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Gene expression

SuperScript IV Template-Switching RT Master Mix

Postvaccination immune repertoire profiling using the SuperScript IV Template-Switching RT Master Mix for 5´ RACE

Keywords

5´ RACE, SuperScript IV master mix, immune repertoire, template switching, next-generation sequencing

Introduction

The Invitrogen™ SuperScript™ IV Template-Switching RT Master Mix is designed for precision and ease of use in gene expression profiling. It enables the synthesis of full-length cDNA and reduces amplification biases, resulting in improved sensitivity and accuracy in downstream applications, including immune repertoire and transcriptome analyses.

The immune repertoire, which encompasses all of the T cell receptors (TCRs) and B cell receptors (BCRs) made by an organism, is integral to the adaptive immune system. Monitoring the immune repertoire is essential for understanding the intricate nature of immune responses and their implications for vaccine efficacy and personalized therapies. Extensive research has demonstrated that genetic variations within immune system molecular interactions contribute to significant variability among individuals in their ability to mount efficient responses to influenza vaccines. This variability highlights the importance of studying the immune repertoire, as even among healthy young individuals, there exists a subset of subjects who do not develop a protective immune response to the influenza vaccine [1].

The complexity and heterogeneity of immune responses pose challenges in identifying and validating correlations between vaccines and immune responses. Therefore, it is vital to employ immune monitoring techniques that allow the detailed tracking of immune system modulations. By studying the patterns of immunoglobulin isotypes in human B cells from vaccinated individuals, valuable insights can be gained into the dynamics of the immune response and can potentially reveal factors influencing vaccine efficacy.

This study demonstrates the use of the SuperScript IV Template-Switching RT Master Mix for 5´ RACE–based immune repertoire analysis following annual vaccination of healthy volunteers with inactivated quadrivalent influenza vaccine containing both A and B strains of the virus.

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Overview of 5′ RACE

Rapid amplification of cDNA ends (RACE) is a procedure for amplification of nucleic acid sequences from a mRNA template between a defined internal site and unknown sequences at either the 3′ or the 5′ end of the mRNA. 5′ RACE strategies are popular because they do not require gene-specific primers, thus allowing new allele variants to be captured.

The 5′ RACE protocol used in this study entails two steps (Figure 1). First, a template-switching reverse transcription (RT) reaction is performed, yielding cDNAs with a known adapter sequence incorporated at the 3′ end. In the second step, the 5′ end of the transcript is preamplified utilizing reverse gene-specific primers that are complementary to constant (C) regions, and a forward primer complementary to the adapter sequence.

In the third step, next-generation sequencing (NGS)-specific adapter sequences are incorporated during cDNA amplification, and the protocol generates indexed libraries that are ready for sequencing.

Materials and methods

Materials

- Whole blood
- Thermo Scientific™ GeneJET™ RNA Purification Kit (Cat. No. K0731)
- SuperScript IV Template-Switching RT Master Mix (Cat. No. A65423)
- Invitrogen™ Template-Switching Oligo (Cat. No. A65427)
- Invitrogen™ Capturing Oligo (dT) Primer (Cat. No. A65429)
- Custom forward primer complementary to template-switching oligo sequence and reverse primers for immune genes
- Invitrogen™ Platinum™ SuperFi™ II Green PCR Master Mix (Cat. No. 12369010)
- Invitrogen™ SuperScript™ IV Single Cell/Low Input cDNA PreAmp Kit (Cat. No. 11752048)
- Invitrogen™ Collibri™ ES DNA Library Prep Kits for Illumina™ Systems with UD indexes (Cat. No. A38606024)
- Invitrogen™ Collibri™ Library Quantification Kit (Cat. No. A38524100)
- Thermo Scientific™ TE buffer (pH 7.6, Cat. No. J62285.EQE)
- Agilent™ RNA 6000 Nano Kit (Agilent, Cat. No. 5067-1511)

