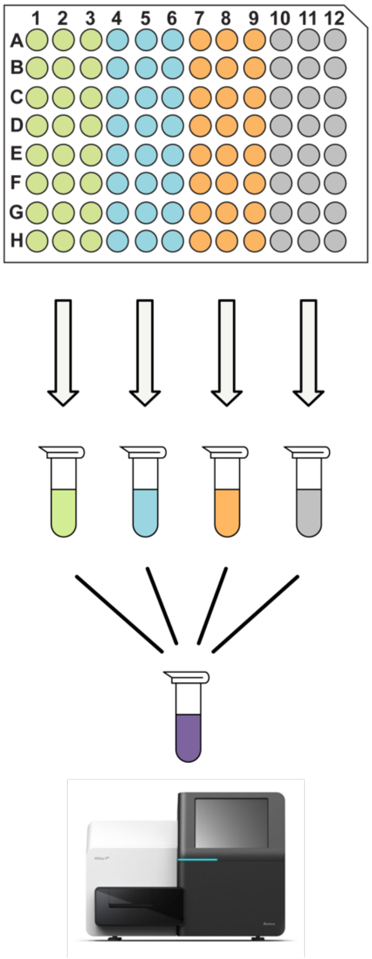
HLA-24S-v6 Protocol

Overview

Scis*Go*™ HLA Typing Kit v6 is designed for use on the Illumina® MiSeq™ Sequencing by Synthesis (SBS) platform to identify HLA alleles for each of HLA-A, B, C, DRB1345, DQA1, DQB1, DPA1, DPB1. All loci are reported at 3-field resolution – exons 1-7 for CI and exons 1-4 for CII – including reporting of all know intron and exon encoded null alleles and detection of novel variants.



1. Stage 1 PCR

Add DNA and Stage 1 mix to pre-plated amplicon mixes.

2. Stage 2 PCR

Add Stage 2 mix directly to wells.

No sample transfer.

3. Pool and Purify

Combine all reactions for each amp and column purify.

4. Quantify and Pool

Combine pools for sequencing.

5. Illumina Sequencing

Reagents

Included in Kit

① Stage 1 PCR – Reaction Plate……...…………..……...…………...10 µl per well (96 well plate)
② Stage 1 PCR – S1 Buffer………………………………………….……...……....1.5 ml (2 ml tube)
③ Stage 2 PCR – S2 Buffer…………………………………………………...…..1.8 ml (2 ml tube)
④ Sequencing Primers – R1, Index, and R2…………………………………30 µl each (2 ml tube)

Not Included in Kit

① Platinum™ Taq DNA Polymerase (5 U/ µl)………….……….Invitrogen™ (Cat. No. 10966018)
② Select-A size DNA Clean & Concentrator™…................Zymo Research® (Cat. No. D4080)[a]
③ Quant-It™ PicoGreen® dsDNA Assay Kit……...Thermo Fisher Scientific® (Cat. No. P11496)[b]
④ MiSeq™ Reagent Kit V2……….………....Illumina® (Cat. No. MS-102-2003 or MS-103-1003)
⑤ 2N Sodium Hydroxide (2N NaOH)…………………….........J.T.Baker™ (Cat. No. 02-004-137)[c]

[a] Alternative size selection kits are applicable, e.g. Qiagen® GeneRead *Size Selection* Kit (Cat. No. 180514).
[b] Alternative quantification methods are applicable, e.g. Qubit™ dsDNA HS Assay Kit (Cat. No. Q32854).
[c] Alternative molecular grade NaOH applicable, e.g. Honeywell® Fluka™ NaOH (2N) (Cat. No. 35254-1L).

Equipment Requirements

① Single- and multi-channel pipettes ranging from 2 µl to 1000 µl
② 96-well thermocycler (e.g. GeneAmp® 9700)
③ Plate centrifuge, microcentrifuge, and vortexer
④ Agarose gel electrophoresis apparatus (optional)
⑤ Flourometer (e.g. BioTek® Synergy™ or Qubit®)
⑥ Illumina® MiSeq® Instrument

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Protocol

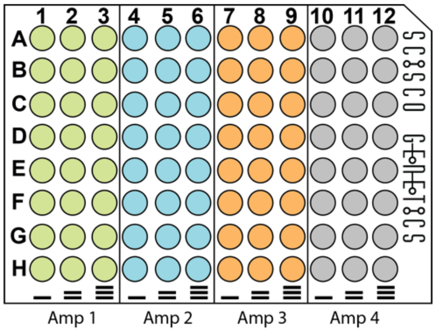
I. Stage 1 PCR

A. Thaw Reaction Plate and S1 Buffer at RT, vortex well and spin down at 1400-2000 rpm for at least 1 min before removing well-caps.

