

independently determined, especially in trials that do not actively modify participant medications.

There are 2 opportunities for further study. First, the moderate test-retest reliability at 6 months may have been influenced by the long time interval, which we note is substantially longer than the 4- to 12-week spans reported for other asthma questionnaires. Second, provider assessment of asthma control was unavailable from this study, which would be an additionally informative criterion.

It is notable that the MSD, ACT/C-ACT, and ATAQ-Control were all poorly correlated to lung function. This finding is compatible with literature reporting inconsistent relationships between spirometry and symptoms in children, and it underscores its complementary value in pediatric asthma.

In conclusion, the MSD is a reliable, responsive, and valid measure of asthma symptomatology that is consistently correlated with other measures of asthma control among inner-city populations. Our results strengthen the level of evidence of studies that have used this outcome and support its continued use in observational and interventional trials of inner-city asthma.

Tianshi David Wu, MD^a

Matthew Perzanowski, PhD^b

Roger D. Peng, PhD^c

Robert A. Wise, MD^d

Susan Balcer-Whaley, MPH^d

Michelle Newman, BSN^d

Amparito Cunningham, MD, MPH^e

Wanda Phipatanakul, MD, MS^e

Elizabeth C. Matsui, MD, MHS^{f,*}

Meredith C. McCormack, MD, MHS^{f,*}

From ^athe Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, Md; ^bthe Department of Environmental Health Sciences, Columbia University Mailman School of Public Health, New York, NY; ^cthe Department of Biostatistics, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Md; ^dthe Division of Pediatric Allergy and Immunology, Johns Hopkins University School of Medicine, Baltimore, Md; ^ethe Division of Pediatric Allergy and Immunology, Harvard Medical School, and Boston Children's Hospital, Boston, Mass; and ^fthe Department of Population Health, The University of Texas at Austin Dell Medical School, Austin, Tex. E-mail: mmccor16@jhmi.edu.

*These authors contributed equally to this work.

This work was supported by grants U01AI083238, K24 AI114769, and K24 AI106822 from the National Institute of Allergy and Infectious Diseases; grants F32 ES028578, P50 ES018176, R01 ES023447, and R01 ES026170 from the National Institute of Environmental Health Sciences; and EPA Agreement Number 83615201.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Available online October 19, 2018.
<https://doi.org/10.1016/j.jaci.2018.10.008>

Elevated IgE M1 prime transcripts in nasal tissues in patients with nasal polyps and asthma



To the Editor:

IgE plays a key role in the pathogenesis of allergic diseases such as asthma, allergic rhinitis, atopic dermatitis, and food allergy. In addition, increased IgE concentrations in tissue and serum have been described in patients with nonallergic diseases such as chronic rhinosinusitis with nasal polyps (CRSwNP)¹ and late-onset asthma unrelated to atopy.² Furthermore, tissue IgE has been demonstrated to be polyclonal and functional.³ Omalizumab, an anti-IgE antibody, binds free tissue and serum IgE and was efficacious in CRSwNP and comorbid asthma as well as in nonatopic asthma, underlining the role of IgE in airway diseases, independent of atopy.²

Class switching to IgE occurs not only in the airways of patients with asthma and allergic rhinitis but also in the nasal mucosa of patients with CRSwNP. In CRSwNP, the discovery of switch circle transcripts revealed ongoing local class switch recombination to IgE.⁴ B and IgE-secreting plasma cells in nasal polyp tissues reexpress recombination-activating gene (RAG)1 and RAG2, which are required for receptor revision. Specifically in CRSwNP mucosal tissue, which is mostly characterized by a type 2-biased, often severe, inflammatory reaction,¹ very high local IgE concentrations that are associated with the presence of IgE antibodies to *Staphylococcus aureus* enterotoxins (SE-IgE) and comorbid asthma⁵ can be measured.

IgE exists in 2 forms, a membrane B-cell receptor (BCR) form expressed on IgE-switched B cells that include several B-cell differentiation intermediates and a secreted form produced by terminally differentiated IgE plasma cells.⁶ Binding of antigen to the BCR on a naive B lymphocyte, in conjunction with signals such as IL-4 or IL-13, causes IgE class switching and maturation to IgE-producing plasma cells.

