HUMAN IG
RNA MULTIPLEX

Human IGH and IGK/L full-length repertoires with UMI

User Manual v.1.6

Cat. # BHRM-001
24 samples

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KIT OVERVIEW

The **HUMAN IG RNA MULTIPLEX** kit is designed for the amplification of IG heavy and light chain cDNA libraries using the combination of highly sensitive multiplex PCR and Unique Molecular Identifiers (UMIs). UMIs are introduced along with 1st strand cDNA synthesis and allow for error-free reconstruction of full-length IG heavy and light chain sequence with hypermutations (except for the FR1 region). In addition, UMIs allow for exact quantification of template cDNA molecules, control for input bottlenecks, and accurate normalization of samples of comparison. Gene-specific primers targeting IG heavy chain constant regions facilitate all IGH isotype discrimination including IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE, and IgA2.

The kit includes a set of reagents sufficient to prepare 24 IGH and 24 IGK/L cDNA libraries starting from 24 RNA samples.

Some B cell populations (e.g. plasma cells, plasmablasts) express much higher IG RNA levels compared to memory or naïve B cells. Thus, for some experiments it can be rational to separate these populations before RNA isolation by FACS or magnetic beads.

The kit allows to start with RNA derived from 100 to 100,000 sorted/purified memory/naïve B cells or from 10 to 1,000 plasma cells/plasmablasts, from PBMC (up to $5x10^5$), or from B cell-containing tissues (see **Appendixes A** and **B** for recommended RNA isolation procedures), and produces indexed ready-to-sequence-on-Illumina libraries.

All steps of IG cDNA libraries construction including reverse transcription, first PCR (multiplex amplification) and second PCR (multiplex amplification and indexing) performed separately for IG heavy chain and IG light chain repertoires (**Figs. 1, 2**). Full-length (without FR1) IG profiling requires 250+250 paired end Illumina sequencing with sufficient reads-per-UMI sequencing coverage (see **Table 2** for details).

The kit contains limited amount of reagents for the synthesis of cDNA and the 1st PCR (for 24 reactions). For the 2nd PCR reagents are provided in sufficient amounts to optimize the number of 2nd PCR cycles if necessary.