B. Transfer 2 µl of DNA (minimum ~1 ng/µl) to the corresponding amplicon wells. For example, if your 24 template DNAs are plated in three columns of a 96 well PCR plate (referred to below as DNA plate), then use an 8-channel pipette to do as follows:

- — Transfer DNA from column 1 of DNA plate to columns 1, 4, 7, and 10 of Reaction Plate. Pipette onto sidewalls and check that every well has a droplet.
- = Transfer DNA from column 2 of DNA plate to columns 2, 5, 8, and 11 of Reaction Plate. Pipette onto sidewalls and check that every well has a droplet.
- ≡ Transfer DNA from column 3 of DNA plate to columns 3, 6, 9, and 12 of Reaction Plate. Pipette onto sidewalls and check that every well has a droplet.

Figure 1. Amplicon Primer Plate Layout for HLA-24S-v6



C. Inspect Reaction Plate to confirm that each well contains DNA, then spin down.

D. Thoroughly vortex S1 Buffer.

E. Add 19 µl of Platinum™ Taq to the S1 Buffer tube and gently vortex.

F. Add 8 µl of the S1 Buffer and Platinum™ Taq mix to each well and spin down.

G. Seal Reaction Plate and run the following thermocycling routine:

Stage 1 PCR Routine

95 °C	→	95 °C	→	60 °C	→	72 °C	→	4 °C
15 min		30 sec		30 sec		1 min		Hold
		↑ 20 cycles						

II. Stage 2 PCR

A. Remove Reaction Plate from thermocycler and spin down at 1400-2000 rpm for at least 1 min.

B. Thaw S2 Buffer immediately before use. After thawing, thoroughly vortex.

C. Add 10 µl of S2 Buffer to each corresponding well of Reaction Plate, then spin down. ***Do not transfer the PCR product to a new plate.***

D. Seal Reaction Plate and run the following thermocycling routine:

Stage 2 PCR Routine

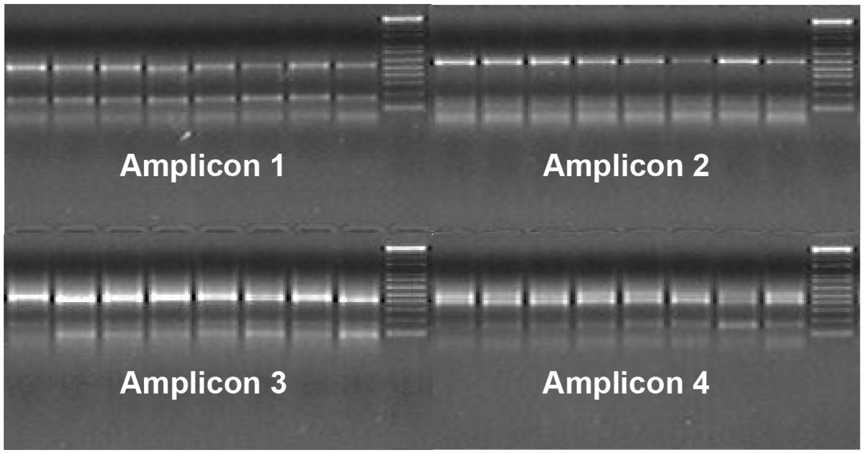
37 °C →	95 °C →	95 °C →	50 °C →	72 °C →	72 °C →	4 °C
60 min	15 min	30 sec	30 sec	1 min	7 min	Hold
		↑ 25 cycles				

E. Proceed to Step III or store at -20 °C for 6 months.

III. Reaction Check (Optional)

A. After Stage 2 amplification is complete, spin down the Reaction Plate and remove 3 µl of amplicon product from several samples, e.g., 8 per amplicon, and visualize on a 1.5% agarose gel. Bands should appear as in Figure 2 (~300 bp):

