Full-length characterization of novel HLA-DRB1 alleles for reference database submission

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Abstract

The prerequisite for successful HLA genotyping is the integrity of the large allele reference database IPD-IMGT/HLA. Consequently, it is in the laboratories' best interest that the data quality of submitted novel sequences is high. However, due to its long and variable length, the gene HLA-DRB1 presents the biggest challenge and as of today only 16% of the HLA-DRB1 alleles in the database are characterized in full length. To improve this situation, we developed a protocol for long-range PCR amplification of targeted HLA-DRB1 alleles. By subsequently combining both long-read and short-read sequencing technologies our protocol ensures phased and error-corrected sequences of reference grade quality. This dual redundant reference sequencing (DR2S) approach is of particular importance for correctly resolving the challenging repeat regions of DRB1 intron1. Until today, we used this protocol to characterize and submit 384 full-length HLA-DRB1 sequences to IPD-IMGT/HLA.

1 Introduction

The human leukocyte antigen (HLA) genes are the most diverse genes in the human genome. As of today, 3,389 HLA-DRB1 alleles are known and encode 2,203 distinct proteins (IPD-IMGT/HLA release 3.50, 10/2022) ***(1)***. Despite the high number of named alleles, the identification of novel HLA-DRB1 alleles is not a rare event during stem cell donor registry genotyping. In contrast to other HLA genes, most laboratories are continuing to submit only the sequences of exons 2 and 3 to the IPD-IMGT/HLA database resulting in poor representation of HLA-DRB1 diversity outside this region. One reason for this is the length of HLA-DRB1, and more specifically the varying length of the different HLA-DRB1 alleles. Due to large sequence variations in intron 1, HLA-DRB1 alleles can span between 11 and 16 kb. While this length in itself poses a substantial challenge for PCR success, preferential amplification will often result in the loss of the longer allele in the presence of a shorter allele. Due to these difficulties, most novel HLA-DRB1 alleles are submitted to the IPD-IMGT/HLA database as partial sequences only. Unfortunately, these partial sequences can then cause

inconsistencies in genotyping algorithms because missing bases need to be imputed. It would therefore be valuable for the IPD-IMGT/HLA database and the HLA genotyping laboratories if novel HLA-DRB1 alleles would be characterized and submitted in full length.

To overcome the challenge of HLA-DRB1 full-gene characterization, several strategies are available. One protocol splits the long gene in two or more PCR amplicons of defined length, which can then be sequenced separately ***(2)***. This will in all likelihood increase PCR success and might be the method of choice if the genomic DNA is of low molecular weight. However, it might not always be possible to phase the individual parts unambiguously. Therefore, one PCR covering the complete gene is preferable. To avoid the length-biased PCR amplification, we developed primer pairs that specifically amplify HLA-DRB1 alleles of similar length. This strategy requires the prior knowledge of the targeted allele in at least allele-group resolution (first field). This information is usually available if the workflow is used for the characterization of novel alleles.

For submission of novel alleles to IPD/IMGT-HLA, sequencing data from two independent PCR reactions per sample are obligatory. While this requirement is generally satisfied by using the same sequencing method twice, we prefer to exploit the strengths of two different technologies: long-read PacBio HiFi- or Oxford Nanopore (ONT) sequencing to obtain a fully phased sequence and short-read Illumina shotgun sequencing to correct for potential sequencing errors. We developed DR2S (Dual Redundant Reference Sequencing) to integrate the analysis of both long and short reads and generate reference quality sequence data ***(3, 4)***.

Within this chapter, we share the protocol for HLA-DRB1 full-length characterization starting at the laboratory and ending at sequence submission to the IPD-IMGT/HLA database. This protocol has been widely tested and used successfully for the characterization of over 300 novel HLA-DRB1 alleles from buccal swab material.