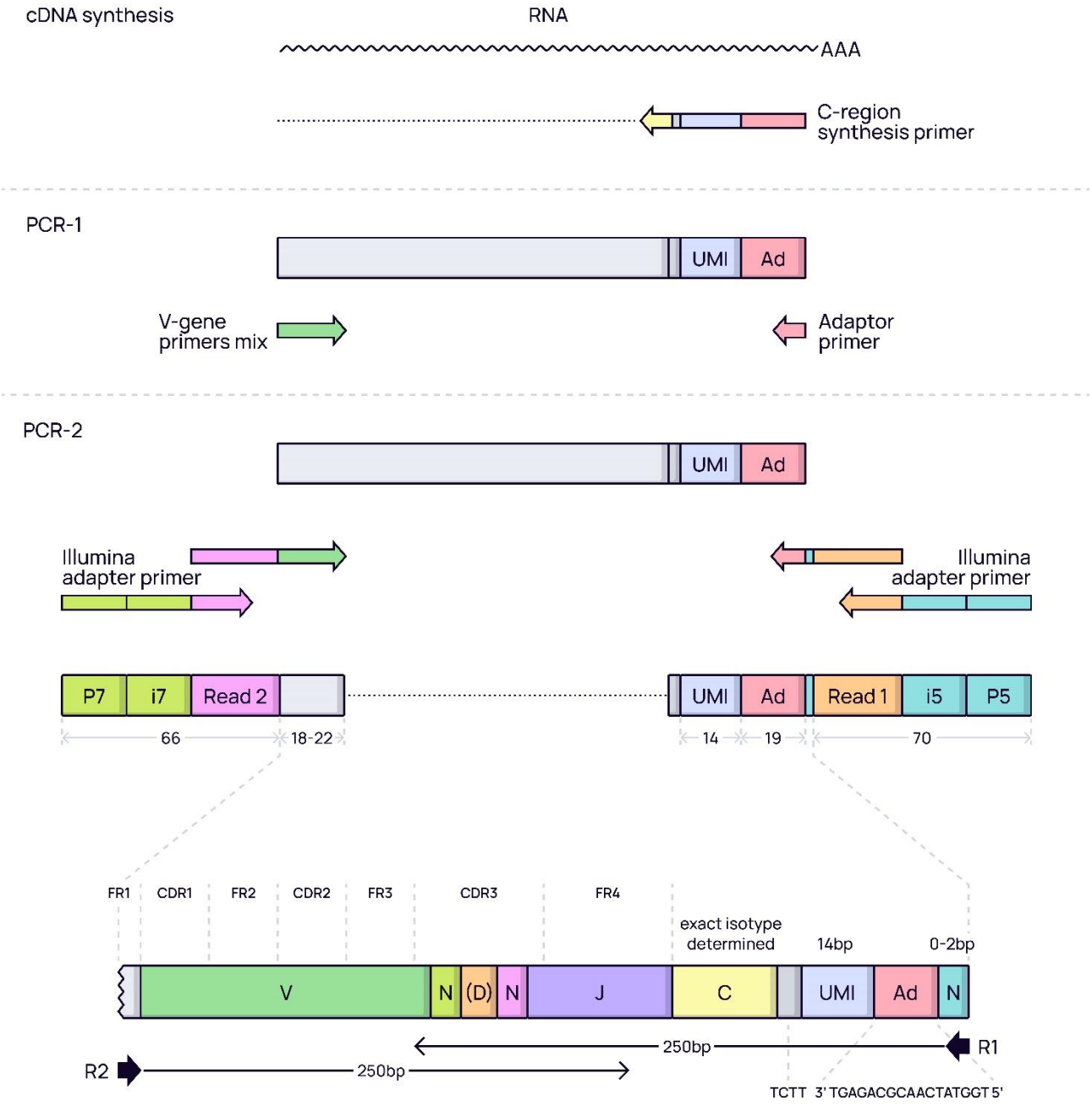


Figure 1. Scheme for IG cDNA libraries generation and sequencing.

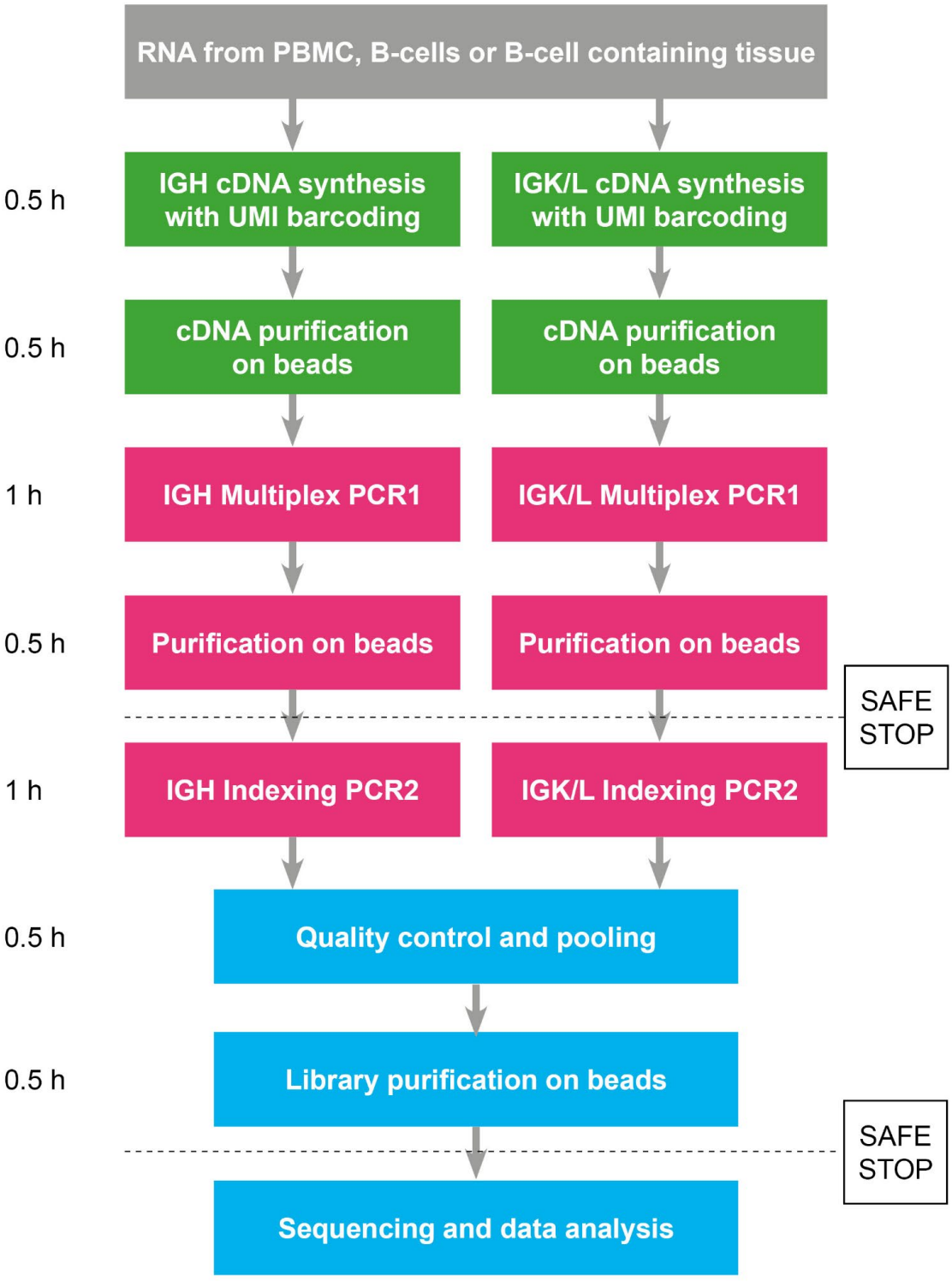


Figure 2. HUMAN IG RNA MULTIPLEX kit pipeline.

KIT CONTENT

Table 1.

