Cloning bovine immunoglobulin V_H and V_L genes from single B cells.

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Abstract

A distinct feature of bovine antibodies that has not been observed in any other species, ultralong CDR H3 regions, has the capacity to access epitopes on structurally complex antigens. Bovine immunoglobulins with ultralong CDR H3s have great potential for development as clinical treatments and research tools targeting a broad variety of antigens. However, unlike other species, techniques for studying bovine immunoglobulin genes at the single cell level have not been comprehensively developed. We established a new method for amplification of bovine immunoglobulin V_H and V_L genes at the single cell level utilizing flow cytometry to sort individual B cells into wells and cDNA production and PCR amplification followed by nested PCR. Optimal primers were designed and ratios of each V_H and V_L primer, along with other conditions, were optimized for production and amplification of cDNA. The amount of template added to nested PCR reactions and the number of subsequent PCR reactions necessary to procure enough DNA for cloning were also optimized. Results from cells sorted by two types of FACS machines were also compared. Both V_H and V_L DNA can be obtained from >50% of sorted B cells using this method. This technique enables the production of monoclonal antibodies with heavy and light chain pairs expressed from individual bovine B cells and will be useful for rapid identification of recombinant bovine antibodies.

Introduction

Individual B cells produce unique heavy and light chain pairs to form functional antibodies; the single cell sorting and PCR approach we developed enables the discovery and production of heavy and light chain pairs from individual B cells. The single cell sorting and PCR strategy utilized for this method has been thoroughly studied using human[1,2] and murine[3] B cells, however this process has not been fully optimized with bovine B cells. There are unique challenges for applying this method to study bovine immunoglobulin genes, such as constraints on PCR primer design and unknown numbers of V_H and V_L RNA molecules relative to one another per single bovine B cell. Genomic leader sequences of bovine V_L genes and constant region sequences for both V_H and V_L genes are comprised of palindromes and regions of multiple G and C nucleotide repeats; thus, there is a high potential for any given 20-30 contiguous nucleotides within these regions to form both self-dimers and heterodimers. A holistic primer design approach, in conjunction with a series of individual and combined primer concentration optimizations were employed to reduce formation of primer dimers and amplify both V_H and V_L from a single B cell's RNA in relatively equal amounts despite likely differences in number of RNA molecule copies.

Figure 1. Schematic of heavy and light chain gene amplification from a single bovine B cell.

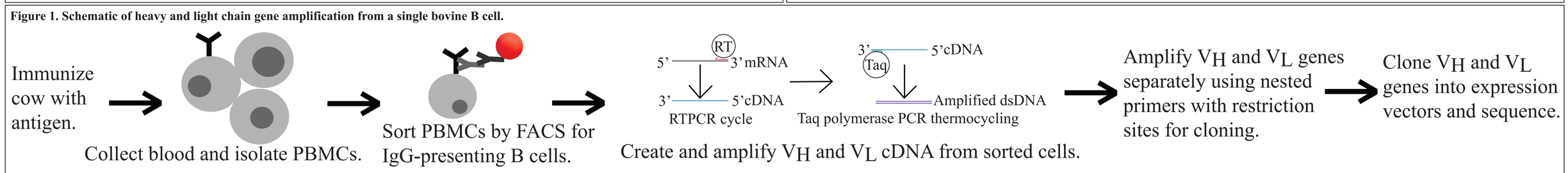

Figure 1 After immunization of a cow with an antigen of interest, blood was collected, PBMCs isolated, and sorted for IgG+ B cells. Qiagen's OneStepRTPCR kit, with primers which anneal to bovine V_H and V_L leader peptides and constant regions, was used to produce V_H and V_L cDNA with reverse transcriptase. V_H and V_L genes were subsequently amplified with HotStarTaq polymerase PCR thermocycling, then further amplified with nested primers containing restriction sites for cloning. Nested primer PCR products were cloned into expression vectors and sequenced.

Figure 2. Primer features.