2 Materials

2.1 PCR Primers

HLA-DRB1 PCR (see note 1):

Forward1 (F1): <Overlap Sequence>CAGATGCTGATTCGTTCTCCAACACT
Forward2 (F2): <Overlap Sequence>TTCTCCAACACGAGATTACCCAACC
Reverse1 (R1): <Overlap Sequence>AGCACAAAACTTGGAAGATGAGGGG
Reverse2 (R2): <Overlap Sequence>GAGCACAAAAGTTGAAGATGAGGCG
Reverse3 (R3): <Overlap Sequence>GAGCACAAAAGTTGAAGATGAGGCAC
Overlap Sequence PacBio forward primer: ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Overlap Sequence PacBio reverse primer: GTGACTGGAGTTCAGA
Overlap Sequence ONT forward primer: ACTTCGTACGTACGGCGTCTTATAC
Overlap Sequence ONT reverse primer: GAGACACGTCCGATTACGACTTGAC

Sample Barcode PCR:

PacBio barcode forward primer:
GGTAG<BARCODE>ACACTCTTTCCCTACACGACGCTCTTCCGATCT
PacBio barcode reverse primer:
CCATC<BARCODE>GTGACTGGAGTTCAGA

For PacBio <BARCODE> sequences, use barcode sequences provided by PacBio (e.g. from SMRTbell barcoded adapter plate 3.0)

ONT barcode forward primer:
<BARCODE>ACTTCGTACGTACGGCGTCTTATAC
ONT barcode reverse primer:
<BARCODE>GAGACACGTCCGATTACGACTTGAC
For ONT <BARCODE> sequences, see Supplementary Table 1

2.2 PCR

- High-quality genomic DNA (HLA-DRB1 pre-genotyped at allele-group resolution) (see note 2)
- Allele-group specific primers (see Table 1)
- Advantage Genomic LA Polymerase Mix (Takara Bio, Mountain View, California) or another long amplification PCR kit
- dNTPs
- 96well plates or reaction tubes
- Thermocycler
- Gel-electrophoresis system

2.3 Purification

- SPRIselect beads
- Magnetic rack
- 80% ethanol

2.4 Library Preparation & Sequencing: Illumina Shotgun

- NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina (NEB)
- NEBNext Ultra II Ligation Module (NEB)
- $MgCl_2$ (2.5 mM)
- NEBNext Multiplex Oligos for Illumina (Dual Index Set 1)
- NEBNext Multiplex Oligos for Illumina (Dual Index Set 2)
- Reagents and system for Illumina library quantification (e.g. qPCR)
- MiSeq
- MiSeq (500 cycle) Reagent Kit

2.5 Library Preparation & Sequencing: PacBio

- HulaMixer Sample Mixer (ThermoFisher Scientific)
- Qubit Fluorometer (ThermoFisher Scientific)
- Qubit 1X dsDNA HS Assay Kit (ThermoFisher Scientific)
- BluePippin (Sage Science)
- 0.75% Agarose Cassettes, dye-free, ext. markers, BluePippin (Sage Science)
- SMRTbell Express Template Prep Kit 2.0 (PacBio)

- AMPure PB Beads
- Sequel/Sequel II (PacBio)
- PreCR Repair Mix (NEB) (optional)

2.6 Library Preparation & Sequencing: ONT

- Qubit Fluorometer (ThermoFisher Scientific)
- Qubit 1X dsDNA HS Assay Kit (ThermoFisher Scientific)
- NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (NEB)
- ONT sequencing platform (MinION, GridION)
- MinION/GridION flowcell (10.4.1 chemistry)
- Ligation Sequencing Kit V14 (SQK-LSK114)

2.7 Genotyping & Sequence Submission to IPD-IMGT/HLA

- DR2S (download from https://github.com/DKMS-LSL/dr2s)
- TypeLoader2 (download from https://github.com/DKMS-LSL/typeloader)

3 Methods

If not stated otherwise in a kit manual, work at room temperature. For long-read sequencing, avoid fast pipetting through thin pipette tips to prevent shearing the DNA. In particular, do not mix by pipetting. Instead, mix by inversion or flicking the tube with a finger.