	Component	Volume (µl)	Description
1	IGH Hum Synt M	60 (after recovery)	Synthesis primer mixes
2	IGK/L Hum Synt M	60 (after recovery)	10X after recovery
3	IGH Hum PCR1	60 (after recovery)	1st PCR primer mixes
4	IGK/L Hum PCR1	60 (after recovery)	25 X after recovery
5	IGH Hum PCR2	30 (after recovery)	2nd PCR primer mixes
6	IGK/L Hum PCR2	30 (after recovery)	250 X after recovery
7	Control RNA	40 (after recovery)	Control RNA final concentration 5 ng/µl
8	Binding Buffer	1 000	On beads DNA resorption buffer
9	Water	1 000 X 2	Deionized nuclease-free water

Components 1-6 are shipped dry.

Transportation and storage until dissolved at RT.

Before use, add water and place the kit in the freezer (at –20°C).

MATERIALS REQUIRED BUT NOT INCLUDED

- SuperScript III™ (ThermoFisher Scientific, Invitrogen™, #18080044, supplied with 5X First-Strand Buffer buffer and DTT)
- RNAsin® 40 U/µl (Promega, #N2515)
- dNTP mix (10mM each)
- Qiagen Multiplex PCR Plus kit (Qiagen, #206152)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63880) or SPRIselect® Reagent Kit (Beckman Coulter, Inc. #B23317)
- Freshly prepared 80% Ethanol (1200 µl per sample)
- Nuclease free water
- Elution Buffer (EB, 10 mM Tris-HCl, pH 8.0-8.5)
- IDT for Illumina Nextera DNA Unique Dual Indexes or IDT for Illumina – DNA/RNA UD Indexes, Tagmentation (any of #20027213, #20027214, #20027215 or #20027216) (recommended)

or

Nextera XT Index Kit (FC-131-1001) or Nextera XT Index Kit v2 (any of Set A, B, C and D, FC-131-2001, FC-131-2002, FC-131-2003, and FC-131-2004)

- Agarose gel electrophoresis system and 50 bp DNA Ladder (NEB, #N3236)
- Thermal Cycler
- Agilent Bioanalyzer/TapeStation
- Appropriate Magnetic Rack
- Low-speed benchtop Mini-centrifuge/vortex
- Qubit® fluorimeter and Qubit dsDNA HS Assay Kit

GENERAL RECOMMENDATIONS TO PREVENT CONTAMINATION

General recommendations to lower the risk of RNA degradation and contamination should be implemented, such as using labcoats, gloves, tips with aerosol filters, certified RNAse/DNAse free reagents.

Performing non-template control reactions is strongly recommended for each experiment.

To prevent cross-sample contamination library preparation should be performed in two separate workspaces (PCR boxes) located in different rooms: pre-PCR and post-PCR. The user should avoid transferring anything (reagents, pipets, racks etc.) from post-PCR to pre-PCR workspace. In the case of possible contamination, use decontaminating procedures with special reagents such as DNA-OFF™ (MP Biomedicals) and UV.

Perform cell isolation, RNA purification, cDNA synthesis, 1st and 2nd PCR Master Mixes preparation in the pre-PCR workspace (Steps 1–8 and 10–12). After the 1st PCR amplification, transfer the tubes to the post-PCR workspace and perform all other steps (9, 13–19).

Perform UV decontamination each time before making up a PCR master-mix.

BEFORE YOU START

Add nuclease-free water to the tubes according to **Table 1** in **Kit Content**. Incubate the tubes at room temperature for 10 min.

Mix by vortexing and spin down.

Store diluted components at –20°C.

STARTING MATERIAL

See **Appendixes A** and **B** for our recommendations on RNA isolation. Use Qubit® fluorometer or other fluorescent based method to determine RNA concentration

For quantitative IG repertoire profiling we recommend to separate B-cell populations with different IG RNA transcription levels (i.e. plasma cells/plasmablasts and memory/naïve B-cells) by FACS or magnetic beads.

The verified minimal number of cells for the protocol is 100 B cells or 10 plasma cells sorted directly into Qiagen RLT® buffer, followed by RNA extraction with TRIzol® (**Appendix B**). To isolate RNA from comparable cell numbers, use co-precipitant and carrier RNA (or both, depending on isolation method) to avoid losses.

High RNA quality is often critical for efficient library preparation. Residual traces of proteins, salts or other contaminants may decrease the efficiency of the enzymatic activity necessary for optimal targeted enrichment.

gDNA significantly affects cDNA synthesis reaction and subsequent PCR amplification. **It is strongly recommended to perform DNAse treatment during RNA extraction.**

Do not use heparin coated tubes for blood collection. Heparin dramatically decreases cDNA synthesis efficiency.

PROTOCOL

NOTE: Perform steps 1–8 in a pre-PCR box/room.

cDNA synthesis (performed separately for IGH and IGK/L)

NOTE: IGK/L mRNA expression levels are usually 2–3 fold higher than IGH. Therefore, we recommend 3/4 of mRNA material to be used for IGH and 1/4 for IGK/L cDNA libraries preparation.

1. In sterile reaction tube(s)/strip(s), mix the following reagents in a final reaction volume of 12 µl:

For **IG heavy chain** (IGH)

Component	**Volume, µl**
RNA, up to 500 ng per reaction*	1–10**
IGH Hum Synt M (10 X)	2
RNAse free water	0-9
Total volume	12 µl

For **IG light chain** (IGK/L)

Component	**Volume, µl**
RNA, up to 500 ng per reaction*	1–10
IGK/L Hum Synt M (10 X)	2
RNAse free water	0–9
Total volume	12 µl

***** For control reaction, take 5 µl of control RNA (25 ng per reaction).
** Do not add less than 1 µl of RNA solution to the reaction. If the RNA solution is highly concentrated (more than 500 ng/µl), we recommend diluting it 2-4 times with RNase free water before starting the work.

