sample(s) of interest. For example the target region could be an exon, a portion of an exon, or a non-coding region of the genome.
- Use standard guidelines for when designing PCR primers for your template of interest. For design assistance, use a web tool such as Primer3**: http://frodo.wi.mit.edu/primer3**
- Design your primers so that any sequence variants of interest are located **between** the primers, so that those variants are not masked by the template-specific part of the primer sequences (see Figure 1C — Example primers and amplicon design on page 5).
- The length of the target region must be considered carefully. Bidirectional sequencing is always recommended for optimal results.

Fusion PCR primer design

The fusion PCR method for preparing an amplicon library requires **four** fusion primers: two pairs of forward and reverse primers per target region, to enable bidirectional sequencing (Figure 1). Design the amplicons so that their length, including the fusion primers with adapter sequences, is shorter than the median library size for the target read length of the library.

Target read length	Median library size
200 bases (200 base-read library)	~330 bp
100 bases (100 base-read library)	~200 bp

One fusion primer pair has the A adapter region followed by the proximal end of the target sequence, and the other has the trP1 adapter region followed by the distal end of the target sequence. The other fusion primer pair has the adapter sequences A and trP1 swapped. See **Figures 1A—1C** for details.

Note: The target-specific portion of each primer should include 15–20 nucleotides of the target region.

Figure 1A — Bidirectional sequencing using the fusion method

Two primer pairs per target region generate two libraries to enable bidirectional sequencing of the target region. For target regions 75-280 bp in length, the resulting library is 125-330 bp in length.

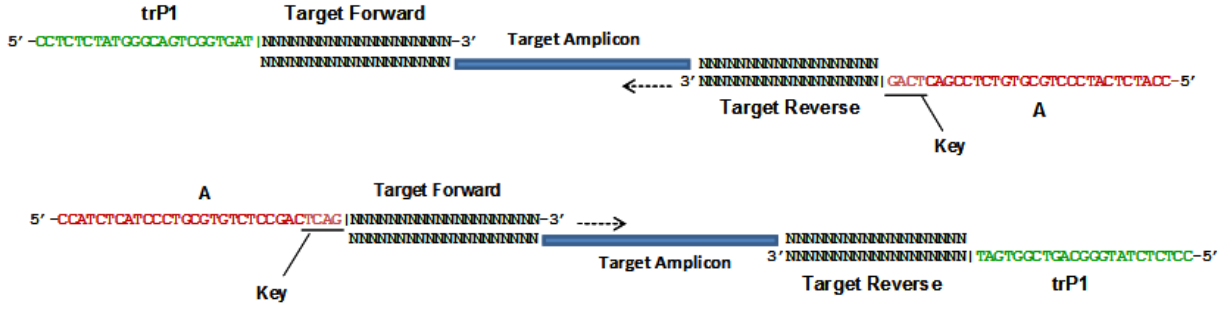


Figure 1B — Fusion PCR primers for bidirectional sequencing

A **Target Forward**
5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG|NNNNNNNNNNNNNNNNNNNN-3′
Key

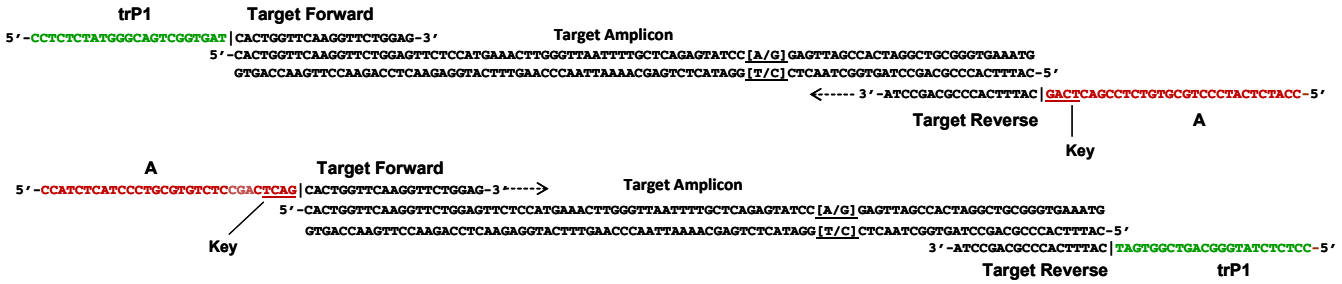
A **Target Reverse**
5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG|NNNNNNNNNNNNNNNNNNNN-3′
Key

trP1 **Target Forward**
5′-CCTCTCTATGGGCAGTCGGTGAT|NNNNNNNNNNNNNNNNNNN-3′

trP1 **Target Reverse**
5′-CCTCTCTATGGGCAGTCGGTGAT|NNNNNNNNNNNNNNNNNNN-3′

Figure 1C — Example primers and amplicon design

Design your primers so that any sequence variants of interest are located **between** the primers, so that those variants are not masked by the template-specific part of the primer sequences.