3.1 PCR

1. Choose the appropriate primers for the HLA-DRB1 allele you want to characterize in full length (Table 1).
2. Perform two independent long-range PCRs with 20-200 ng of genomic DNA in a 25 µl reaction using 0.2 µM primers, 0.4 mM dNTPs, 1x Advantage Genomic LA Buffer and 1.25 U Advantage Genomic LA Polymerase Mix. Cycling protocol: 94°C 1 min, 35 cycles: 98°C 10 sec/68°C 12 min, 72°C 10 min. Verify amplification success and specificity by gel-electrophoresis (Figure 1, see notes 3 and 4).
3. Use one PCR reaction for Illumina shotgun sequencing and the other for long-read sequencing (PacBio or ONT).

3.2 Illumina Library Preparation & Sequencing

1. Fragment 10 µl of PCR product by adding 0.5 mM $MgCl_2$ (final concentration), 2.5 µl NEBNext Ultra II FS Reaction Buffer and 0.1 µl NEBNext Ultra II FS Enzyme Mix in a 25 µl reaction. Incubate at 37°C for 30 min and 65°C for 20 min.
2. Attach barcoded sample adapters by adding the following reagents to the 25 µl fragmentation reaction: 7.5 µl NEBNext Ultra II Ligation Master Mix, 0.25 µl NEBNext Ligation Enhancer, 0.63 µl NEBNext Adaptor for Illumina. Incubate at 20°C for 15 min.
3. Add 0.75 µl USER enzyme and 0.75 µl water to the reaction. Incubate at 37°C for 15 min.

152 4. Purify the DNA by adding 15.5 µl water and 40 µl SPRIselect beads to the reaction. Make sure
153 the beads are suspended completely by vortexing. Mix by inversion and spin down only for a
154 short time such that the beads remain dispersed. Incubate for 5 min. Separate beads on a
155 magnetic rack. Discard the supernatant. Perform a wash step with 100 µl 80% ethanol leaving
156 the tube on the magnetic rack. Discard the supernatant. Repeat the wash step. Spin down
157 shortly to gather the ethanol at the bottom of the plate/tube. Separate the ethanol from beads
158 using a magnetic rack and remove completely. Air-dry the pellet for 2 min. Elute with 17 µl of
159 water, mix by inversion and spin down shortly. Incubate for 5 min. Separate beads using the
160 magnetic rack. Transfer the supernatant to a fresh plate/tube.
161 5. Now add the individual sample indexes to the fragmented, adapter-ligated and purified DNA.
162 First, prepare the Index Primer Mix (NEBNext Multiplex Oligos for Illumina, see kit protocol
163 for preparation of index combinations) by mixing 5 µl of the i5 primer and 5 µl of the i7 primer
164 with 15 µl water. In a new plate/tube, attach the sample index by mixing 3.75 µl DNA with
165 2.5 µl of the prepared Index Primer Mix (NEBNext Multiplex Oligos for Illumina, fold adapter
166 dilution, see kit protocol for preparation of index combinations) and 6.25 µl NEBNext Ultra II
167 Q5 Master Mix. Cycling protocol: 98°C 30 sec, 7 cycles: 98°C 10 sec/65°C 75 sec, 65°C
168 5 min.
169 6. Prepare pools of up to 48 samples (5 µl each).
170 7. Purify and remove small fragments by adding 0.7x SPRIselect beads (e.g. 168 µl beads to a
171 pool of 240 µl). Make sure the beads are suspended completely by vortexing. Mix by inversion
172 and spin down only for a short time such that the beads remain dispersed. Incubate for 5 min.
173 Separate beads on a magnetic rack. Discard the supernatant. Perform a wash step with 500-
174 1000 µl 80% ethanol leaving the tube on the magnetic rack. Discard the supernatant. Repeat the
175 wash step. Spin down shortly to gather the ethanol at the bottom of the tube, separate the
176 ethanol from beads using a magnetic rack and remove completely. Air-dry the pellet for 2 min.
177 Elute with 30 to 75 µl of water (depending on the number of samples in the pool). Mix by
178 inversion and spin down shortly. Incubate for 5 min. Separate beads using the magnetic rack.
179 Transfer the supernatant to a fresh tube.
180 8. Quantify the sequencing libraries using an appropriate method for Illumina sequencing (e.g.
181 qPCR).
182 9. Load samples on an appropriate Illumina MiSeq flow cell (500 cycle kit) to achieve a coverage
183 of 50,000 reads per sample and run according to the manufacturer's instructions (see note 5).