Figure 2, A Schematic representation of heavy chain (HC) and light chain (LC) genes including variable and constant regions (size not to scale). Primer locations are indicated by arrows. All primers were designed to anneal with sequences on indicated regions that are homologous between frequently used bovine alleles. Two sets of primers were added to each OneStepRTPCR reaction. Forward primers were designed to anneal with leader peptide sequences of HC (dark green) and LC (light green) genes. Reverse primer locations for OneStepRTPCR reactions are HC IgG1 constant region (red) and lambda LC constant region (magenta). Product from OneStepRTPCR reactions was added as template to two separate nested primer PCR reactions for the HC and LC. Restriction sites for cloning into pFUSE vectors were included in the nested primers. The HC forward primer (blue) is located on the 5' end of the V_H region and contains a 5' EcoRI site; the HC reverse primer (dark purple) is on the 3' end of the J_H region with a 3' NheI site. The LC forward nested primer (cyan) is located on the 5' end of the V_L region and has a 5' EcoRI site; the LC reverse nested primer (light purple) anneals to the 3' end of J_L and contains a 3' AvrII site.

B

LC forward GCTGGTCGCTCTCTGCACAGGATCCTGGGC
HC forward CTGTCCTGCGGAGACCCCCGACTGTCGTGTTTCT
LC forward GCTGGTCGCTCTCTGCACAGGATCCTGGGC
HC reverse GCCGTCGTCGAGTCCTGTCTGGAAGTGGACGTTGC

Figure 2, B Predicted heterodimer structures of the OneStepRTPCR LC forward primer with each HC primer (left is HC forward, right is HC reverse, base-pairing nucleotides in red). The potential of each OneStepRTPCR primer to form both homo and hetero dimers was analyzed (using IDT OligoAnalyzer tool) during the primer design process. Template for each OneStepRTPCR reaction is single cell mRNA; primer dimers are substantially more problematic with this low amount of template than with higher template concentrations that are typically used in PCR reactions. Each forward primer was designed to anneal with portions of leader sequences that are homologous with different alleles. These regions of homology are relatively short and heavily comprised of multiple contiguous G/C nucleotides and palindromes. All primer-length (20-30 nucleotides) combinations of sequences that include regions of homology have the capacity to form self-dimers as well as dimers with other OneStepRTPCR primers. The region of homology within the LC leader sequence is 19 nucleotides long. Due to the limited target sequence length in conjunction with the other challenges discussed, the LC forward primer has inevitable capacity to form dimers.

Figure 3. Higher annealing temperature results in lower amounts of non-specific product.

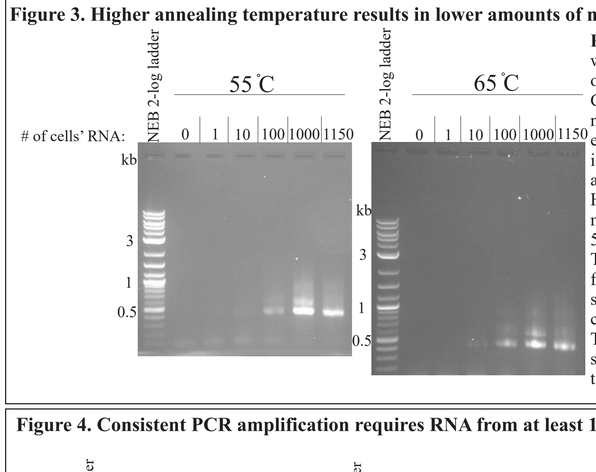

Figure 3 Bulk-sorted cells were used for single cell PCR optimization experiments. Concentration, in units of number of cells' RNA/µl in each OneStepRTPCR reaction is noted above gel lanes. Two annealing temperatures for HotStarTaq polymerase thermocycling (OneStepRTPCR), 55 ˚C and 65 ˚C, were tested. The amount of desired product formed at each temperature is similar however less non-specific product is formed at 65 ˚C. The annealing temperature selected for all subsequent optimization experiments is 65 ˚C.

Figure 4. Consistent PCR amplification requires RNA from at least 100 cells.