Materials and equipment needed

Use common commercial kits for all procedure steps. To analyze the amplicon library size distribution, use the BioAnalyzer™ 2100 instrument or conventional gel electrophoresis.

The following table lists required materials and equipment:

✓	Description	Supplier	Part number	Quantity
	PCR primers, 2 pairs per amplicon (10 µM each forward and reverse; see Figure 1 for design)	MLS	Varies	10 µM stock per primer
	Platinum® PCR SuperMix High Fidelity	Invitrogen	12532016 12532024	100 reactions 5000 reactions
	Nuclease-free Water	Applied Biosystems	AM9932	1000 mL
	TE, pH 8.0	Applied Biosystems	AM9849	0.5 L
	Ethanol, absolute	MLS	N/A	N/A
	Agencourt® AMPure® XP Kit	Beckman Coulter	A63880 or A63881	1
	Agencourt® SPRIPlate 96R Magnet Plate *or* Magna-Sep™ 96 Magnetic Particle Separator	Beckman Coulter Invitrogen	A32782 K1585-96	1 each
	(*Optional*) MinElute PCR Purification Kit	Qiagen	28004	1 kit
	Agilent BioAnalyzer™ 2100 instrument	Agilent	G2939AA	1
	Agilent DNA 1000 Kit	Agilent	5067-1504	1 kit
	Agilent High Sensitivity DNA Kit	Agilent	5067-4626	1 kit
	0.2-mL PCR strip tubes *or* 96-well PCR plate	MLS	N/A	1 box
	(*Optional*) 1.5-mL LoBind Tubes	Eppendorf	022431021	1 box
	Pipettors 1–1000 µL	Major Laboratory Supplier (MLS)	N/A	1 each
	Barrier pipette tips	MLS	N/A	1 box each
	Microcentrifuge	MLS	N/A	1
	Thermal cycler	MLS	N/A	1 each

Procedural guidelines

- Use good laboratory practices to minimize cross-contamination of products. If possible, perform library construction in an area or room that is distinct from that of template preparation.
- Perform all steps requiring 1.5-mL tubes with 1.5-mL Eppendorf LoBind Tubes (Eppendorf Part no. 022431021).
- Thaw reagents on ice before use, and keep enzymes at –20°C until ready to use.

CAUTION! A primary source of contamination is spurious DNA fragments from previous sample processing steps. Do not introduce amplified DNA into the library preparation laboratory or work area.

Workflow

PCR amplify genomic DNA targets

▼

Purify the amplicon libraries

▼

Prepare an equimolar pool of the amplicon libraries

▼

Determine the dilution required for Template Preparation

Procedure

PCR amplify genomic DNA targets

A singleplex PCR reaction is described in this section. To save time and reagents, you can perform multiplex PCR if the primers have been specifically designed for multiplex PCR.

IMPORTANT! Multiplexing may increase the probability of unwanted PCR products and of over- or under-represented amplicons in the pool.

Note: Remember that for bidirectional sequencing, each target region has two sets of primers, and must be amplified in separate PCR reactions.

Materials required for this procedure

- 10 µM of forward and reverse primers
- 0.2-mL strip tubes or 96-well Eppendorf® plate
- Platinum® PCR SuperMix High Fidelity
- High-quality genomic DNA

1. Thaw the PCR primers, Platinum® PCR SuperMix High Fidelity, and high-quality genomic DNA on ice.
2. For each primer pair, mix equal volumes of 10 µM forward and 10 µM reverse primers to prepare a 10-µM primer stock mix (5 µM of each primer).
3. Add the following reagents to 0.2-mL strip tubes or to the wells in a 96-well PCR plate exactly in this order:

Component	Volume
Platinum® PCR SuperMix High Fidelity	45 µL
20–50 ng genomic DNA	4 µL
10-µM primer stock mix	1 µL
Total	**50 µL**

4. Load the tubes or plates in a thermal cycler and run the program to amplify the target DNA:

   Note: Amplification conditions may vary according to primer design and DNA input. Adjust the cycling conditions and number of cycles for your specific experiment to achieve optimal results.