184 **3.3 PacBio Library Preparation & Sequencing**

185 1. Perform an indexing PCR to add barcodes to each sample. Mix 1-3 µl of the initial PCR
186 product (estimation, use thickness of band in gel-electrophoresis), 0.2 µM barcode primers,
187 0.4 mM dNTPs, 1x Advantage Genomic LA Buffer and 1.25 U Advantage Genomic LA
188 Polymerase Mix in a 25 µl PCR reaction. Cycling protocol: 94°C 1 min, 15 cycles: 98°C
189 10 sec/68°C 12 min, 72°C 10 min. Verify amplification success and specificity by gel-
190 electrophoresis (Figure 1).
191 2. Pool up to 96 samples. Use 20 µl for samples with weak amplification and 5 µl for samples
192 with strong bands. (Use 25 µl if only few samples are pooled.) Do not include samples that did
193 not yield PCR products of the expected size.
194 3. Reduce the volume by mixing the pool 1:1 with AMPure PB Beads (see note 6). Briefly
195 centrifuge and rotate the tube on a HulaMixer for 10-15 min. Briefly centrifuge and put the
196 tube on the magnetic rack. Incubate for 2-3 min and remove the supernatant. Perform a wash
197 step with 1000 µl 80% ethanol leaving the tube on the magnetic rack. Discard the supernatant.

198 Repeat the wash step. Spin down shortly to gather the ethanol at the bottom of the tube,
199 separate the ethanol from beads using a magnetic rack and remove completely. Air-dry the
200 pellet for 2 min. Elute with 30 to 200 µl of water (depending on the number of samples in the
201 pool). Mix by inversion and spin down carefully. Incubate for 5 min. Separate beads using the
202 magnetic rack. Transfer the supernatant to a fresh tube.
203 4. Quantify the pool using Qubit.
204 5. Use 2500 ng of DNA and follow the "Procedure & Checklist – Preparing HiFi SMRTbell
205 Libraries using the SMRTbell Express Template Prep Kit 2.0" protocol (PacBio), starting at
206 step "Repair DNA Damage". For HLA-DRB1 amplicons, select a range from 8,000 to
207 50,000 bp using the BluePippin system. Elute the final purification step in 40 µl water if you
208 want to perform another step of DNA damage repair (continue at step 6) or in 11 µl PB Elution
209 buffer to proceed at step 8.
210 6. Another step of DNA damage repair can improve the results. Add 5 µl Thermopol-buffer 10x,
211 0.1 mM dNTPs, 1x NAD^+ and 2x PreCR Mix to a final volume of 50 µl. Tap the tube and spin
212 down briefly. Incubate at 37°C for 15 min and at 4°C for 10 min.
213 7. Add 50 µl water and 60 µl AMPure PB Beads (0.6x) and repeat the final purification step
214 according to PacBio's protocol. Elute in 11 µl PB Elution buffer.
215 8. Check the library by Qubit and DNA fragment sizing system.
216 9. Sequence the libraries on a Sequel or Sequel II system. Up to 192 samples can be multiplexed
217 on a SMRT Cell 1M.
218

