Redefining the Identification of Knops Blood Group Alloimmunization

Jennifer O’Connor

BloodCenter of Wisconsin

Abstract

BACKGROUND

Antibodies directed against antigens in the Knops blood group system can be difficult to identify due to variable weak reactivity with most red cells tested. Although generally thought to be clinically insignificant, these antibodies need to be identified correctly, because they can mimic other antibodies. Therefore, algorithms that include new techniques in addition to serologic hemagglutination, are important to ensure proper identification and patient safety.

STUDY DESIGN AND METHODS

Three different approaches to Knops antibody identification were explored. First, donors were genotyped using real-time PCR to create a selected cell panel capable of definitively ruling-in the specific antibody and ruling-out all other Knops antibodies. This same methodology was explored to genotype individuals with Knops antibodies. Finally, soluble complement receptor 1 (sCR1) substance was utilized to neutralize Knops antibodies.

RESULTS

A panel of donors was created capable of ruling in Anti-Kn^{a}, Anti-McC^{a}, Anti-Sl1, or Anti-KCAM while ruling-out other Knops antibodies. Additional donors would need to be screened if the goal is to rule out all routinely tested clinically significant antibodies. Genotyping of patients with previously identified Knops antibodies confirmed several antibodies, but two donors with identified Anti-Kn^{a} tested Kn(a+b-). Finally, at high concentrations sCR1 was capable of neutralizing Knops antibodies.

CONCLUSION

A three-pronged approach utilizing these three methods would be effective to identify Knops antibodies. First, the sCR1 substance could be used to pinpoint specificity to the Knops blood group system, followed by running the selected cell panel to determine specific identification. Finally, patient genotyping would confirm specificity.

Introduction

The Knops blood group system is localized to the transmembrane protein complement receptor 1 (CR1/CD35), whose main function is the regulation of the complement system through the binding of complement components 3b and 4b in immune complexes. Four structurally different types of CR1 have been identified ranging from 190kDa to 280kDa resulting from deletions and duplications. [1] The most common form is a 220kDa protein, which contains the Knops blood group system antigens.[2]

Antibodies directed against antigens in the Knops blood group system were included in the now obsolete group referred to as high-titer, low-avidity antibodies. Although generally thought to be clinically insignificant, alloantibodies formed against Knops blood group system antigens can be difficult to identify due to variable weak reactivity with most red cells. Therefore, they can mask the presence of other clinically significant antibodies, cause difficulty in identification of other alloantibodies, and can mimic clinically significant antibodies, such as anti-Yt^a, -Lu^b, -Vel, and -Ge.

The polymorphisms that cause all nine known Knops blood group system antigens have been identified in either exon 26 or exon 29 of the gene that encodes for CR1/CD35. [2-6]. Using this information, assay have been created for genotyping eight Knops antigens. If enough donors were genotyped to find those negative for high prevalence Knops blood group system antigens, a "Knops panel" could be created to help identify these antibodies.

Additionally, the use of patient genotyping for Knops blood group antigens was explored due to difficulties in phenotyping patients. Commercially-available antisera are not available to phenotype individuals for Knops antigens. As a result, the red cells used as selected cells to help

identify these antibodies are suspect since they are typically typed using somewhat unpredictable patient antibodies previously identified. Therefore, all current identification of these antibodies relies on the proper identification of previous antibodies.

Finally, although neutralization using plasma (as is done with Chido/Rodgers antibodies), is not possible for Knops blood group system antibodies due to very low concentrations of CR1 in plasma, Moulds and Rowe have shown that use of recombinant DNA technology can create soluble CR1 (sCR1) substance, which is capable of neutralizing these antibodies.[7] Due to the high prevalence of many of the Knops antigens that antibodies are formed against, neutralization allows easier identification of underlying antibodies and aids in confirmation of Knop blood group specificity.

Due to the difficulty of identifying these antibodies, some immunohematology reference labs do not definitively identify these antibodies, but rather titrate and report them as high-titer, low avidity antibodies. However, the use of other techniques in addition to traditional serologic antibody identification could be beneficial. In this study, three techniques were explored, which included patient genotyping, antibody neutralization, and the creation of a panel of selected donors negative for the high prevalence Knops antigens. Our goal was to find the best way to utilize these techniques to more accurately and efficiently identify Knops blood group system antibodies to improve patient safety.