Figure 2. ***Gel image to check reactions after Stage 2 PCR***



B. Proceed to Step IV or store at -20 °C for 6 months.

IV. Amplicon Sample Pooling

A. For each amplicon, transfer 20 µl from each reaction well into a microcentrifuge tube.

B. Thoroughly vortex each of the 4 amplicon pools and proceed to Step V. or store at – 20 °C for up to 6 months.

V. Clean Up and Size Selection of Amplicon Pools

The objective of this step is to remove short DNA fragments (< 250 bp) from the amplicon pools to optimize the percentage of usable clusters within a MiSeq™ run. Of the available silica-based columns for size selection, we recommend the Zymo Research® *Select-A-Size DNA* Clean and Concentrator™ (Cat. No. D4080). Alternative size selection options include GeneRead™ Size Selection Kit (Qiagen® Cat. No. 180514) and 0.7X AMPure XP Magnetic SPRI Beads (Beckman Coulter® Cat. No. A63880).

For each of the 3 options below, use 100 µl of each amplicon pool for purification followed by visualization of the products on a 1.5% agarose gel to confirm removal of bands < 200 bp (see Figure 3).

- **Option 1:** *Select-A-Size DNA* Clean and Concentrator™ (Zymo Research® Cat. No. D4080)

A. Follow Zymo Research® guide (Instruction Manual *Select-A-Size DNA* Clean and Concentrator™). We recommend the following parameter changes:

a. Prepare Size Selection Binding Buffer Mix according to Table 1:

Table 1: Zymo Size Selection Binding Buffer Mix

Component	Zymo DNA Binding Buffer	Molecular Grade NaOH (2N)	Molecular Grade 100% Ethanol	*Total Volume*
Total (5 rxns)	2480 µl	20 µl	150 µl	*2650 µl*

b. Add 530 µl of Binding Buffer Mix to 100 µl of pooled samples.

c. Elute with 40 µl of elution buffer.

- **Option 2:** GeneRead™ Size Selection Kit (Qiagen® Cat. No. 180514)

A. Follow Qiagen® guide (Protocol: GeneRead Size Selection of DNA Libraries Prepared with the GeneRead™ DNA Library Prep I Kit). We recommend the following parameters:

a. Allow column to incubate at RT **5 min** after adding Buffer TE to membrane.

b. For elution, add **30 µl** Buffer EB and allow column to incubate at RT for **5 min**.

Note: If the above protocol does not produce adequate yield, use a higher volume of DNA or try a single pass approach as detailed in “Protocol: GeneRead Size Selection of Sheared DNA in Common Elution Buffers”.

- **Option 3:** 0.7X AMPure XP Magnetic Bead Cleanup (Beckman Coulter® Cat. No. A63880)

A. Follow the protocol below:

  a. Ensure Ampure beads have equilibrated to room temperature before use.

  b. Aliquot 0.7X volume of AMPure beads into a fresh 2.0 ml tube, add 1x volume of each amplicon pool to AMPure beads (e.g. 100 ul amplicon pool to 70 μl AMPure beads).

  c. Thoroughly mix each amplicon pool and beads via vortex and quick spin to gather contents at bottom of tube. Incubate at RT for 5 min.

  d. Place each 2.0 ml tube onto the magnetic tube rack until solution has completely cleared (~5min), carefully aspirate supernatant and dispose.

  e. Remove tubes from magnetic tube rack, and add 1 ml of 70% ETOH, gently vortex to resuspend beads and quickly spin to gather contents at tube bottom.

  f. Place tubes on magnetic tube rack until solution has completely cleared (~5 min), carefully aspirate supernatant and dispose.

  g. Repeats steps e. and f.

  h. Remove tubes from magnetic rack and spin for ~30 seconds to collect all beads and residual ETOH into bottom of tube, and place each tube on rack.

  i. Carefully aspirate away any residual ETOH from bottom of tube and then remove tube from magnetic stand.

  j. Allow bead pellet to dry at RT for 3-5 min. Be careful to not over-dry bead pellet.