Stage	Step	Temperature	Time
Holding	Activate the enzyme	94ºC	3 min
Cycling (40 cycles)	Denature	94ºC	30 sec
	Anneal	58ºC	30 sec
	Extend	68ºC	1 min/kb
Holding	—	4ºC	∞

Purify the amplicon libraries

IMPORTANT! If the total fragment size, including amplicon and fusion primer sequence, is <100 bp, use a different purification method such as Qiagen MinElute PCR Purification Kit.

Note: Use 1.8 volumes of Agencourt® AMPure® XP Reagent for every volume of pooled DNA.

Materials required for this procedure

- Agencourt AMPure® XP Reagent
- Amplified genomic DNA
- TE, pH 8.0
- Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator
- 70% ethanol

1. Resuspend the Agencourt® AMPure® XP Reagent and allow the mixture to come to room temperature (~30 minutes).
2. Prepare 70% ethanol:

Component	Volume
Nuclease-Free Water	300 µL
Ethanol, Absolute	700 µL
Total	**1000 µL**

IMPORTANT! Use *freshly prepared* 70% ethanol. A higher percentage of ethanol causes inefficient washing of smaller-sized molecules. A lower percentage of ethanol could cause sample loss.

3. Add Agencourt® AMPure® XP Reagent to each sample as indicated below, mix the bead suspension with the DNA by pipetting up and down several times, and incubate the samples at room temperature for 5 minutes.

Component	Volume
Amplified genomic DNA	50 µL
Agencourt® AMPure® XP Reagent	90 µL*

* Equal to 1.8 volumes of genomic DNA

4. Place each plate or tube on a magnet (such as the Agencourt® SPRIPlate 96R Magnet Plate or Magna-Sep™ 96 Magnetic Particle Separator) for 2 minutes. After the solution clears, carefully remove and discard the supernatant from each sample without disturbing the pellet.
5. Without removing the samples from the magnet, add 30 µL of freshly prepared 70% ethanol to each well or tube and incubate the samples at room temperature for 30 seconds. After the solution clears, remove and discard the supernatant without disturbing the pellet.
6. Repeat step 5 for a second wash.
7. To remove residual ethanol, keep the samples on the magnet and carefully aspirate remaining supernatant with a 20-µL pipet without disturbing the pellet.

8. Air-dry the beads on the magnet at room temperature for ≤5 minutes.
9. Remove the samples from the magnet, add 20 µL of TE to each well or tube, and pipet the samples up and down to mix.
10. Place the plate or tube on the magnet for at least 1 minute until the solution clears and the beads are pelleted. Transfer the supernatant containing the amplicon library to a new well or tube.

IMPORTANT! The ***supernatant*** contains the amplicon library. **Do not discard!**

Prepare an equimolar pool of the amplicon libraries

Pooling the amplicon libraries in equimolar amounts for Ion library construction ensures even coverage of the target regions. In the following procedure, each amplicon library is analyzed on the Agilent Bioanalyzer™ instrument to:

- Quantitate each amplicon library for pooling
- Assess the size distribution of each library

Materials required for this procedure

- Agilent Bioanalyzer™ 2100 instrument
- Agilent DNA 1000 Kit
- Amplified genomic DNA
- TE, pH 8.0

1. Analyze an aliquot of each amplicon library with a Bioanalyzer™ instrument and Agilent DNA 1000 Kit. Follow the manufacturer's instructions.

   If a library is too dilute to detect with a DNA1000 Kit, use an Agilent High Sensitivity Kit.

IMPORTANT! Ensure that excessive amounts of primer-dimers (immediately adjacent to the marker) or over-amplification products (concatemers) are not present. See **Figures 2–4** in the Appendix for example Bioanalyzer™ traces. For more information, contact Life Technologies Technical Support.

2. Determine the molar concentration (nmol/L) of each amplicon library using the Bioanalyzer™ software. If necessary, use manual integration to place the entire range of library fragments within a single peak.
3. Prepare an equimolar pool of amplicon libraries at the highest possible concentration.
4. Calculate the combined concentration of the pooled amplicon library stock.