Materials and Methods

Real-time PCR to Genotype Patients with Knops Antibodies

Patients and donors with previously identified Knops blood group system antibodies were obtained from the immunohematology reference lab, including 4 donors with anti-Kn^{a}, one

patient with anti-McC^a, and 2 patients with anti-Sl^a. DNA from these patients/donors was isolated using a QIAamp DNA Mini Kit (Qiagen). The DNA was then genotyped for the antigen that corresponded to the alloantibody that they had presumably developed to confirm or refute proper identification. Those donors whose genotype did not support the antibody identified by serology were then further genotyped for other antigens in the Knops blood group system.

Genotyping was performed using assays designed in-house or by Life Technologies, Inc. (LTI) to detect the SNPs that cause the alleles of interest (Table 1). The initial assay for *Yk*^{a} genotyping failed due to non-specific reactions resulting from base changes adjacent to the *Yk*^{a} SNP and therefore was not included in this study.

All assays were designed to detect nucleotide changes using a 5' nuclease hydrolysis SNP genotyping assay on a LightCycler480 (Roche Applied Bioscience, Indianapolis, IN) real-time PCR instrument. Each assay used 12.5 µL of LTI's ready-master mix, 1 µL of DNA, 10.25 µL water, and 1.25 µL of the pre-formulated assay mix specific for the SNP of interest which included: 2 amplification primers flanking exon 29 and two fluorogenic probes. One probe is 5' labeled with VIC dye and detects one allele; the other is 5' labeled with FAM dye and detects the other allele. Each probe is labeled at the 3' end with a non-fluorescent quencher. FAM/VIC ratios were calculated to determine the donors' zygosity for the allele of interest.

Use of Soluble CR1 to Neutralize Knops Antibodies

Soluble CR1 substance was created in the research laboratory by transfecting Chinese hamster ovarian cells with recombinant DNA. It was purified using a heparin column. The final product was then electrophoresed using a Phastgel system and stained with Coomassie to ensure

that a 220kDa band was present indicating that the process was successful and the purification left the intended product behind (Figure 1). Pool3 and Pool4 were shown to have a concentration of approximately 1mg/mL.

Once purified, the sCR1 substance (pool3 and pool4) was diluted 1:100 in 6% albumin and doubling dilutions were created up to 1:51200. One drop of each of these dilutions was then mixed with two drops of patient's serum that contained a strongly reactive (2+) Anti-Kn^a. A dilution control was run simultaneously by adding 1 drop of 6% albumin to 2 drops of the same serum. They were incubated at room temperature for 15 minutes and then one drop of a 2-5% cell suspension of a cell, known to show strong positive reactivity with Anti-Kn^a, was added and incubated at 37°C for 30 minutes. The cells were then washed four times and polyspecific AHG was added. Although noticeable reduction in antibody strength was noted with some of the lesser dilutions, none of the dilutions resulted in complete neutralization. As a result, the procedure was repeated starting with a 1:10 dilution and performing a doubling dilution up to 1:160 with pool3, because it was the pool with the slightly higher concentration of sCR1 substance. The procedure and control remained the same, but varied incubation times (15 and 30 minutes) and temperatures (25°C and 37°C) were attempted to see if it helped neutralization.

Real-time PCR to Genotype Donors

DNA samples used were obtained from blood donors previously isolated for routine donor genotyping. DNA isolations were from Caucasian donors with a history or at least three previous donations over the last three years, or any African American, Hispanic and Asian donor. Each week, a total of 180 donors DNA isolations were performed, which consisted of mostly African American donors and other ethnicities on one plate and Caucasians on another. Genotyping was performed using the same methods/assays mentioned above. When running

Knops genotyping to identify those negative for the high prevalence Knops antigens, donor plates were preferentially selected to maximize the greatest number of rare negative genotypes. For example, when running a Kn(a/b) assay, donors were chosen to maximize the number of Caucasian donors genotyped, since the Kn^b/Kn^b genotype is more prevalent in Caucasians.

After preferential genotyping to find the donors that were negative for the high prevalence antigens (i.e. Kn(a-), McC(a-), Sl1-, Sl3-, and KCAM-), these donors were then genotyped for four other Knops antigens in order to obtain a Knops genotype for these donors. Results of routine blood group antigen genotyping were then reviewed to determine the donors' red cell antigen profiles for common antigens. Antigen profiles of all of these donors were then evaluated to select the best donors to make a red cell panel capable of ruling out underlying clinically significant alloantibodies in the presence of a Knops antibody.