219 **3.4 ONT Library Preparation & Sequencing**

220 1. When using DNA isolated from buccal swabs, gel-electrophoresis bands from the initial PCR
221 (3.1 step 2) are usually weak to medium. If this is the case, proceed directly to step 2. However,
222 in case of very strong bands, dilute the PCR products 1:10 with water before proceeding to
223 step 2 (see note 7).
224 2. Perform an indexing PCR to add barcodes to each sample. Mix 1-3 µl of the initial PCR
225 product (estimation, use thickness of band in gel-electrophoresis), 0.2 µM barcode primers,
226 0.4 mM dNTPs, 1x Advantage Genomic LA Buffer and 1.25 U Advantage Genomic LA
227 Polymerase Mix in a 25 µl PCR reaction. Cycling protocol: 94°C 1 min, 15 cycles: 98°C
228 10 sec/68°C 12 min, 72°C 10 min. Verify amplification success and specificity by gel-
229 electrophoresis (Figure 1).
230 3. Pool up to 96 samples. Use 20 µl for samples with weak amplification and 5 µl for samples
231 with strong bands. (Use 25 µl if only few samples are pooled.) Do not include samples that did
232 not yield PCR products of the expected size.
233 4. Clean the pool by adding 0.6x SPRIselect beads (e.g. 180 µl beads to a pool of 300 µl). Mix by
234 inversion and spin down only for a short time such that the beads remain dispersed. Incubate
235 for 5 min. Separate beads on a magnetic rack. Discard the supernatant. Perform a wash step
236 with 800 µl 80% ethanol leaving the tube on the magnetic rack. Discard the supernatant. Repeat
237 the wash step. Spin down shortly to gather the ethanol at the bottom of the tube. Separate the
238 ethanol from beads using a magnetic rack and remove the ethanol completely. Air-dry the
239 pellet for 2 min. Elute with 30 to 200 µl of water (depending on the number of samples in the
240 pool). Mix by inversion and spin down carefully. Incubate for 5 min. Separate beads using the
241 magnetic rack. Transfer the supernatant to a fresh tube.
242 5. Quantify the pool using Qubit.

6. It is recommended to use the latest chemistry (V14 SQK-LSK114) together with a R10.4.1 flowcell. Consequently, prepare the sequencing library following the “Ligation Sequencing Kit V14 (SQK-LSK114) protocol” by Oxford Nanopore Technologies. When eluting with Elution Buffer (EB), use the given option for high molecular weight DNA (incubation at 37°C instead of room temperature).
7. Sequence the library on a MinION or GridION system. Up to 192 samples may be multiplexed on one MinION flowcell.

3.5 Data Analysis: DR2S

`DR2S` (Dual Redundant Reference Sequencing) is an R package designed to facilitate generating full-length phase-defined haplotype sequences in reference quality from a combination of long reads and short reads. It can be installed and run in a Linux environment and depends on local installations of `samtools`, `bwa` (version 0.7.11 or later), `minimap2`, and `IGV`. The latest version of `DR2S` can be installed in R from https://github.com/DKMS-LSL/dr2s using the commands:

```
> install.packages("devtools") # if not already installed

> devtools::install_github("DKMS-LSL/DR2S")
```

For ease of use we recommend, however, to use the DR2S docker image provided at docker hub. The image can be loaded and used with the following commands:

```
$ docker pull dkmslsl/dr2s:<version>

$ docker run --rm -p 8788:8787 -e PASSWORD=<pwd> -it dkmslsl/dr2s:<version>
```

Make sure to replace `<pwd>` with a password of your choice and `<version>` with the latest version of the software available (`1.1.21` at the time of writing). An RStudio-session can now be accessed in your browser at `localhost:8788`. Log into RStudio using the username rstudio and the password `<pwd>`. A detailed tutorial on how to use `DR2S` is shipped as an R package vignette “DR2S”. Example data to follow the tutorial are also shipped as part of the docker image.