Human membrane IgE contains M1 prime, an extracellular 52-amino acid segment that is not expressed in secreted IgE.⁷ Although germline M1 prime transcription can occur from un-rearranged heavy chain loci before class switch recombination, these transcripts do not lead to protein expression.⁶ IgE class switch recombination removes portions of the epsilon heavy chain locus from the chromosome and joins the gene segments surrounding the deleted portion. This process creates a functional IgE gene that gives rise to a mature IgE mRNA transcript leading to IgE protein expression, which is required for generating IgE-specific B cells and plasma cells.⁶

To target IgE-mediated diseases, anti-M1 prime antibodies were developed to deplete membrane IgE-expressing B cells and prevent IgE production. In multiple preclinical models of IgE immune responses, these antibodies reduced IgE-producing plasma cells and serum IgE levels.⁸ Quiluzumab, a humanized monoclonal anti-M1 prime antibody that depletes M1 prime-expressing B-cell subsets, reduced serum IgE in early clinical studies in healthy volunteers and patients with allergic rhinitis

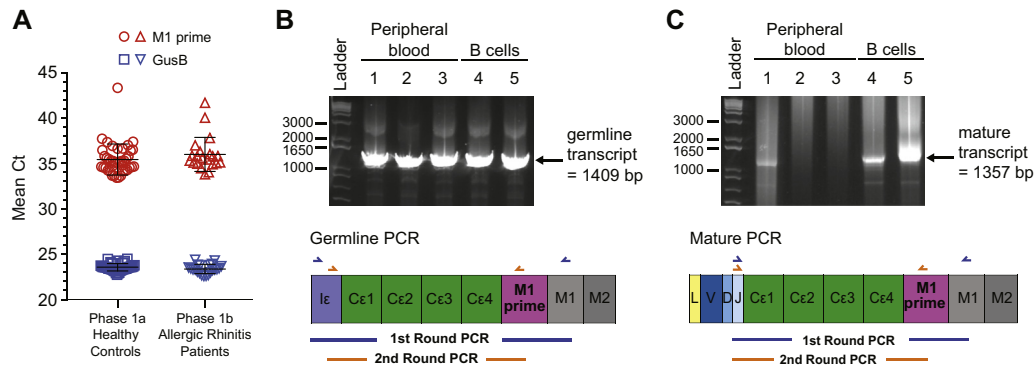


FIG 1. IgE M1 prime mRNA transcripts in peripheral blood samples are mostly germline transcripts. **A**, M1 prime quantitative RT-PCR of peripheral blood RNA samples from healthy controls and patients with allergic rhinitis. Quantitative RT-PCR was normalized to the amount of input RNA. Error bars, SD; *GusB*, beta glucuronidase housekeeping gene; symbols, mean threshold cycle (Ct) values for individual subjects. Nonquantitative PCR of germline (**B**) and mature (**C**) M1 prime transcript in peripheral blood and purified B-cell RNA samples from healthy volunteers (samples 1-5).