Results

When donors/patients with previously identified Kn^a antibodies were genotyped, only two of the four confirmed negative for Kn^a. While the one patient with anti-McC^a and two with anti-Sl^a confirmed negative for their respective antigens. Due to the unexpected results obtained using the Kn(a/b) assay, known Kn^b/Kn^b and Kn^a/Kn^b samples were obtained (a gift from Dr. J Moulds) to evaluate whether the assay gave expected results. All controls performed as expected.

As previously mentioned, when testing sCR1 substance at dilutions greater than 1:100 a decrease in reactivity was noted, but the reactions remained positive. Serologic results of 1:10 through 1:160 with varying incubation conditions (Table 2) show that only the 1:10 dilution of the sCR1 substance was able to neutralize the Anti-Kn^a when incubated for thirty minutes at room temperature. Unfortunately, not enough sCR1 substance remained after testing to do any

further testing at a 1:10 dilution to determine if the neutralization was specific and would not neutralize other common antibodies.

The number of donors genotyped using each assay, as well as the number of rare homozygous negative donors and those heterozygous for the genes, are summarized in Table 3. Based on these results, we were able to calculate the observed allele frequency, which were used to determine if the populations were in Hardy-Weinberg equilibrium by determining the expected number of each genotype and using a Chi-square test to determine a p-value. The p-values for McC^a/McC^b, *Sl1/Sl2,* and *KCAM* were less than 0.05 meaning the null hypothesis can be rejected, and they are not in Hardy-Weinberg equilibrium (Table 4).

Of particular interest is that out of all the donors run using the Kn(a/b) assay, only 3 donors were Kn^b/Kn^b, 2 of which were already known to have anti-Kn^a. Therefore, at random only of 1 out of 849 donors was negative for Kn^a. Due to these unexpected results, self-declared ethnicities of the donors genotyped were analyzed to try to determine a cause for this discrepancy (Table 5). Based on this data, it would appear that the frequency of Kn^b/Kn^b in our Caucasian donor population 1 in 528 or approximately 0.19%.

With the donors screened, we were able to create a panel that would definitively identify any of the five Knops antibodies that were included in this study (Figure 2). Two additional A cells were included as extra panel cells due to their ability to help rule out certain other clinically significant antibodies.

Discussion

Although serologic phenotyping with licensed reagents is a regulatory requirement for donor unit labeling, genotyping has revolutionized blood banking. It has allowed for widespread

screening of donors for antigens, which allows donor centers to have a large stock of "pre-screened" units to select for serological phenotyping.[8] In addition, as shown in this study, it is also possible to utilize genotyping to assist in the identification of antibodies for which we don't have very effective typing sera. To alleviate difficulties surrounding properly identifying Knops antibodies, we integrated the use of patient genotyping, sCR1 substance neutralization, and a Knops selected cell panel.

Serologically Misidentified Antibodies

Out of the seven patients/donors with identified Knops blood group system antibodies, two with serologically identified anti-Kn^a genotyped *Kn^a/Kn^a*, which likely indicates that the antibodies were misidentified. Upon further genotyping, one of these donors was negative for the *KCAM* while the other was positive for all the high prevalence Knops antigens tested. Therefore, anti-KCAM should be a suspect for the first donor and additional testing should be performed. If we are to believe the second donor's antibody was in the Knops system, there is the possibility they may have an anti-Yk^a, because genotyping was not completed for *Yk^a* as the assay was still under development at the time this study.

Unfortunately, as a result of the difficulty in identification of Knops blood group system antibodies, it is a good possibility some of them are misidentified. This difficulty stems from the variable weak strength of positive reactions and a lack of commercial antisera or quality patient antibodies to use to type patients. However, misidentification within the Knops blood group system (i.e. identifying an anti-KCAM as an anti-Kn^a) will not typically adversely affect the patient since neither antibody is considered clinically significant,[10] but it is of the utmost importance to differentiate them from other clinically significant antibodies that they may mimic.

sCR1 Substance Benefits

A widely used technique to help identify Knops blood group system antibodies is the use of DTT/AET to destroy the corresponding antigens to help confirm the identity of the antibody and allow for the identification of underlying antibodies. However, if sCR1 substance was used in conjunction, it would allow for a more definitive confirmation of these antibody identifications and also allow for the exclusion of other antibodies whose antigens are sensitive to DTT/AET, such as antibodies directed against Kell blood group system antigens. Although we were able to effectively neutralize a Knops blood group system antibody, there was insufficient quantities of the sCR1 substance to do additional testing to prove that it only neutralizes Knops antibodies. In order for this procedure to be feasible, production of the sCR1 substance would need to be scaled-up to produce a large enough quantity to perform validation and to support the workload of suspected Knops antibodies.