2. Place the reaction tube(s)/strip(s) into a thermal cycler with a heated lead and incubate for 2 minutes at 70ºC, followed by 2 minutes at +4ºC.

3. While incubating, prepare Master Mix of the following components in a final volume of 8 µl per reaction (16 µl per IGH+IGK/L):

Component	Volume, µl
5X First-Strand Buffer (Thermo Fisher)	4
DTT (100 mM, Thermo Fisher)	1
dNTPs (10 mM each)	1
RNAsine®, (40 U/µl, Promega)	1
SuperScript III® Reverse Transcriptase (200 U/µl, Thermo Fisher)	1
Total volume	8 µl

NOTE: Add SuperScript III Reverse Transcriptase to the Master Mix just before use and mix gently by pipetting.

4. Add 8 µl of Master Mix to each reaction, pipette gently, spin briefly, and incubate for **30 minutes at 55ºC**.

5. Purify the obtained cDNA synthesis products using **modified** AMPure® XP Beads protocol (Beckman Coulter) with **Binding Buffer** included in the kit, as follows:

- Homogenize beads by vortexing.
- Add 20 µl of beads to each reaction and mix well by pipetting up and down 10 times.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.
- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.
- Remove tubes from the magnetic rack. Add 10 µl of EB (10 mM Tris-HCl, pH 8.0-8.5) and mix well by pipetting.
- Add 10 µl of **Binding Buffer** and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.

- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.
- Remove tubes from the magnetic rack. Briefly spin down and place back on the magnetic rack. Remove all the residual ethanol.

NOTE: It is important to remove all the traces of ethanol at this step as it could inhibit subsequent PCR reaction.

- Remove tubes from the magnetic rack. Add 11 µl EB for elution and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear.
- Keep tubes on the magnetic rack. Take 10 µl of cDNA solution into a new tube.

NOTE: During the elution steps, beads clumping may significantly reduce the cDNA yield, which is critical at the cDNA purification step. To maximize the cDNA yield, we recommend breaking up the clumps using pipetting and heating at 50ºC for 10-30 minutes until complete dissolving.

1st PCR amplification

6. In a sterile tube, prepare two separate PCR1 Master Mixes in a final volume of 40 µl per reaction:

For **IG heavy chain** (IGH)

Component	**Amount, µl**
Nuclease free water	13
2x Multiplex PCR Master Mix (Qiagen)	25
IGH Hum PCR1 (25 X)	2
Total volume	40 µl

For **IG light chain** (IGK/L)

Component	**Amount, µl**
Nuclease free water	13
2x Multiplex PCR Master Mix (Qiagen)	25
IGK/L Hum PCR1 (25 X)	2
Total volume	40 µl

7. Add the appropriate Master Mix to purified cDNA samples:

7a. Add 40 µl of PCR1 IGH Master Mix to each of 10 µl IGH cDNA samples. Mix gently by pipetting.

7b. Add 40 µl of PCR1 IGK/L Master Mix to each of 10 µl IGK/L cDNA samples. Mix gently by pipetting.

8. Perform 1st PCR using the following parameters:

95ºC for 5 min	
94ºC for 30 s	
60ºC for 1 min 30 s	18–24 cycles*
72ºC for 30 s	
68ºC for 4 min	
4ºC hold	

* Choose the number of cycles depending on the amount of RNA/B cell count at start (**Table 2** below):

NOTE: Perform step 9 in the post-PCR box/room.

9. Purify the obtained 1st PCR products using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:0.8 sample:beads ratio:

- Homogenize beads by vortexing.
- Add 40 µl of beads to each reaction and mix well by pipetting up and down 10 times.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.
- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.
- Repeat washing by 80% ethanol without taking off the magnetic rack and remove the ethanol.
- Remove tubes from the magnetic rack. Briefly spin down and place back on the magnetic rack. Remove all the residual ethanol.

NOTE: It is important to remove all the traces of ethanol at this step as it could inhibit subsequent PCR reaction.

- Remove tubes from the magnetic rack. Add 21 µl of EB for elution and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear.
- Keep tubes on the magnetic rack. Take 20 µl of purified 1st PCR products into a new tube.

SAFE STOP POINT: Purified product of the 1st PCR can be stored up to 1 month at −20°C.

2nd PCR amplification

NOTE: perform steps 10-12 in the pre-PCR box/room.

10. Prepare working primer solutions for IGH and IGK/L PCR2.

For this, calculate the volume of PCR2 primers required to process N number of samples based on 1 µl per sample. Dilute 250 X stocks of **IGH Hum PCR2** and **IGK/L Hum PCR2** primer mixes with nuclease-free water 1:9 according to the calculation, to obtain 25 X solutions. Discard diluted aliquots after use.