Briefly, the following steps constitute a DR2S workflow:

1. Place the demultiplexed FASTQs from a short-read and a long-read experiment into two separate directories, respectively, following the naming convention: `<sampleid>_<locus>_*.fastq.gz`
2. Prepare a `yaml`-based configuration file (or provide the relevant information via `R` in an interactive session) that provides information on the paths to input and output, the sample ids, the expected HLA locus, the type of long reads (`pacbio` or `nanopore`) as well as a couple of workflow parameters. A default configuration file and detailed documentation of the various options are provided as part of the docker image.
3. In an interactive R session, initialize a DR2S driver object:

```
> library(DR2S)
> dr2sDriver <- InitDR2S(readDR2SConf(<path/to/configFile>))
```

4. Create an initial sample-specific consensus sequence by mapping the short reads to a generic reference:

```
> dr2sDriver <- mapInit(dr2sDriver)
```

5. Partition long reads into haplotype-specific read clusters based on heterozygous positions in the initial mapping:

```
> dr2sDriver <- partitionLongreads(dr2sDriver)
```

6. Iteratively, map long-read clusters to generate haplotype-specific consensus scaffolds:

```
> dr2sDriver <- mapIter(dr2sDriver)
```

7. Assign short reads to haplotype clusters:

```
> dr2sDriver <- partitionShortreads(dr2sDriver)
```

8. Polish the long-read-derived scaffold consensus sequences using the more accurate short reads:

```
> dr2sDriver <- mapFinal(dr2sDriver)
```

9. Report the polished consensus sequences as well as diverse quality diagnostics to a `report/` subdirectory.

```
> dr2sDriver <- report(dr2sDriver)
```

10. To aid visual inspection and correction of possibly remaining problems, the DR2S package provides the functions `checkAlignmentFile()`, `remapAndReport()`, and `reportCheckedConsensus()`. For detailed instructions on their usage within a generic quality-checking and postprocessing workflow consult the R package vignette "DR2S".

For further details about the D2RS algorithm and its performance see Reference ***(3)***.

3.6 Submission to IPD/IMGT-HLA: TypeLoader2

Once the polished HLA-DRB1 consensus sequences are generated, novel allele sequences are ready to be submitted to the ENA and IPD/IMGT-HLA databases. While you could also follow the instructions for manual submission on the databases' websites, we developed TypeLoader2 for better performance, quality assurance and convenience ***(5)***. For a stepwise instruction on how to install and use TypeLoader2 see the chapter of Schöne et al. in this book.

4 Notes

1. The overlap sequences are not required for short-read sequencing. However, since they do not interfere, it is more convenient to use the same target-specific primers for both long- and short-read sequencing.
2. Genomic DNA can be isolated from blood or buccal swabs. As DNA from buccal swabs is usually more fragmented, higher PCR failure rates are to be expected. If in doubt, check DNA quality and fragmentation with a suitable method before PCR.
3. PCR failure rates for 624 buccal swabs (DNA concentrations > 20 ng/µl, no prior fragmentation analysis): 15%, 6%, 44% (long-range PCR groups S, M, L, respectively).
4. For long-read sequencing, weak bands are fine at this point as the initial PCR product is still subjected to the barcode PCR. If insufficient PCR product is obtained for Illumina library

329 preparation, barcoding PCR (3.3 step 1) with any barcoding primers may be performed to
330 increase PCR product quantity before continuing the Illumina library preparation protocol.
331 5. Other Illumina instruments in combination with 300 cycle kits have not been tested but are
332 expected to provide similar results.
333 6. Washed SPRIbeads can be used instead of AMPure PB Beads.
334 7. Dilution ensures that every molecule is barcoded. Too much of the initial PCR in the barcoding
335 PCR will result in a high number of sequencing reads that cannot be demultiplexed. Try
336 diluting the PCR product even more if you experience this issue.