Donor Genotype Anomalies

After genotyping over 2600 donors, two interesting topics for further research were identified. As previously mentioned, the frequency of Kn^b/Kn^b donors in our Caucasian donor population seems to be much lower than currently published values of one to two percent [9, 10]. However, when originally describing the first anti-Kn^a, Helgeson et al. published values very similar to our own with 0.2% Kn^b/Kn^b donors.[11] Additionally, our data is supported by allele frequency information found in National Center for Biotechnology Information's SNP database, which cites two studies in which they found the allele frequencies for Kn^a and Kn^b to be 0.976 and 0.023 respectively in one study and 0.974 and 0.026 in the other.[12] This discrepancy between reported antigen and allele frequency may be due to the inaccuracy of phenotyping especially in

those patients with low copy numbers of the CR1 molecule who can falsely type Kn(a-) or due to the use of patient antibodies as typing sera.

Additionally, all *Kn^b/Kn^b*, *McC^b/McC^b* and *Sl2/Sl2* donors that we identified were also *KCAM*-. As far as we are aware, this relationship has yet to be reported. This observation is likely due to the closeness of these SNPs in exon 29 which results in recombination being very rare. Out of these antigens the farthest SNP from the *KCAM* SNP is that which encodes for *Kn^a/Kn^b*, which is only 54 amino acids away. However, this finding is worthy of additional research. Finally, the *McC^a/McC^b*, *Sl1/Sl2*, and *KCAM* frequency results were not in Hardy-Weinberg equilibrium, which may be due to bias in the sample tested. One such source of bias was that many additional samples were tested for these antigens due to their negative results for another Knops blood group system antigen and preferential selection of donors to test based on reported race.

Creating Knops Antigen Negative Selected Cell Panel

With the possibility of screening large number of donors for Knops blood group system antigen, it is possible to find enough antigen negative donors to create a Knops antigen negative selected cell panel. However, if the goal was to be able to rule out all other clinically significant antibodies in the presence of said antibodies many additional donors would need to be screened due to the prevalence of these negative phenotypes in certain races and the prevalence of other significant antigens in those races (i.e. McC(a-) is found mostly in blacks; double dose Fy^b is rare in blacks). For example, our panel is unable to rule out anti-Jk^b in the presence of Anti-Kn^a. Using the prevalence of the *Kn^a* in our donor population and published Jk^b frequencies, it can be determined that in order to find *Jk^b/Jk^b* & *Kn^b/Kn^b* cell we would need to genotype approximately 2300 more Caucasian donors. In addition, donors included in the panel would

need zygosity testing run for the D antigen and genotyping assays would need to be analyzed to determine if the black donors carried the *Fy* allele. By doing so, we would be able to determine if the red cells were single dose or double dose for D and Fy^a and Fy^b antigens.

Creation of such a panel of cells to help with the identification of Knops antibodies could be invaluable in definitively ruling out other antibodies. In larger reference labs that routinely identify Knops blood group system antibodies, this panel could be a supplemental panel prepared weekly and run only when other testing or patient history indicates that the antibody being tested is likely in the Knops system. However, even in smaller reference labs, this type of panel could be extremely beneficial and worthwhile. After donors are identified, units or large samples of blood could be drawn and frozen in small aliquots. Then whenever a Knops antibody is suspected a set of these aliquots that makes up the panel could be thawed and used to confirm the identity and rule out other clinically significant antibodies.

Conclusion

Once all three methods (Knops panel, sCR1 substance, and patient genotyping) are validated and implemented, identification of Knops antibodies will be significantly improved (Figure 3). If a technologist suspects a sample has a Knops antibody, the sample can be tested using the sCR1 substance to prove it is directed against that system. Then, the sample could be run against the Knops antigen negative selected cell panel to determine the exact specificity and help rule out other clinically significant antibodies. Finally, in order to prove the specificity identified is correct, the patient's sample could be genotyped for the corresponding antigen to prove the patient is antigen negative. However, if the patient is positive for the corresponding antigen, sequencing exons 26 and 29 of the CR1/CD35 gene could help classify these Knops related antibodies. This algorithm for identification is much improved versus traditional

serologic methods, because it lessens the doubt and guesswork that currently is involved in identification of Knops antibodies. As a result, more immunohematology reference labs may choose to do full identification of "HTLA" antibodies. Using these methodologies, technologists' insecurity when identifying these antibodies can be alleviated providing the patient with the safest transfusion possible.