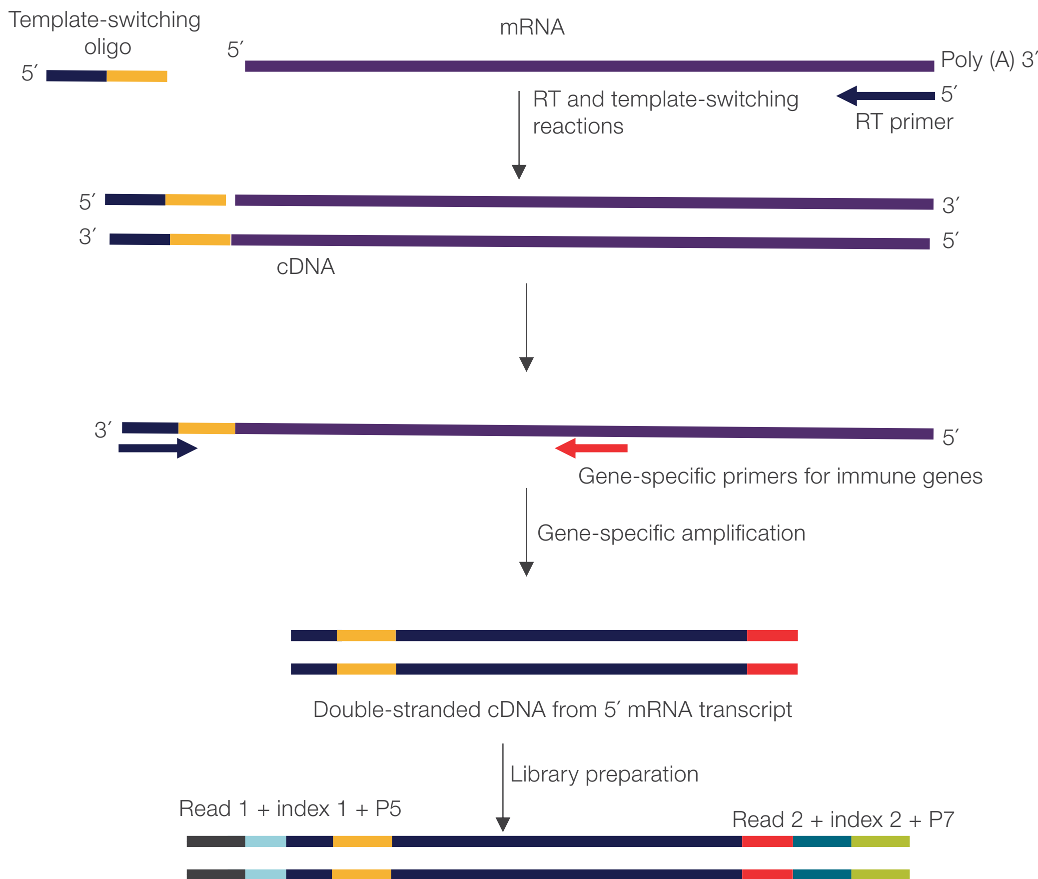

Figure 1. Overview of the protocol for 5′ RACE–based library preparation.

Sample preparation

Venous blood from 3 healthy donors was collected 2 weeks postvaccination with quadrivalent influenza vaccine. Total RNA extraction was performed using the GeneJET RNA Purification Kit according to the protocol for human blood cells total RNA purification provided in the user guide. RNA quantity and purity was monitored using the Agilent RNA 6000 Nano Kit. The final concentration of RNA sample was 15.5–26.1 ng/μL depending on the tested sample. For the RT reaction, each RNA sample was diluted with Thermo Scientific TE buffer (pH 7.6) to a final concentration of 10 ng/μL, and 2 μL of the diluted RNA was used in RT reactions.

RT and preamplification

cDNA was synthesized using the SuperScript IV Template-Switching RT Master Mix. For the RT and template-switching reactions, Capturing Oligo (dT) Primer and Template-Switching Oligo were used. All cDNA synthesis reaction conditions were applied according to the user guide. Obtained cDNA was amplified using reverse IgG, IgK, IgL, and IgM immune gene–specific primers and forward primer complementary to the template-switching oligo sequence. For PCR amplification, Platinum SuperFi II Green PCR Master Mix was used according to the user guide. After amplification, all PCR assays were pooled together in one microtube for subsequent purification and NGS library preparation.

Purification

After amplification, reaction cleanup was performed using AMPure™ XP SPRI Reagent (Beckman Coulter, Cat. No. A63880). Purification is required to remove excess primers and achieve sufficient concentration of cDNA product for NGS. Purification was performed according to the protocol provided in the user guide of the SuperScript IV Single Cell/Low Input cDNA PreAmp Kit.

NGS and data analysis

The cDNA samples were converted to sequencing-ready libraries through enzymatic fragmentation using the Collibri ES DNA Library Prep Kit for Illumina Systems and unique dual (UD) indexes. Following purification and size selection according to the Collibri ES DNA Library Prep Kit manual, libraries were validated using the 2100 Bioanalyzer™ system (Agilent). The final concentrations of the sequencing libraries were determined using the Collibri Library Quantification Kit. Libraries were sequenced on the MiSeq™ Sequencing System (Illumina) by paired-end sequencing of 2 x 150 bp with 300-cycle v2 cartridges (Cat. No. MS-102-2002). NGS data analysis was carried out using MiXCR™ software (https://mixcr.com/) [2].