11. Prepare two separate PCR2 Master Mixes in a final volume of 22 µl per reaction without indexes:

For **IGH**

Component	**Amount, µl**
Nuclease free water	8.5
Multiplex PCR Plus Mix (Qiagen)	12.5
IGH Hum PCR2 (25 X)	1
Total volume	22 µl

For **IGK/L**

Component	**Amount, µl**
Nuclease free water	8.5
Multiplex PCR Plus Mix (Qiagen)	12.5
IGK/L Hum PCR2 (25 X)	1
Total volume	22 µl

12. For each reaction, add 22 µl of PCR2 Master Mix to a nuclease-free 0.2 ml tube/strip. Two separate reaction volumes are required for **IGH** and **IGK/L**.

NOTE: Perform steps 13-19 in the post-PCR box/room.

13. Add 2 µl of purified 1st PCR product to each **IGH** and **IGK/L** reaction. Mix gently by pipetting.

14. Add Index Primers:

14a. If using **IDT for Illumina Nextera Unique Dual Indexing primers** (recommended): Add 1 µl of IDT for Illumina Nextera DNA UD Index to each reaction. Use a unique Index for each reaction. The final reaction volume is 25 µl.

14b. If using **Nextera XT Indexing primers**: Add 1 µl of Nextera N70X and 1 µl of Nextera S50X primer to each reaction. Use a unique Indexing primers combination for each reaction. The final reaction volume will be 26 µl, but it will not affect the efficiency of the reaction.

15. Perform 2nd PCR using the following parameters:

95ºC for 5 min	
94ºC for 30 s	
55ºC for 1 min 30 s	10-15 cycles*
72ºC for 30 s	
68ºC for 4 min	
4ºC hold	

* Choose the number of cycles depending on the amount of RNA/B cell count at start (**Tables 2-1** and **2-2**):

Table 2-1. Guidelines for choosing of appropriate number of PCR cycles for sorted memory/naïve B cells or PBMC*,**,***.

Estimated count of naive/memory B cells in a sample	Amount of template RNA extracted from sorted B cells or PBMC	Number of 1st PCR cycles	Number of 2nd PCR cycles	Recommended number of paired end 250+250 nt sequencing reads****
10^2 – 5x10^2	0.1 ng – 5 ng	24	12–15	50–100 reads per input B cell
5x10^2 – 5x10^3	0.5 ng – 50 ng	21	12–15	
5x10^3 – 5x10^4	5 ng – 500 ng	18	12–15	
5x10^4 – 5x10^5	50 ng – 500 ng	18	10–13	

* B cells may constitute 5-10% of PBMC.
** Plasma cells may prominently increase in PBMC after infection or vaccination. Take into account that plasma cells may have 100-1,000-fold higher levels of IG mRNA expression, that requires appropriate sequencing coverage as described in **Table 2-2**.
*** IGK/L mRNA expression levels are usually 2-3 fold higher than IGH. Therefore, we recommend 3/4 of mRNA material to be used for IGH and 1/4 for IGK/L cDNA libraries preparation (see **Step 1**).
**** Recommended coverage that allows for UMI-based elimination of amplification biases and accumulated errors.

Table 2-2. Guidelines for choosing appropriate number of PCR cycles for sorted plasma cells and plasmablasts. Note that plasma cells express 100-1,000 times more IG mRNA compared to other B cells populations*.

Number of plasma cells ina sample	Number of 1st PCR cycles	Number of 2nd PCR cycles	Recommended number of paired end 250+250 nt sequencing reads**
10-200 plasma cells	22	12-15	25,000 reads per input plasma cell
200 -2,000 plasma cells	20	12-15	

* IGK/L mRNA expression levels are usually 2-3 fold higher than IGH. Therefore, we recommend 3/4 of mRNA material to be used for IGH and 1/4 for IGK/L cDNA libraries preparation (see **Step 1**).
** Recommended coverage that allows for UMI-based elimination of amplification biases and accumulated errors.

NOTE: The optimal number of 2nd PCR cycles may vary for different templates, cell types, thermal cyclers, and sample amounts. We recommend that you determine the minimal number of PCR cycles required to obtain a sufficient amount (at least 40 ng of each library) empirically for each experiment. Furthermore, we recommend that you generate parallel libraries of similar nature (e.g., ten samples of 2,000-20,000 sorted B cells each) using the same number of PCR cycles, and mix the obtained libraries in equal volume proportion for sequencing. This allows obtaining an even coverage in terms of reads-per-cDNA molecule.

For example, the library, which started from 20,000 B cells, may produce more PCR product than the one which started with 2,000 B cells, after the same number of PCR cycles. However, the former library would also carry proportionally more IG cDNA molecules, and thus requires more sequencing reads to achieve a comparable coverage.

16. Verify quality of the obtained 2nd PCR product on the agarose gel (2.0%) next to the DNA ladder. The resulting IGH cDNA library should have a size at approximately 600 bp. The resulting IGK/L cDNA library should have a size at approximately 500 bp.

SAFE STOP POINT: Purified PCR product can be stored for 1-3 months at −20°C.

17. Pool the obtained 2nd PCR libraries separately for IGH and IGK/L chains. To do this, combine equal volume portions from each individual sample after the 2nd PCR. If 1-4 libraries are prepared at the same time, the whole reaction volumes of the 2nd PCRs can be pooled. If there are many samples, pool 5-10 µl from each 2nd PCR. The total amount of pooled DNA should be at least 50 ng.