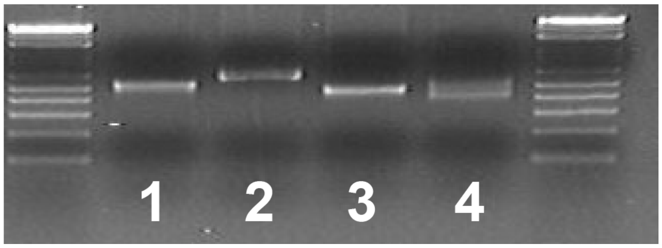
  k. Add 30 μl Qiagen® elution buffer to bead pellet and flick/vortex to completely resuspend beads.

  l. Quick spin to collect contents at bottom of tube and incubate for at least 5 min.

  m. Place tubes on magnetic tube rack until solution has completely cleared (~5 min).

  n. Slowly aspirate supernatant. Do not disturb bead pellet. Aliquot supernatant into clean 1.5 ml tube. This supernatant contains the purified amplicon pool and is ready to be quantified, combined with other amplicon pools, and run on the MiSeq™.

Figure 3. NGS library after final purification



VI. Reaction Product Quantification

The objective of this step is to achieve an optimal balance among the 4 amplicon reaction products. Any method that accurately measures mass and/or molar quantities will provide the information needed to perform this step. Concentrations are derived from the chosen quantification method with derivative values entered directly into the HLA-v6-Pooling-Workbook.xlsx, enabling calculation of optimal volumes of each amplicon for combination before MiSeq™ loading.

- **Option 1:** Quant-iT™ PicoGreen® dsDNA Kit (Thermo Fisher Scientific® Cat. No. P11496)
  A. Follow PicoGreen® guide (Quant-iT™ PicoGreen® dsDNA Kit Reagent and Kits). We recommend the following parameters:
    a. Add 200 µl PicoGreen® dilution mix to each of the 10 wells of a 96-well measurement plate (e.g., Nunclon®, Cat. No. 137101).
    b. Take duplicate readings. Add 2 µl of each purified amplicon pool. Add 2 µl Lamba Standard to adjacent wells for comparison (Ex/Em: 480/520 nm).
    c. Allow to incubate at RT for 5 minutes.
  B. Enter the measured value (RFU) into the "Quant and Pool Picogreen" worksheet in the HLA-v6-Pooling-Workbook.xlsx.

- **Option 2:** Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific® Cat. No. Q32854)
  A. Follow Qubit® guide (User Guide: Qubit® dsDNA HS Assay Kit). We recommend the following parameter:
    a. Measure 2 µl of each purified amplicon pool.
  B. Enter the measured value (ng/µl) into the "Quant and Pool Qubit HS" worksheet in the HLA-v6-Pooling-Workbook.xlsx.

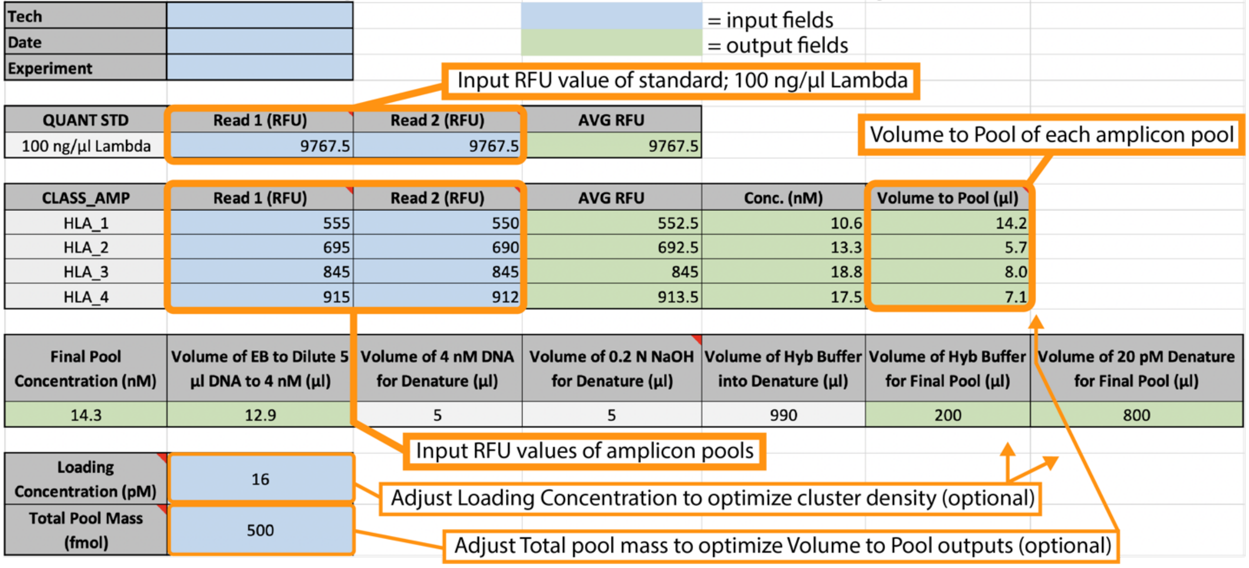
- **Option 3:** User-defined method for quantitating DNA concentrations
  A. Enter measured DNA concentration (ng/µl) into the "Quant and Pool Qubit HS" worksheet in the HLA-v6-Pooling-Workbook.xlsx.