337 **5 References**

338 1. Robinson J, Barker DJ, Georgiou X, et al (2020) IPD-IMGT/HLA Database. Nucleic Acids Res
339 48:D948–D955

340 2. Gerritsen KEH, Groeneweg M, Meertens CMH, et al (2015) Full-length HLA-DRB1 coding
341 sequences generated by a hemizygous RNA-SBT approach. Tissue Antigens 86:333–342

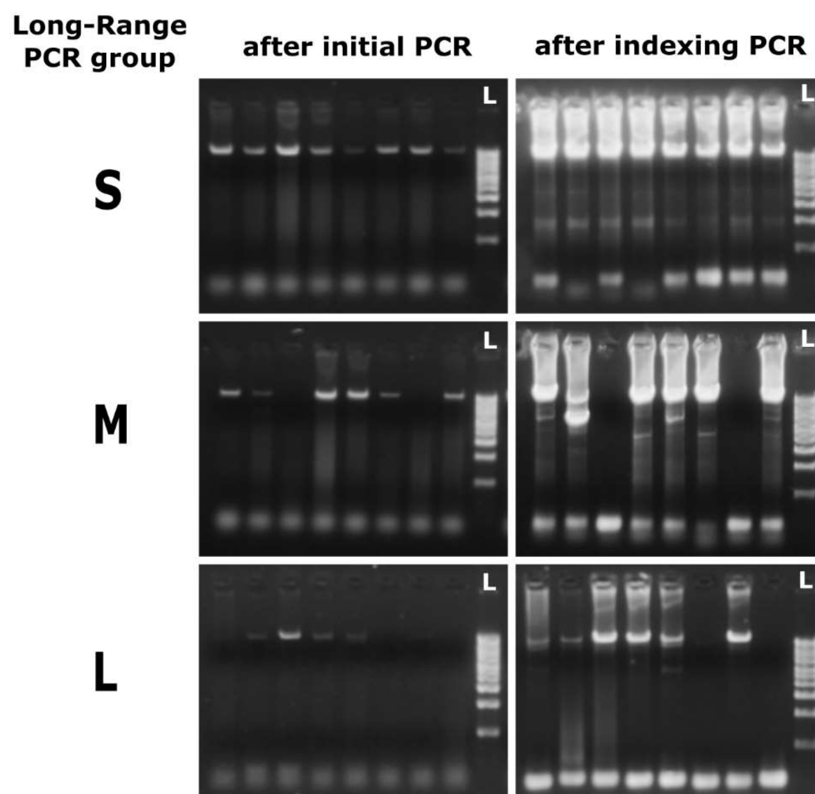
342 3. Klasberg S, Schmidt AH, Lange V, et al (2021) DR2S: an integrated algorithm providing
343 reference-grade haplotype sequences from heterozygous samples. BMC Bioinformatics 22:236

344 4. Albrecht V, Zweiniger C, Surendranath V, et al (2017) Dual redundant sequencing strategy: Full-
345 length gene characterisation of 1056 novel and confirmatory HLA alleles. HLA 90:79–87

346 5. Schöne B, Fuhrmann M, Surendranath V, et al (2019) TypeLoader2: Automated submission of
347 novel HLA and killer-cell immunoglobulin-like receptor alleles in full length. HLA 93:195–202

348

349 **6 Figures**



350

351 Figure 1: Gel-electrophoresis control of eight exemplary samples of Long-Range PCR groups S, M
352 or L after the initial PCR and after indexing PCR, respectively. DNA was isolated from buccal
353 swabs. DNA ladder (lane L) ranges from 500 bp to 10 kb.

354

355 **7 Tables**

Long-Range PCR Group	Alleles	Length	Primer
S	DRB1*01/15/16	11 – 12 kB	F2 + R2 + R3
M	DRB1*03/08/10/11/12/13/14	13 – 14 kB	F1 + R2 + R3
L	DRB1*04/07/09	15 – 16 kB	F1 + R1

356 Table 1: HLA-DRB1 allele groups targeted by the respective primer combinations. Depending on the
357 targeted allele a particular set of primers is chosen for amplification resulting in an amplification
358 product of the given length.