Results

Immune repertoire structure analysis

B and T cells are key players of the adaptive immune system and are composed of many different clones defined by their specific BCR or TCR sequence rearrangement. A comparative analysis of the general immune repertoire structure across all individuals in the study revealed that the transcripts of immunoglobulin heavy chain (IGH) accounted for 21.74% to 23.74% of the repertoire. Transcripts of immunoglobulin kappa light chain (IGK) ranged from 18.95% to 23.55%, while transcripts of immunoglobulin lambda light chain (IGL) ranged from 10.19% to 16.39%. Transcripts of T cell receptor α locus (TRA) were found to range from 9.03% to 20.20%, whereas transcripts of T cell receptor β locus (TRB) constituted the majority of all expressed genes, ranging from 20.20% to 24.34% (Figure 2).

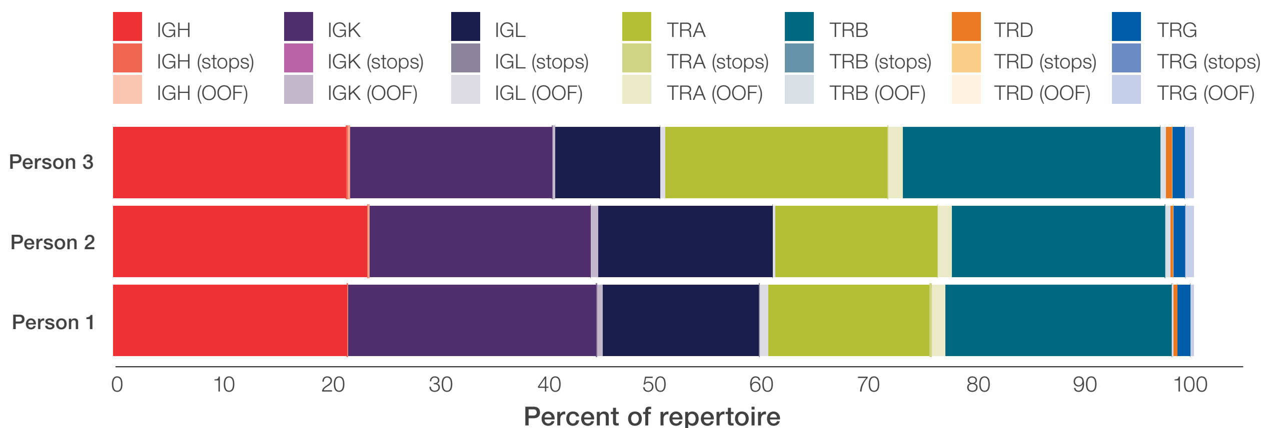

Figure 2. General immune repertoire structure in individuals vaccinated with quadrivalent influenza vaccine. IGH: immunoglobulin heavy chain, IGK: immunoglobulin kappa light chain, IGL: immunoglobulin lambda light chain, TRA: T cell receptor α locus, TRB: T cell receptor β locus, TRD: T cell receptor δ locus, TRG: T cell receptor γ locus, stops: gene that contains a stop codon (indicates that the resulting protein may be truncated or nonfunctional), OOF: out-of-frame rearrangements of the immunoglobulin gene. Immune profiling with NGS data analysis was carried out using MiXCR software.

Clonotype distribution

Multivalent vaccines assume equal immunogenicity of the vaccine components to induce a balanced immune response to each vaccine strain [3]. However, more often, there is a subtype immunodominance to one or more components following influenza vaccination [4]. According to the clonotype distribution of immunoglobulins, in all three individuals the IgA type comprises the majority (53–57%) of clonotypes (Figure 3). IgA antibodies that are specific to the virus are generated by plasma cells in the mucosal-associated lymphoid tissues (MALTs), such as the tonsils, and are secreted into the respiratory tract. These antibodies play several crucial roles in mucosal defense, including preventing entry of the virus and reducing transmission [5].

The second most abundant clonotype is IgM. IgM antibodies are produced rapidly within two weeks of vaccination, but they decline more rapidly than IgG antibodies. However, IgM antibodies can persist for extended periods of time.