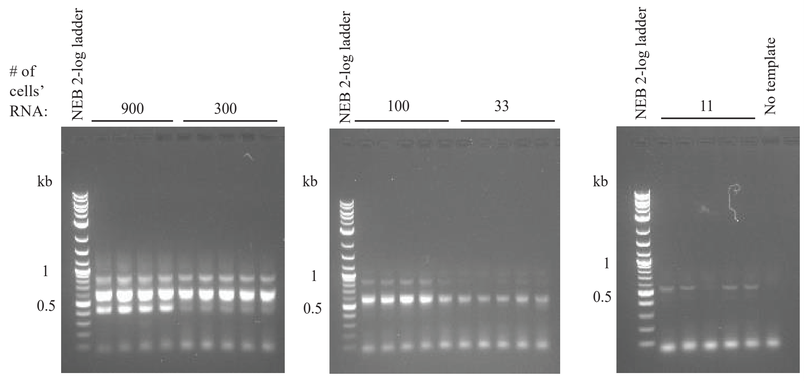

Figure 4 The number of cells' RNA per OneStepRTPCR reaction (indicated above lanes) was titrated in 3-fold dilutions, with 5 replicates per template dilution, in order to determine the lowest number of cells' RNA per reaction that yields the most consistent results in replicates under the same conditions. An uneven distribution of antibody-encoding RNA in higher-fold dilutions of the stock is a potential source of inconsistent results amongst replicates. It is imperative that template concentration is as consistent as possible in OneStepRTPCR optimization experiments in order to draw meaningful conclusions about the effect of conditions tested on product formation. The lowest number of cells' RNA per reaction with consistent product in replicates is 100; this template concentration was used for all following optimization experiments with the bulk-sorted cell stock as template.

Figure 5. Light chain forward primer forms dimers with both heavy chain primers.

Figure 5 Figure 2B illustrated the potential of the OneStepRTPCR LC forward primer to form dimers with each OneStepRTPCR HC primer. Each OneStepRTPCR primer, alone and combined pair-wise, was added to OneStepRTPCR reactions with and without template to determine which primer and/or primer pair formed dimers. Primer and primer pairs added to each reaction are indicated above gel lanes (H= heavy chain, L= light chain, F=forward, R=reverse). The presence and intensity of bands at around ~100 bp in each lane were evaluated; high-intensity primer dimer bands are present in lanes with the light chain forward primer paired with each heavy chain primer, indicating that the light chain forward primer forms dimers with each heavy chain primer. Due to limitations of primer design, it was more feasible to proceed by optimizing ratios and concentrations of each primer than to redesign any of the primers (Figure 6, Figure 7).

Figure 6. Specific product was formed at each reduced forward primer concentration.

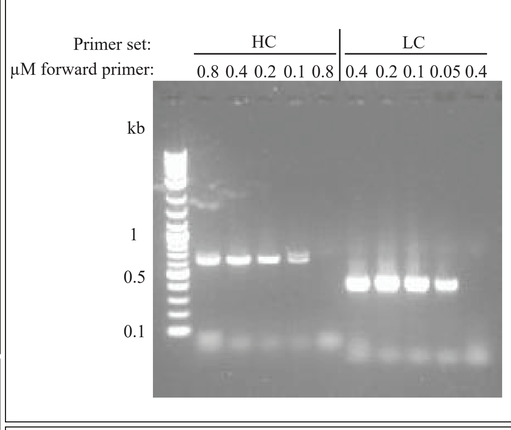

Figure 6 In order to reduce formation of primer dimers and increase amount of desired product, the concentration of each forward primer was optimized. OneStepRTPCR reactions were performed with each primer set separately (primer set indicated above gel lanes) and the concentration of each forward primer was titrated 2-fold while the concentration of each reverse primer was constant in each reaction (0.8 µM for heavy chain reverse primer, 0.4 µM for light chain reverse primer). Lanes with equal concentrations of forward and reverse primer with no template added served as negative controls. The lowest forward primer concentrations of each primer set that yielded comparable amounts of product formation with higher forward primer concentrations were selected for further primer concentration optimization with combined primer sets (Figure 7); these concentrations are 0.2 and 0.1 µM for the heavy chain forward primer, 0.1 and 0.05 µM for the light chain forward primer.

Figure 7. Primer dimer formation was reduced by lowering forward primer concentrations.