   Alternatively, analyze an aliquot of the library pool on the Bioanalyzer™ with an Agilent DNA 1000 Kit, and use the Bioanalyzer™ software to determine the molar concentration of the pooled library stock. If necessary, use manual integration to place the entire distribution of library molecules within a single peak. Follow the manufacturer's instructions.

STOPPING POINT Store the pooled library stock at –20°C. Before use, thaw the storage stock on ice. To reduce the number of freeze-thaw cycles, store the pooled stock in several aliquots.

Determine the dilution required for Template Preparation

Determine the Template Dilution Factor that gives a concentration of ~26 pM (~15.5 × 10^6 molecules per µL). This concentration is suitable for template preparation using either the Ion Xpress™ Template Kits or the Ion OneTouch™ System. Use the following formula:

Template Dilution Factor = (Library concentration in pM)/26 pM

Example: The library concentration is 10,000 pM.

Template Dilution Factor = 10,000 pM/26 pM = 385

Thus, 1 µL of library mixed with 385 µL of Low TE (1:385 dilution) yields approximately 26 pM (15.5 × 10^6 molecules per µL) for either the Ion Xpress™ Template Kits or the Ion OneTouch™ System procedure.

Note: When you perform the Template Preparation procedure, you will need to prepare three serial dilutions of the library at ½× Template Dilution Factor, Template Dilution Factor, and 2× Template Dilution Factor, to ensure that one or more dilutions are in the optimized concentration range.

Note: Do not quantitate fusion PCR amplicon libraries with the Ion Library Quantitation Kit (Part no. 4468802). Libraries prepared using trP1 and A sequences are incompatible with this qPCR method.

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Proceed to Template Preparation

The libraries are ready for the downstream Template Preparation procedure for clonal amplification on Ion Spheres™ using one of the following methods:

- **For 200 base-read libraries**: The Ion Xpress™ Template 200 Kit (Part no. 4471253).
- **For 100 base-read libraries**:
  - The Ion Xpress™ Template Kit (Part no. 4469001) for non-automated template preparation.
  - The automated Ion OneTouch™ System (Part no. 4470001).

The user guides are available at the Ion Torrent users community. Visit **http://ioncommunity.iontorrent.com** and **www.appliedbiosystems.com/iontorrent**.

Appendix: Bioanalyzer™ instrument analysis of amplicon libraries

Figure 2: Example Bioanalyzer™ analysis of amplicon library pool

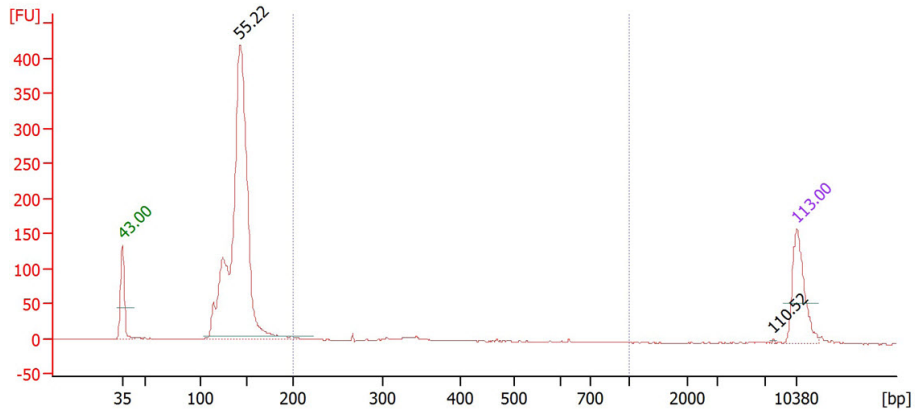


Figure 3: Example Bioanalyzer™ analysis of a single amplicon library before pooling