In the context of influenza vaccination, if IgG accounts for approximately 15% of all immunoglobulin clonotypes, it could suggest that IgA and IgM play a more prominent role in the immune response to 2023/2024 quadrivalent influenza vaccine: influenza viruses primarily infect the respiratory tract, and IgA antibodies, which are secreted into the respiratory tract, are crucial for mucosal defense against the virus. IgM antibodies may also be produced rapidly in response to influenza vaccination, providing early protection. Therefore, if there is a lower representation of IgG clonotypes, it may suggest that IgA and IgM antibodies are the predominant types involved in the early immune response against influenza. Even though IgG accounts for a smaller proportion of clonotypes, it may still have a specific role in providing long-term protection against influenza or have specialized functions within the immune system.

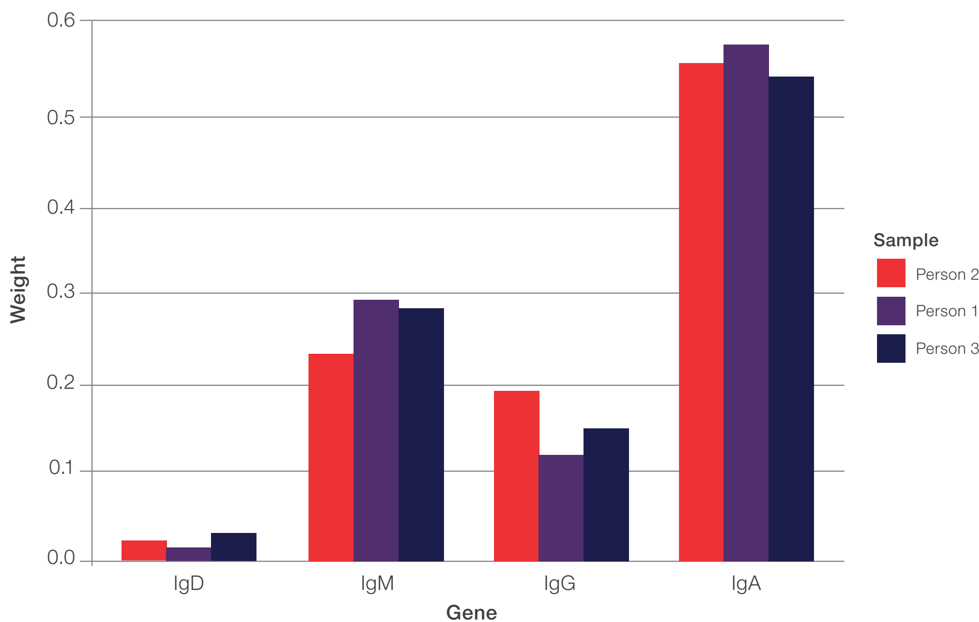

Figure 3. Clonotype calls identified from 20 ng of total RNA. BCR profiling of IgG, IgM, IgD, and IgA libraries were generated from human total RNA. After quality control, filtering, and alignment to the region of interest, sample sizes for person 1, person 2, and person 3 were 5,315, 7,834, and 5,712 reads, respectively. Downsampling to the same number of reads in MiXCR software was used to calculate cloneset abundances and compare between persons.

Immunoglobulin isotype patterns

Detailed analysis of immunoglobulin isotype patterns in human B cells revealed 32 possible V regions within the variable domain of immunoglobulin lambda light chain (IGLV), 34 possible V regions within the variable domain of immunoglobulin kappa light chain (IGKV), and 54 possible V regions within the variable domain of immunoglobulin heavy chain (IGHV) (Figure 4). The most pronounced immunoglobulin patterns (darker blue) are described by IGLV2-14, IGLV3-21, and IGLV1-40 within IGLV; IGKV1-5, IGKV3-20, IGKV3-15, IGKV4-1, IGKV1D-39, IGKV3-11, IGKV3D-20, and IGKV2-28 within IGKV; and IGHV3-23 within IGHV. In general, a similar immunoglobulin pattern predominates among the B cells of the individuals studied. These findings shed light on the diversity and complexity of the immunoglobulin repertoire in human B cells, highlighting the importance of these isotypes in immune responses.