VII. Combine Quantified Pools

A. Using volumes given by the HLA-v6-Pooling-Workbook.xlsx, combine the amplicon pools in a new 1.5 ml microcentrifuge tube.

B. Thoroughly vortex the pooled library and use the HLA-v6-Pooling-Workbook.xlsx for MiSeq™ library dilution to calculate EB Buffer volume. The HLA-v6-Pooling-Workbook.xlsx contains two worksheets titled “Quant and Pool Picogreen,” and “Quant and Pool Qubit HS.” They perform calculations for use with the Quant-iT™ PicoGreen® dsDNA Kit and the Qubit® dsDNA HS Assay Kit, respectively. The “Quant and Pool Qubit HS” worksheet can also be used with any user-defined method for quantitating DNA concentrations. Refer to the screenshots below for additional information.

HLA-v6-Pooling-Workbook.xlsx, “Quant and Pool Picogreen” worksheet



Tech	
Date	
Experiment	

= input fields
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Input RFU value of standard; 100 ng/µl Lambda

Volume to Pool of each amplicon pool

QUANT STD	Read 1 (RFU)	Read 2 (RFU)	AVG RFU
100 ng/µl Lambda	9767.5	9767.5	9767.5

CLASS_AMP	Read 1 (RFU)	Read 2 (RFU)	AVG RFU	Conc. (nM)	Volume to Pool (µl)
HLA_1	555	550	552.5	10.6	14.2
HLA_2	695	690	692.5	13.3	5.7
HLA_3	845	845	845	18.8	8.0
HLA_4	915	912	913.5	17.5	7.1

Final Pool Concentration (nM)	Volume of EB to Dilute 5 µl DNA to 4 nM (µl)	Volume of 4 nM DNA for Denature (µl)	Volume of 0.2 N NaOH for Denature (µl)	Volume of Hyb Buffer into Denature (µl)	Volume of Hyb Buffer for Final Pool (µl)	Volume of 20 pM Denature for Final Pool (µl)
14.3	12.9	5	5	990	200	800