References

1. Hourcade D, Miesner DR, Bee C, Zeldes W, Atkinson JP. Duplication and divergence of the amino-terminal coding region of the complement receptor 1 (CR1) gene. An example of concerted (horizontal) evolution within a gene. Journal of Biological Chemistry 1990 January 15;265(2):974-980.
2. Moulds JM, Thomas BJ, Doumbo O, Diallo DA, Lyke KE, Plowe CV, et al. Identification of the Kna/Knb polymorphism and a method for Knops genotyping. Transfusion 2004 02;44(2):164-169.
3. Moulds JM, Zimmerman PA, Doumbo OK, Kassambara L, Sagara I, Diallo DA, et al. Molecular identification of Knops blood group polymorphisms found in long homologous region D of complement receptor 1. Blood 2001;97(9):2879-2885.
4. Moulds JM, Zimmerman PA, Diallo OK, Atkinson JP, Krych-Goldberg M, Hourcade DE, et al. Expansion of the Knops blood group system and subdivision of Sl[sub a]. Transfusion 2002 02;42(2):251-256.
5. Veldhuisen B, Ligthart PC, Vidarsson G, Roels I, Folman CC, van dS, et al. Molecular analysis of the York antigen of the Knops blood group system. Transfusion 2011 07;51(7):1389-1396.
6. Moulds JM, Pierce S, Peck KB, Tulley ML, Doumbo O, Moulds JJ. KAM: a new allele in the Knops Blood Group System (abstract). Transfusion 2005;45(Supplement s3):27a.
7. Moulds JM, Rowe KE. Neutralization of Knops system antibodies using soluble complement receptor 1. Transfusion 1996 06;36(6):517.
8. Denomme GA, Johnson ST, Pietz BC. Mass-scale red cell genotyping of blood donors. Transfusion Apheresis Sci 2011 2;44(1):93-99.

9. Molthan L. The serology of the York-Cost-McCoy-Knops red blood cell system. Am J Med Technol 1983;49(1):49-56.
10. Moulds JM. The Knops blood group system: a review. Immunohematology 2010 03;26(1):2-7.
11. Helgeson M, Swanson J, Polesky HF. Knops-Helgeson (Kna), a High-frequency Erythrocyte Antigen. Transfusion 1970;10(3):137-138.
12. Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. dbSNP accession:{rs41274768}, (dbSNP Build ID: {139}). Available from:http://www.ncbi.nlm.nih.gov/SNP/

TABLE 1. SNP position and Amino Acid Change for Knops Antigens

Antigen	Position	Amino Acid Change
Kn(a/b)	*CR1* 4681G>A	V1561M
McC(a/b)	*CR1* 4786A>G	K1590E
Sl1/Sl2	*CR1* 4801A>G	R1601G
Sl3+/Sl3-	*CR1* 4828T>A/4801A	S1610T/R1601
KCAM+/KCAM-	*CR1* 4843A>G	I1615V

TABLE 2. Serologic results of Anti-Kn[a] sCR1 inhibition with dilutions of Pool3

	Incubation Condition		
	15 minutes 25°C	30 minutes 25°C	15 minutes 37°C
1:10	+/-	0	+/-
1:20	1+	NT	NT
1:40	1+	NT	NT
1:80	2+	NT	NT
1:160	2+	NT	NT
Dilution Control	2+[s]	2+	2+

TABLE 3. Donor Genotyping Results

Assay	Donors Run	Negative for High	Heterozygous	Wild Type
Kn(a/b)	849	3	48	798
McC(a/b)	492	10	62	420
Sl1/Sl2	382	50	43	288
Sl3+/Sl3-	463	0	15	448
KCAM+/KCAM-	364	89	155	120

TABLE 4. Calculated Allele Frequencies & Hardy-Weinberg Equilibrium Expected Results

	Observed		H-W Equilibrium Results			
	p	q	Negative for High	Heterozygous	Wild Type	p-value
Kn(a/b)	0.970	0.030	0.738	48.524	797.738	0.7532
McC(a/b)	0.917	0.083	3.417	75.167	413.417	0.0001
Sl(a/b)	0.812	0.188	13.418	116.164	251.418	9.77E-35
Sl3(+/-)	0.984	0.016	0.121	14.757	448.121	0.7231
KCAM(+/-)	0.543	0.457	76.160	180.680	107.160	0.0067

p = high prevalence allele frequency; q = low prevalence allele frequency

TABLE 5. Ethnicities of Donors Genotyped for Kn(a/b)