18. Purify the obtained pooled DNA using standard AMPure® XP Beads protocol (Beckman Coulter) with 1:0.8 sample:beads ratio:

- Homogenize beads by vortexing.
- Add beads to each reaction in a volume equal to 0.8 of the pooled DNA and mix well by pipetting up and down 10 times.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear (which should take approximately 2–5 minutes).
- Keep tubes on the magnetic rack. Carefully remove and discard the cleared solution from the tubes.
- Keep tubes on the magnetic rack. Add 200 µl of freshly prepared 80% ethanol. Wait for 30 seconds and remove the ethanol.
- Repeat washing by 80% ethanol without taking off the magnetic rack and remove the ethanol.
- Remove tubes from the magnetic rack. Briefly spin down and place back on the magnetic rack. Remove all the residual ethanol.

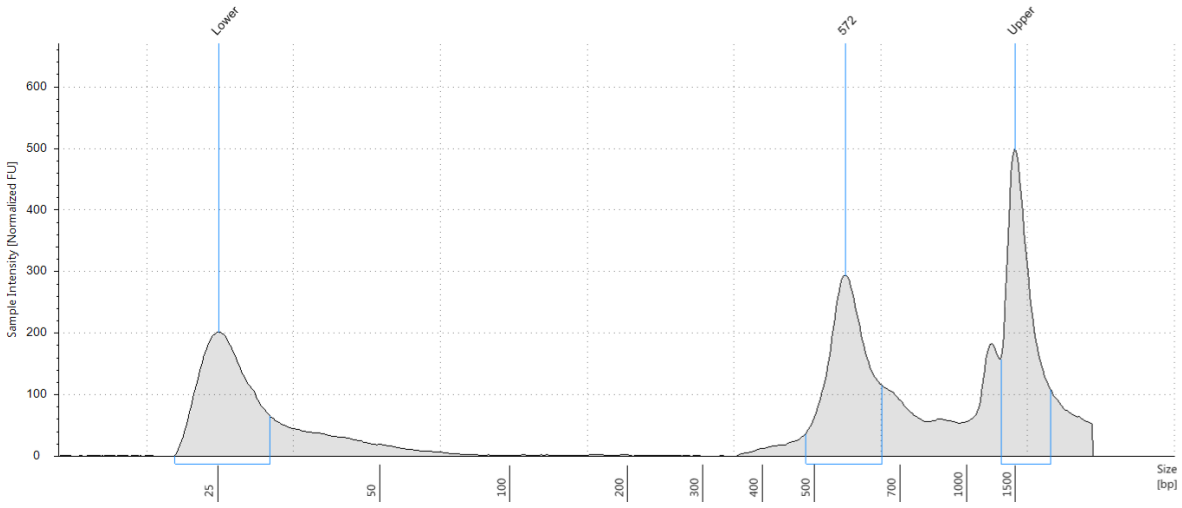
NOTE: It is important to remove all the traces of ethanol at this step as it could inhibit subsequent sequencing procedures.

- Remove tubes from the magnetic rack. Add 21 µl of EB for elution and mix well by pipetting.
- Incubate for 5 minutes at RT and place on the magnetic rack.
- Wait for the solution to clear.
- Keep tubes on the magnetic rack. Take 20 µl of purified 2nd PCR libraries into a new tube.

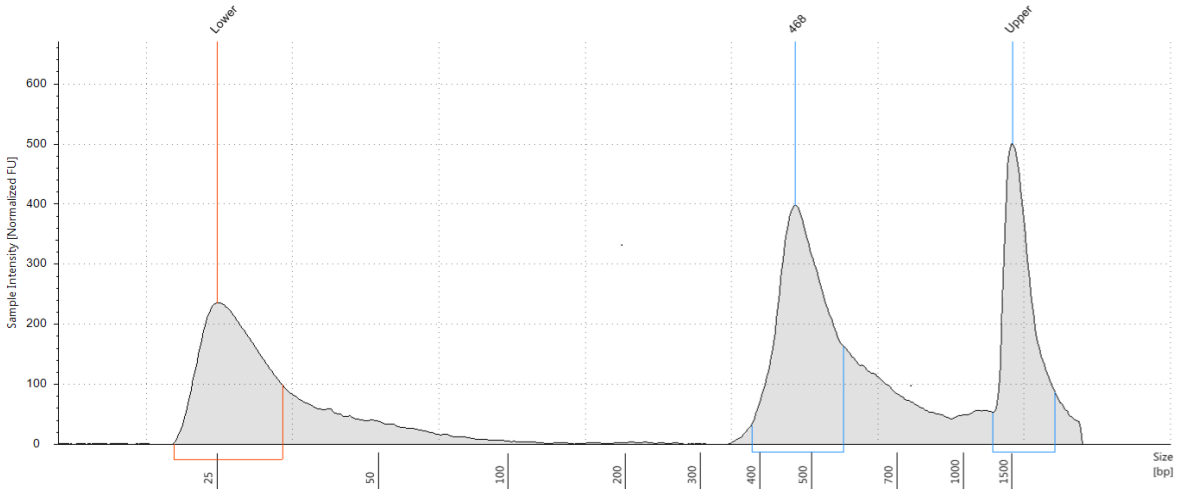
NOTE: The elution volume can be changed to obtain a DNA library with the desired concentration.