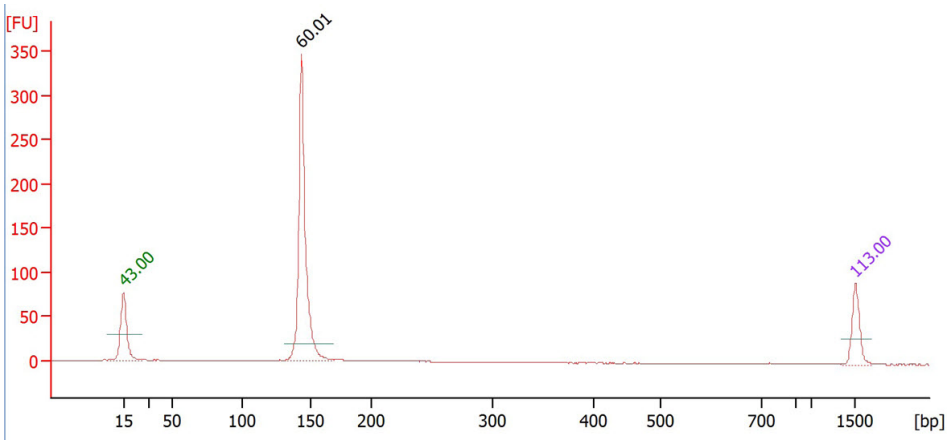
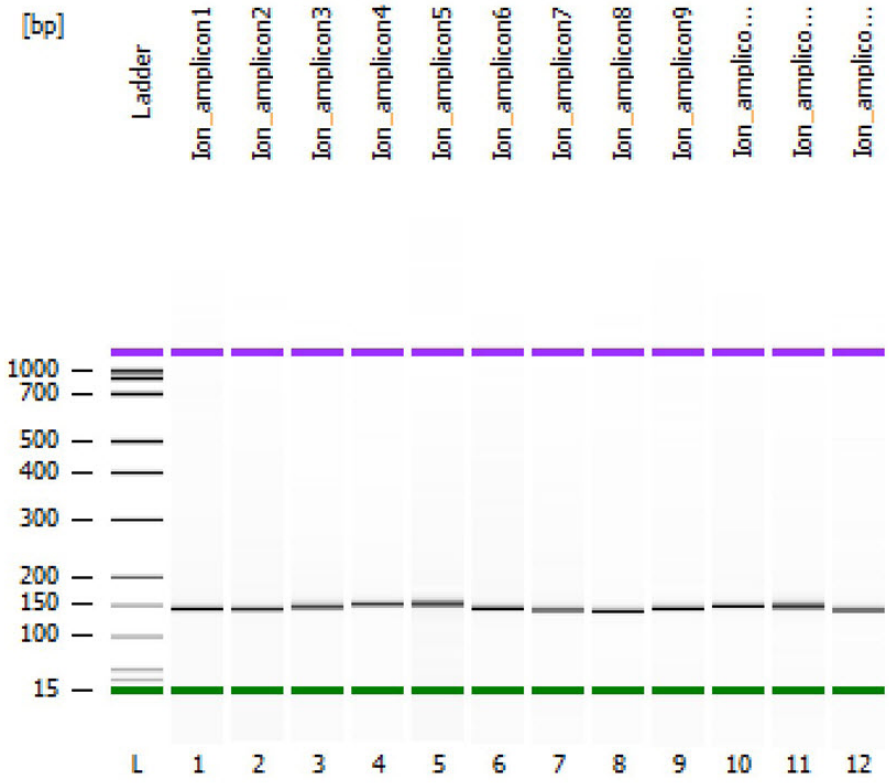


Figure 4: Example Bioanalyzer™ analysis of an amplicon set before pooling



Safety

Safety information

WARNING! General safety. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc). To obtain SDSs, see the "Documentation and Support" section in this document.

Chemical safety

WARNING! General chemical handling. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! Hazardous waste (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! Biohazard. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the information below:

In the U.S.:

- U.S. Department of Health and Human Services guidelines published in Biosafety in Microbiological and Biomedical Laboratories found at:
  www.cdc.gov/biosafety
- Occupational Safety and Health Standards, Bloodborne Pathogens
  (29 CFR§1910.1030), found at: **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at: **www.cdc.gov**

In the EU:

Check local guidelines and legislation on biohazard and biosafety precaution and refer to the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition, found at:
www.who.int/csr/resources/publications/biosafety/ WHO_CDS_CSR_LYO_2004_11/en/

Documentation and support

Obtaining SDSs

Safety Data Sheets (SDSs) are available from **www.lifetechnologies.com/support**

For the SDSs of chemicals not distributed by Life Technologies, contact the chemical manufacturer.

Obtaining support

For the latest services and support information for all locations, go to:

www.iontorrent.com/support

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Search for user documents, application notes, and other product support documents

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