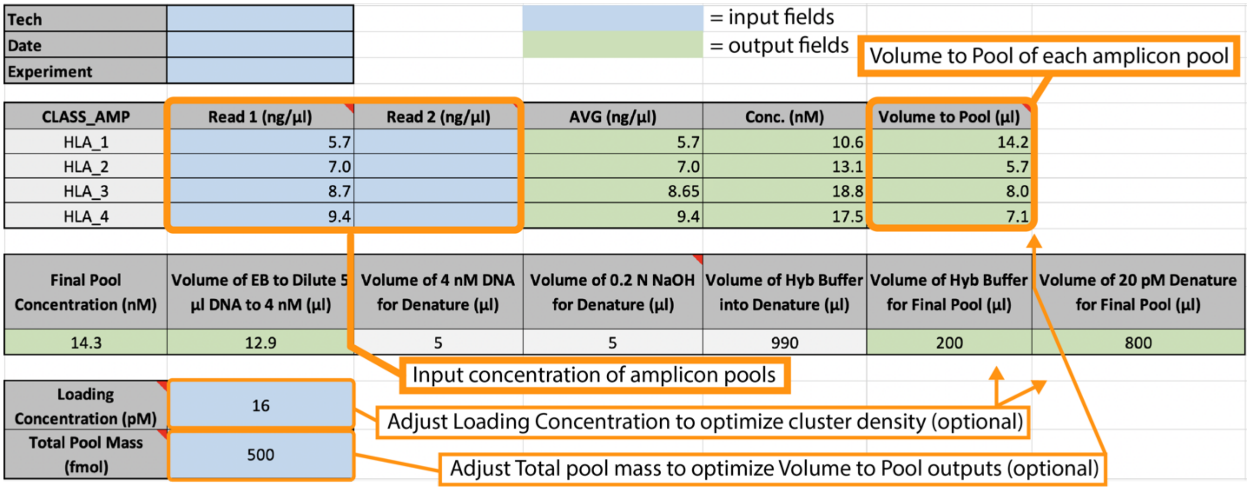
Input RFU values of amplicon pools

Loading Concentration (pM)	16
Total Pool Mass (fmol)	500

Adjust Loading Concentration to optimize cluster density (optional)

Adjust Total pool mass to optimize Volume to Pool outputs (optional)

HLA-v6-Pooling-Workbook.xlsx, “Quant and Pool Qubit HS” worksheet



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Experiment	

= input fields
= output fields

Volume to Pool of each amplicon pool

CLASS_AMP	Read 1 (ng/µl)	Read 2 (ng/µl)	AVG (ng/µl)	Conc. (nM)	Volume to Pool (µl)
HLA_1	5.7		5.7	10.6	14.2
HLA_2	7.0		7.0	13.1	5.7
HLA_3	8.7		8.65	18.8	8.0
HLA_4	9.4		9.4	17.5	7.1

Final Pool Concentration (nM)	Volume of EB to Dilute 5 µl DNA to 4 nM (µl)	Volume of 4 nM DNA for Denature (µl)	Volume of 0.2 N NaOH for Denature (µl)	Volume of Hyb Buffer into Denature (µl)	Volume of Hyb Buffer for Final Pool (µl)	Volume of 20 pM Denature for Final Pool (µl)
14.3	12.9	5	5	990	200	800

Input concentration of amplicon pools

Loading Concentration (pM)	16
Total Pool Mass (fmol)	500

Adjust Loading Concentration to optimize cluster density (optional)

Adjust Total pool mass to optimize Volume to Pool outputs (optional)

VIII. MiSeq Preparation and Loading

A. Follow the Illumina® MiSeq™ preparation instructions with the below parameters:

Step	Details
Dilute to 4 nM	5 µl pooled library See pooling template for EB Buffer volume
Denature	5 µl 4 nM library 5 µl 0.2N NaOH mix thoroughly
Incubate	5 min @ RT
Dilute denatured DNA	990 µl HT-1 Buffer 10 µl denatured library
Dilute to loading concentration (16 pM)*	800 µl 20 pM denatured library 200 µl HT-1 Buffer
Dilute MiSeq Primers	770 µl HT-1 Added directly to each Scis*Go*™ R1, Index, and R2 vial
Add to tray	Port 17: add 600 µL of diluted library Port 18: add 600 µL of diluted R1 primer Port 19: add 600 µL of diluted Index primer Port 20: add 600 µL of diluted R2 primer

*Cluster density can be adjusted directly proportional to loading concentration.

B. Follow Illumina® guidelines to rinse and dry the flowcell.

C. Create a sample sheet using the MiSeq™ sample sheet template corresponding to the Scis*Go*™ kit in use (HLA-24S-v6) and the specific index system (A1, A2, or both).

  a. Fill in cells adjacent to cells labeled “Investigator Name”, “Project Name”, and “Experiment Name” (do not use commas, slashes, or spaces when filling these cells).

  b. Input sample ID names into cells under cell labeled “Sample_Name”.

  c. Click “save as” and use the MS Number on the MiSeq cartridge to name the file (MSxxxxxxx-500V2).

  d. Save as .csv file.

D. Load the sample sheet on the MiSeq™ instrument.

E. Go to the Illumina® MiSeq™ Control Software and follow the on-screen instructions to load the MiSeq™ and begin sequencing.

Note: Integrity of kit components is guaranteed for up to 6 months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.