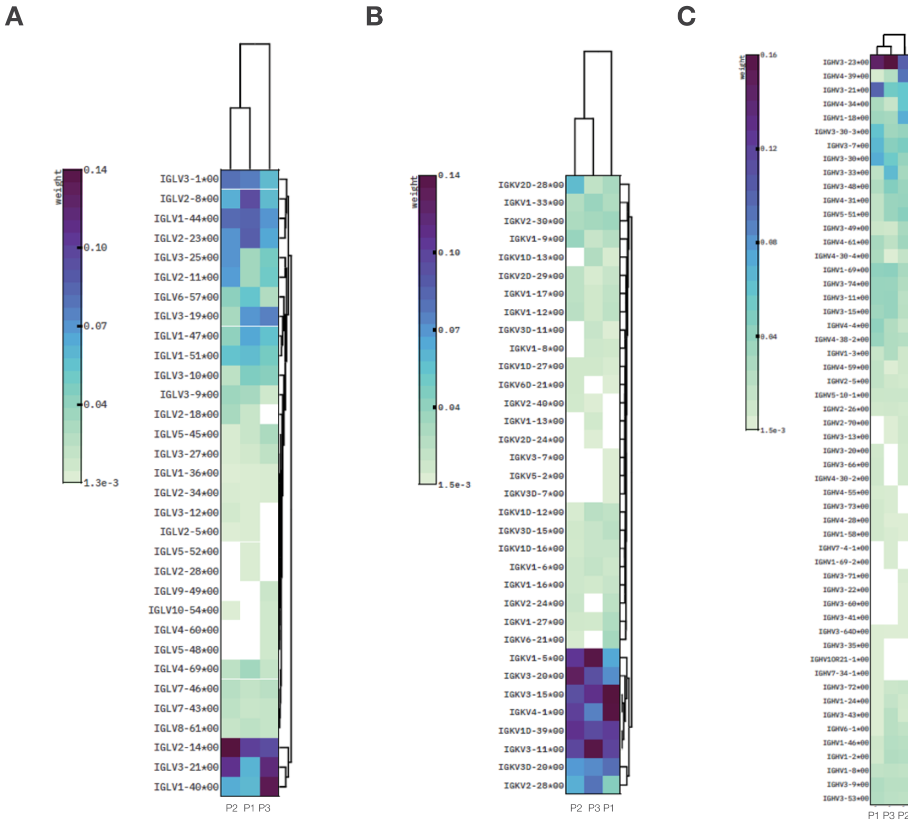

Figure 4. Patterns of immunoglobulin isotypes in human B cells. The prevalence of different V regions are shown within **(A)** IGLV, **(B)** IGKV, and **(C)** IGHV.

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Conclusions

The 5´ RACE–based sequencing described here is a highly effective tool for analyzing the human immune repertoire. It utilizes template-switching technology and combines a 5´ RACE method with gene-specific amplification to capture the complete variable regions of B cell receptors. This method provided highly sensitive and specific clonotype detection.

By incorporating NGS-specific adapter sequences into the primers during cDNA amplification, the protocol generates indexed libraries that are ready for sequencing on Illumina™ platforms. This method also includes a unique PCR cycling and pooling workflow, which helps reduce sequencing costs while maintaining accurate clonotype identification.

One notable advantage of this method is that it avoids the use of multiplex PCR, which can introduce biases in amplification of certain sequences. Template switching contributes to improved sensitivity and accuracy and helps ensure a comprehensive and precise view of human immune receptor repertoires using the 5´ RACE application.

Ordering information

Product	Quantity	Cat. No.
GeneJET RNA Purification Kit	50 preps	K0731
SuperScript IV Template-Switching RT Master Mix	48 reactions	A65423
Template-Switching Oligo	48 reactions	A65427
Capturing Oligo (dT) Primer	48 reactions	A65429
Platinum SuperFi II Green PCR Master Mix	100 reactions	12369010
Collibri ES DNA Library Prep Kits for Illumina Systems	24 preps	A38606024

References

1. Castrucci MR (2018) Factors affecting immune responses to the influenza vaccine. *Hum Vaccin Immunother* 14:637–646. doi: **10.1080/21645515.2017.1338547**
2. Bolotin DA et al. (2015) MiXCR: software for comprehensive adaptive immunity profiling. *Nat Methods* 12:380–381. doi: **d10.1038/nmeth.3364**
3. Abreu RB et al. (2020) IgA responses following recurrent influenza virus vaccination. *Front Immunol* 11:902. doi: **10.3389/fimmu.2020.00902**
4. Abreu RB et al. (2020) Preexisting subtype immunodominance shapes memory B cell recall response to influenza vaccination. *JCI Insight* 5:e132155. doi: **10.1172/jci.insight.132155**
5. Tyagi R et al. (2023) Role of immunoglobulin A in COVID-19 and influenza infections. *Vaccines* 11:647. doi: **10.3390/vaccines11111647**

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