19. Verify quality of obtained pooled libraries by analyzing the aliquot by agarose gel or capillary electrophoresis (e.g. BIOANALYZER® or TapeStation®, Agilent Technologies). The library should have a peak at approximately 600 bp and 500 bp for IGH and IGK/L chains, respectively **(Fig. 3)**. Quantify the obtained purified pools using Qubit fluorometer and Qubit dsDNA High Sensitivity Assay Kit.

IGH and IGL/K pools can be mixed in equimolar ratio, considering that initially 3/4 of mRNA material was used for IGH and 1/4 for IGK/L cDNA libraries preparation (see **Step 1**).



IGH



IGL/K

Figure 3. A typical library peaks for IGH and IGK/L libraries. The shape and length distribution of a library may vary depending on the repertoire composition.

SAFE STOP POINT: Store purified libraries at –20ºC.

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SEQUENCING RECOMMENDATIONS

The obtained purified pooled library is ready for Illumina sequencing.

Determine library concentration according to the Illumina recommendations.

Spike with 10% of PhiX or any other random library (e.g. RNA-Seq, Exome-Seq etc.).

Recommended coverage that allows for optimal UMI-based elimination of amplification biases and accumulated errors:

- 50-100 sequencing reads per input naive/memory B cell
- 25,000 sequencing reads per input plasma cell/plasmablast

Analyze the resulting pooled library using at least 250+250 paired end Illumina sequencing with standard sequencing primers. For comparative analysis of the obtained IGrepertoire data, use the same sequencing platform. Ideally, use the same sequencing run for the samples under comparison, or mix control and experimental samples in order to minimize batch effects.

DATA ANALYSIS

Data analysis can be performed using MiXCR software for UMI group assembly and clonotype extraction from raw sequencing reads.

20. Extract repertoires using MiXCR™ software, a universal tool for fast and accurate analysis of T- and B- cell receptor repertoire sequencing data.

NOTE: License is required to use MiXCR. Academic users can quickly get a license at https://licensing.milaboratories.com. Commercial license may be requested at https://licensing.milaboratories.com or by email: licensing@milaboratories.com. Information regarding installing the software as well as the detailed pipeline describing how to process the data can be found at https://docs.milaboratories.com/ in sections “Getting Started” and “Guides”, respectively.

21. MiXCR software also provides a broad and rapidly growing range of repertoire postanalysis tools with table and graphical outputs. Please refer to documentation for more information.

APPENDIX A: PREPARING STARTING MATERIAL

BCR cDNA libraries can be generated starting from RNA isolated from sorted/isolated B cells, PBMCs or any tissue containing B cells.

When choosing a purification kit, ensure that it is appropriate for your sample type, input mass, and includes DNase treatment. The last point is critical because gDNA significantly affects cDNA synthesis and subsequent PCR amplification. We recommend RNeasy kits (Qiagen) or TRIzol reagent (Invitrogen). TRIzol reagent provides DNA-free RNA samples. RNeasy kits are compatible with gDNA removal via on-column DNAse treatment, gDNA eliminator columns or RNA cleanup after DNAse treatment in solution. For large numbers of cells (> 200,000 cells) on-column DNAse treatment protocol can be used without modification. For small samples (<200,000 cells) it is recommended to dilute DNAse I stock solution 4 times before treatment to decrease losses.

Control for the counts of B cells in a sample is desirable for the downstream data analysis.

It is preferable to use a freshly isolated cell sample. In the case of frozen samples, culture thawed cells overnight in RPMI-1640 supplemented with 10% human serum.

Lysed cells/tissues can be stored in an RLT® buffer (QIAGEN) at -70°C for at least 6 months.

Large samples (>50,000 cells) can be also stored in TRIzol® at -70°C for up to 6 months.

For large samples (>50,000 cells): verify quantity and quality of the extracted RNA using Qubit and then Agilent Bioanalyzer or gel electrophoresis. RNA Integrity Number > 7, or correct 28S rRNA:18S rRNA ratio (around 1.5-2.5:1) and a low number of shadow bands above and below 18S band are indicative of high quality RNA.

For small samples (i.e. <50,000 cells): it is not necessary to check the quantity and quality of the extracted RNA.

Degraded RNA may indicate that the samples were stored too long before processing, isolated RNA was stored at an incorrect temperature or RNAse contamination is present.

Carrier poly(A) RNA can be used during the RNA extraction procedure to increase the yield of isolated RNA. Carrier RNA will not interfere with primers for cDNA synthesis reaction.

RNA can be stored in 75% ethanol for at least 1 year at -20°C, or at least 1 week at 4°C. For small RNA amounts (less than 100 ng), it is preferable to start cDNA synthesis immediately after RNA extraction.

Further recommendations should help to choose the appropriate strategy for RNA isolation in most situations.

PBMC

Do not use heparin coated tubes for blood collection. Heparin dramatically decreases cDNA synthesis efficiency.

Perform isolation of mononuclear cells from whole blood using Ficoll Paque density gradient centrifugation. Spin down the cells at 350 g for 15 min, remove the supernatant.