Ethnicity	Number of Donors
African American	175
Asian	13
Caucasian	528
Hispanic	82
Native American	1
Other	50

FIGURE 1: Gel electrophoresis of sCR1 substance pools

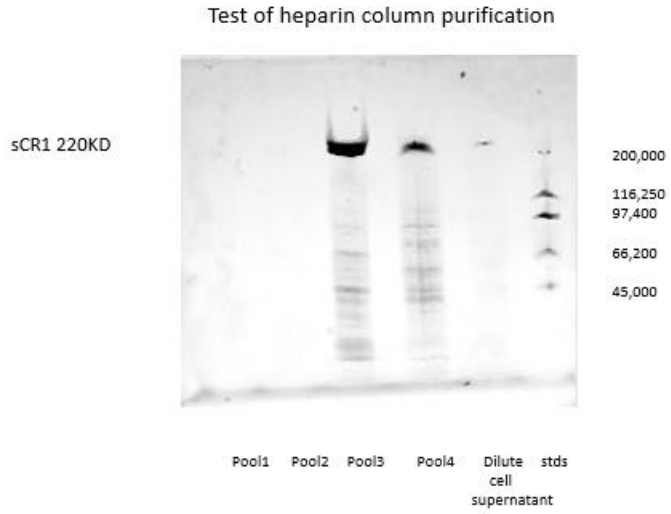

FIGURE 2. Knops Antigen Negative Selected Cell Panel (Negatives for high prevalence antigens highlighted)

Cell #	Kna	Knb	McCa	McCb	Sl1	Sl2	Sl3	KCAM	ABO	Rh haplo	D	C	E	c	e	K	k	Fya	Fyb	Jka	Jkb	M	N	S	s
1	0	+	+	0	+	0	+	0	O	R1R2	+	+	+	+	+	0	+	+	+	+	0	+	0	0	+
2	0	+	+	0	+	0	+	0	O	R1r	+	+	0	+	+	0	+	+	+	+	0	+	+	0	+
3	+	0	0	+	0	+	+	0	O	R0r	+	0	0	+	+	0	+	0	0	+	0	+	0	0	+
4	+	0	0	+	0	+	+	0	O		+	0		+		0	+	+	0	+	0	+	+		
5	+	0	0	+	0	+	+	0	O	R0r	+	0	0	+	+	0	+	0	0	+	+	0	+	0	+
6	+	0	+	+	0	+	+	0	O	r'r	0	+	0	+	+	0	+	+	0	+	+	+	+		
7	+	0	+	0	0	+	+	0	O	R0r	+	0	0	+	+	0	+	0	+	+	0	+	+	+	0
8	+	0	+	+	0	+	+	0	O	R2r/R2R0	+	0	+	+	+	0	+	0	0	+	+	0	+	0	+
9	+	0	+	0	+	0	+	+	O	R1R1	+	+	0	0	+	0	+	0	+	0	+	+	+	+	0
10	+	0	+	0	+	0	+	+	O	R2r	+	0	+	+	+	0	+	+	0	+	0	+	0	+	+
11	+	+	+	0	+	0	+	+	O	rr	0	0	0	+	+	0	+	0	+	+	+	0	+	0	+
12	+	0	+	0	+	0	+	0	O	R1R1	+	+	0	0	+	0	+	+	0	+	+	+	0	+	+
13	+	0	+	0	+	0	+	0	O	R2R2	+	0	+	+	0	0	+	+	+	+	0	+	+	0	+
14	+	0	+	0	+	0	+	0	O	rr	0	0	0	+	+	0	+	+	+	+	+	+	+	+	+
15	+	0	+	0	+	0	+	0	O	R1r	+	+	0	+	+	+	+	+	+	+	0	+	0	0	+
Extra 16	+	0	0	+	0	+	+	0	A	R2r/R2R0	+	0	+	+	+	0	+	0	0	+	+	+	0	0	+
Extra 17	+	0	+	+	0	+	+	0	A	rr	0	0	0	+	+	+	+	0	+	+	+	0	+	+	+

FIGURE 3. Knops Antibody Identification Flowchart