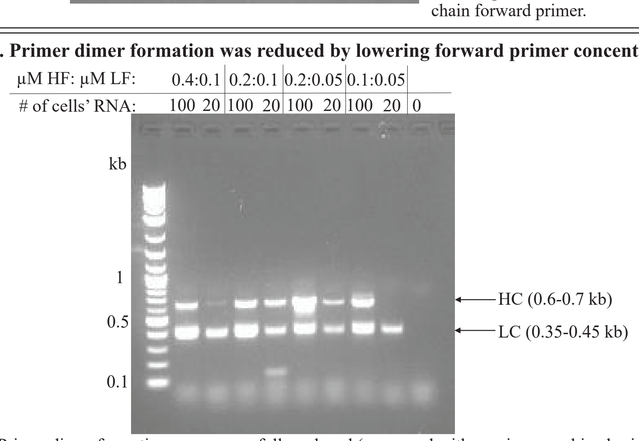

Figure 7 Primer dimer formation was successfully reduced (compared with previous combined primer set experiments, Figure 3 and Figure 4) by lowering the concentrations of forward primers in OneStepRTPCR reactions. Four combinations of forward primer concentrations, with consistent reverse primer concentrations in each reaction (0.8 µM heavy chain reverse, 0.4 µM light chain reverse), were tested to determine which condition resulted in the most robust formation of both heavy and light chain product in relatively equal amounts. Each forward primer concentration combination was added to reactions with 100 and 20 cells' RNA as template. Forward primer concentrations and number of cells' RNA used as template for each reaction are indicated. Expected band migration distances for each target product is noted to the right of the gel. The forward primer concentration combinations that yielded the most robust, and approximately equivalent, heavy and light chain products were 0.2 µM heavy chain forward with 0.1 µM and 0.05 µM light chain forward. The concentrations of each forward primer selected for OneStepRTPCR reactions with single-sorted cells as template were 0.2 µM heavy chain forward and 0.05 µM light chain forward. Preference was given to the lower light chain forward primer concentration due to its capacity to form primer dimers.

Table 1. Type of cell sorter used resulted in differential PCR success rates.

FACS machine used to sort cells	BD FACS Aria2U	NanoCellect WOLF
Total number of single cells	16	21
% of cells with visible OneStepRTPCR product	43.75	80.95
% of cells with visible HC nested primer PCR product	68.75	80.95
% of cells with visible LC nested primer PCR product	75	90.5
% of cells both heavy and light chain sequences obtained from	37.5	52.94

Table 1 Results obtained from cells sorted by BD FACS Aria2U versus NanoCellect's WOLF Cell Sorter were compared in terms of proportion of total number of cells sorted that yielded visible PCR results and full antibody sequences (GeneWiz) were recovered from. The percent of cells with visible OneStepRTPCR product includes HC only, LC only and both HC and LC. Based on these results, percent of cells yielding visible PCR products and full antibody sequences are overall higher when cells are sorted by NanoCellect's WOLF Cell Sorter than by BD FACS Aria2U.

Figure 8. The cell sorter impacts PCR success.