Add at least 50 ul per $2x10^5$ cells of RLT® buffer and mix by pipetting for:

- storage at -70°C for up to 6 months, or
- RNA purification using RNeasy Micro or Mini kit (QIAGEN), or
- RNA purification using 4 volumes of TRIzol® added per 1 volume of RLT® buffer (Appendix C).

Alternatively, place cells in at least 300 ul (or 300 ul per 3 x 106 cells) of TRIzol® and extract RNA using TRIzol® protocol (Appendix B).

From 100 to 50,000 sorted or purified B cells

Sort/place directly in 50-300 ul RLT® buffer. The volume of RLT® buffer should not be diluted more than 20% during sorting. 1000 sorted cells may carry the volume around 1 ul when using 70 mkm nozzle for cell sorting. The cells are lysed immediately in the collection tube and mRNA is protected from degradation. Lysed cells can be stored in RLT® buffer at -70°C for at least 6 months.

Use RNeasy Micro kit (QIAGEN) for RNA purification.

To obtain maximum number of cDNA molecules for small samples (100-10,000 cells), add 4 volumes of TRIzol® to RLT® cellular lysate and extract RNA using TRIzol® protocol **(Appendix B)**.

More than 50,000 sorted or purified B cells

Sort/place cells into 300 ul of PBS, then spin down the cells at 350 g for 15 min, remove the supernatant.

Add at least 50 ul per $2x10^5$ cells of RLT® buffer and mix by pipetting for:

- storage at -70°C for up to 6 months, or
- RNA purification using RNeasy Micro or Mini kit (QIAGEN), or
- RNA purification using 4 volumes of TRIzol® (Appendix C).

Alternatively, place cells in at least 300 ul (or 300 ul per 3 x 10^6 cells) of TRIzol® and extract RNA using TRIzol® protocol **(Appendix B)**.

B cell-containing tissue

Homogenize fresh tissue. Ideally, obtain a single cell suspension, for which incubation with DNAse and with proteases mixes (such as Liberase™ TL from Roche) can be recommended. Optionally, wash cells with PBS. Immediately proceed with the RNeasy Micro kit (QIAGEN) using RLT® buffer or use TRIzol® (Appendix B) for RNA extraction.

APPENDIX B: RNA ISOLATION USING TRIZOL®

Lyse cell pellet in TRIzol® reagent by repetitive pipetting. Use at least 300 ul of the reagent per 10^2 – 3x10^6 cells. For larger cell amounts increase the volume of the reagent according to the proportion of 1 ml per 107 cells.

If you have cell lysate in the RLT buffer add **4 volumes** of TRIzol® reagent and mix well by vortexing.

Use carrier RNA (Qiagen # 1068337 or analog). Add carrier RNA directly to the lysate.

Incubate the homogenized samples for 5 min at room temperature.

Add 1/5 volume of chloroform and mix well by vortexing. Incubate tubes at room temperature for 3 min.

Centrifuge the samples at > 10,000 g for 10 min at 2 to 8°C. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

Transfer the colorless aqueous phase without disturbing the interphase to a fresh tube. Add 1 ul of co-precipitant (Pellet Paint® #70748 Merck Millipore or analog) and mix well by pipetting.

Precipitate the RNA from the aqueous phase by mixing with equal volume of isopropyl alcohol. Incubate samples at 15 to 30°C for 10 min and centrifuge at > 10,000 x g for 10 min at 2 to 8°C. RNA with co-precipitant forms a visible pellet on the bottom of the tube.

Remove the supernatant without disturbing the pellet.

If starting from <50 000 cells

Add 1 ml of freshly prepared 75% ethanol to the RNA pellet. Mix the sample by vortexing and centrifuge at >10,000 x g for 5 min at 2 to 8°C. Remove the supernatant.

Dry the RNA pellet completely. It is important, however, not to overdry the pellet as this will decrease its solubility. Dissolve RNA in 10 ul of RNase-free water and immediately proceed to cDNA synthesis.

If starting from >50,000 cells

Add 1 ml of freshly prepared 75% ethanol to the RNA pellet. Mix the sample by vortexing and centrifuge at >10,000 x g for 5 min at 2 to 8°C. Remove the supernatant.

Repeat this step.

Dry the RNA pellet completely. It is important, however, not to overdry the pellet as this will decrease its solubility. Dissolve RNA in 10 ul of RNase-free water.

Proceed to cDNA synthesis or store at –70°C for up to one week. For longer storage add 1/10 volume of sodium acetate (3M pH=5,5) and 3 volumes of 96% ethanol, mix well by vortexing. Store at –70°C.

TROUBLESHOOTING

Table 4

Problem	Possible reason	Possible solution
Low IGH/IGK/L product yield	RNA contains impurities that inhibit cDNA synthesis	In some cases, ethanol precipitation or additional column-based purification of RNA can remove impurities. If this does not help, re-isolate the RNA.
	RNA is heavily degraded	Re-isolate the RNA.
	PCR undercycling	Repeat the 2nd PCR amplification, using two or three more PCR cycles.
IGH/IGK/L bands and background smear are very intense	PCR overcycling	Repeat the PCR amplification, using two or three fewer 2nd PCR cycles.
IGH/IGK/L bands are present but background smear is intense or alternative length fragments are visible	Low IG RNA content in the initial RNA sample	Purify the target library using AMPure XP beads or agarose gel purification.