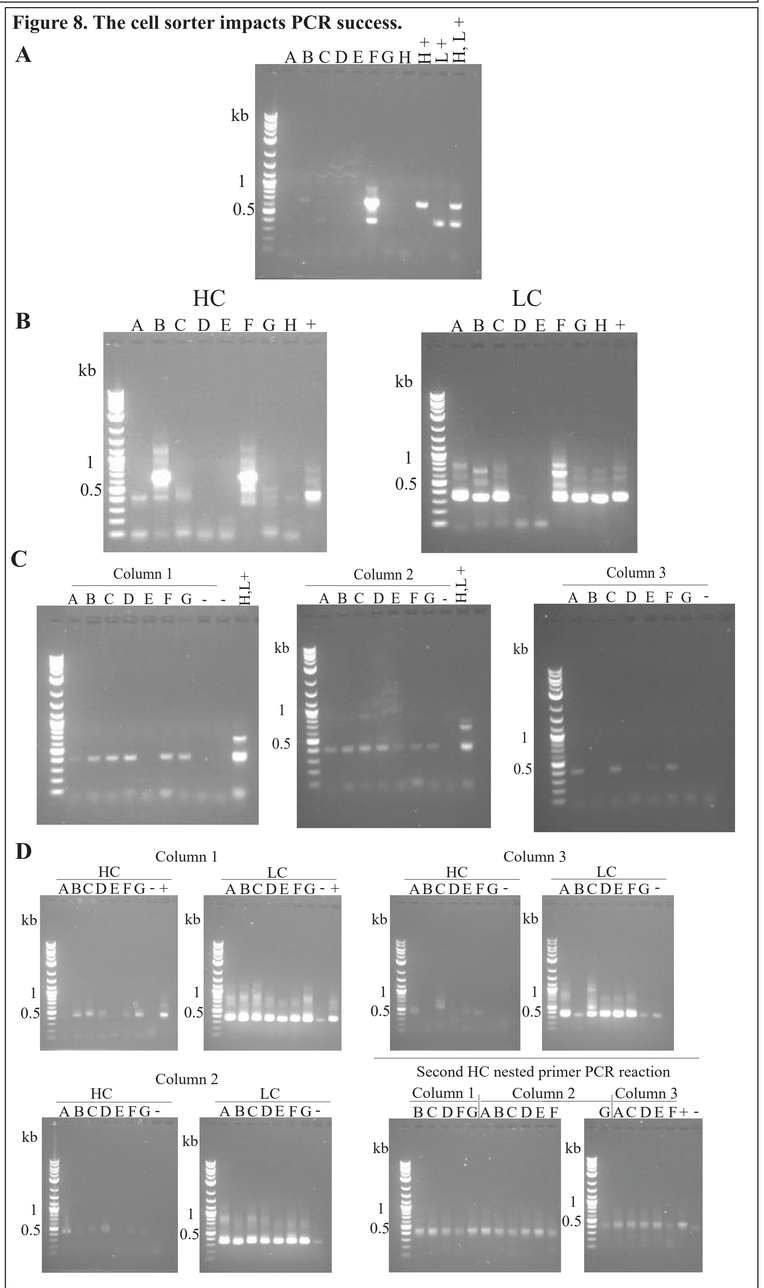

Figure 8 Single cells, sorted by BD FACS Aria2U (A-B) or the NanoCellect WOLF cell sorter (C-D) were used as template for OneStepRTPCR reactions (each template is assigned a letter, noted above lanes of Figure 8 gels). **A** Optimized primer concentrations (Figure 7) were added to single-cell template OneStepRTPCR reactions. The percent of reactions with visible product for LC only, HC only, both HC and LC are 12.5% for each. Positive controls (+) included 100 cells' RNA as template with heavy chain primers alone (H+), light chain primers alone (L+) and both primer sets (H,L+). **B** OneStepRTPCR products were added as template for separate nested primer PCR (Invitrogen HiFi Platinum Taq polymerase) reactions (nested primer sets are labeled above each gel, HC for heavy chain and LC for light chain). Expected nested primer product gel migration distances are 0.4-0.5 kb for heavy chains and 0.3-0.4 kb for light chains. Product is visible in 75% of both heavy and light chain nested primer gel lanes. Substantially higher amounts of product were formed with LC nested primers than HC nested primers. In order to amplify enough HC nested primer PCR products for cloning, HC nested primer PCR products were added as template for a subsequent round of HC nested primer PCR (data not shown). **C** Cells were sorted into individual wells of a 96-well PCR plate (Biorad) using NanoCellect's WOLF cell sorter; OneStepRTPCR products in columns 1-3 were visualized on 1% agarose gels. Visible product for LC is present in 81% of reaction lanes. **D** Initial heavy chain nested primer PCR products are visible in 81% of reaction lanes and are present in the same sample lanes with visible OneStepRTPCR light chain products. Light chain nested primer PCR products are visible in 90.5% of reaction lanes. Samples with visible HC nested primer PCR products were amplified in a second round of HC nested primer PCR with a 100% success rate.

Conclusions

1. This is the first protocol to comprehensively optimize amplification of antibody genes from single bovine B cells. Sequences encoding both heavy and light chain genes can be obtained from >50% of sorted cells using this method.
2. Conditions for single cell PCR to amplify bovine heavy and light chain genes from sorted B cells that were optimized include type of FACS machine used to sort cells, annealing temperature for OneStepRTPCR HotStarTaq polymerase thermocycling, concentration and ratios of OneStepRTPCR primers, amount of nested primer PCR reactions necessary to procure enough DNA for cloning and ideal amount of template for nested primer PCR reactions.
3. A correlation exists between type of FACS machine used to sort cells and success rate of single cell PCR results, which may be due to lower stress of the B cells.

Future Directions

1. Application of this method for antigen-specific antibody discovery projects. This protocol could be useful for functional antibody discovery when antibody genes are subsequently recombinantly expressed.
2. Little is known about bovine antibody gene repertoires; this method will enable us to evaluate bovine heavy and light chain genetic repertoires at the single cell level.

References

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