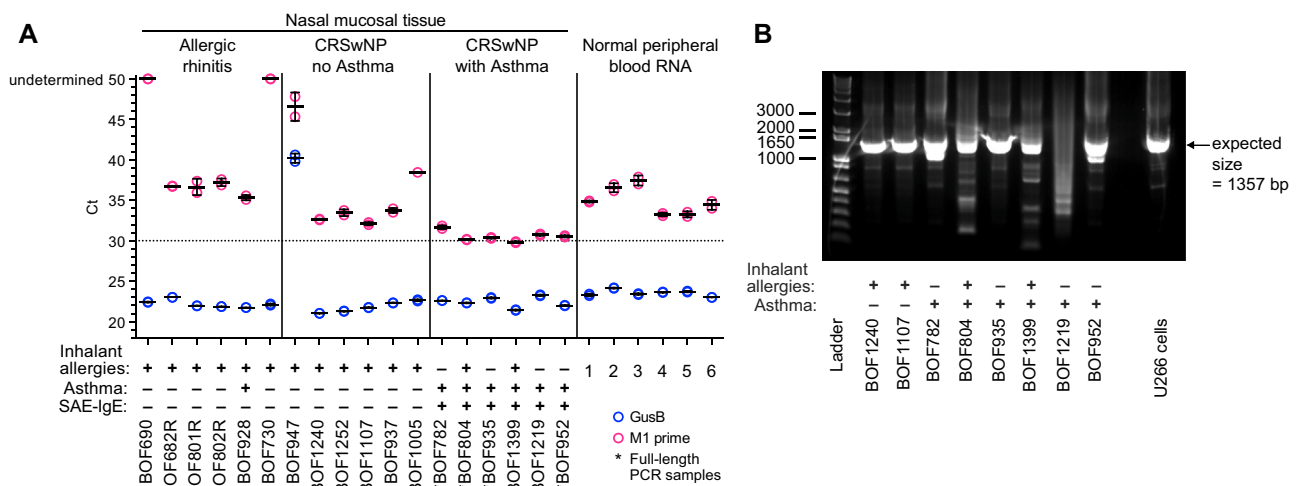


FIG 2. Total M1 prime mRNA transcripts are higher in nasal polyp tissue of patients with CRSwNP with asthma compared with patients with CRSwNP without asthma as shown by the quantitative RT-PCR assay to detect both germline and mature transcripts (**A**). **B**, Mature M1 prime transcript detected in nasal polyp tissues using the nested, nonquantitative PCR assay. Asterisks indicate patient samples in Fig 2, **A** corresponding to full-length PCR samples shown in Fig 2, **B**. The shorter band in samples BOF782 and BOF952 is likely to be the shorter isoform of membrane IgE² that would have been amplified by the primers in the first stage of PCR. PCR bands from Fig 2, **B** were purified and confirmed by Sanger sequencing. Ct values for Fig 2, **A** are provided in Table E1. *GusB*, Beta glucuronidase housekeeping gene; U266, membrane IgE-expressing myeloma used as a positive control.

or mild asthma.⁹ However, we were unable to correlate this effect to the actual depletion of M1 prime-expressing B cells in peripheral blood using a flow cytometry assay, likely due to their low frequency, but also possibly due to the limitations of staining reagents.⁹ In this letter, we describe the development of PCR assays for the detection of M1 prime mRNA (see Fig E1 in this article's Online Repository at www.jacionline.org) as an alternative, and possibly more sensitive, method to measure membrane IgE-expressing B cells and enable the assessment of therapies targeting these cells.

By using quantitative RT-PCR, we first tested our ability to detect M1 prime mRNA in all peripheral blood samples from healthy volunteers and subjects with allergic rhinitis. In both subgroups, the threshold cycle (Ct) generally ranged from

33 to 38 (Fig 1, A; see Table E1 in this article's Online Repository at www.jacionline.org). However, this assay could not distinguish between mature and germline transcripts because of the limitations of amplicon size for a TaqMan assay, the length of the IgE locus, and the position of the M1 prime sequence within the locus (Fig E1). Subsequently, we developed a nonquantitative, nested and seminested PCR assay to determine whether mature and/or germline M1 prime mRNA transcripts could be detected in peripheral blood (see this article's Online Repository at www.jacionline.org; Fig 1, B and C). We detected germline M1 prime transcripts in both peripheral blood and in purified B cells from the blood of healthy controls (Fig 1, B), but could detect mature M1 prime transcripts only in RNA isolated from purified B cells (Fig 1, C). Given that only peripheral blood samples were

available from the quilizumab clinical studies, we could not use this assay to directly determine whether quilizumab had acted by depleting M1 prime-expressing cells.

To then validate the assay in the context of an IgE-associated disease and test whether it could detect membrane IgE-expressing B cells in upper airway tissues in contrast to peripheral blood, we assessed levels of M1 prime transcripts in nasal polyp tissue from patients with CRSwNP with or without asthma, and from subjects with allergic rhinitis. We preselected 6 patients with CRSwNP and inhalant allergies without asthma, 6 patients with CRSwNP and asthma with elevated levels of IgE and SE-IgE, and 6 patients with allergic rhinitis with/without asthma or inhalant allergies. Patients with CRSwNP and asthma all had elevated levels of total IgE and SE-specific IgE (see Table E2 in this article's Online Repository at www.jacionline.org). Using the quantitative assay, we initially detected total M1 prime transcripts (germline and mature) in 11 of 12 patients with CRSwNP (Fig 2, A; patient BOF947 possibly had compromised RNA, based on housekeeping gene expression). Expression of M1 prime transcripts was approximately 16-fold higher in patients with CRSwNP with asthma when compared with patients with CRSwNP without asthma or with patients with allergic rhinitis (>4 Δ Ct difference; see Table E2). Nested PCR then confirmed that mature transcripts were present in all but 1 of the confirmed samples (Fig 2, B). These results demonstrate that M1 prime-expressing cells can be detected locally in tissue at sites of inflammation. Specifically, levels of M1 prime transcripts appeared elevated in the nasal mucosa of patients with CRSwNP with asthma comorbidity and SE-IgE expression, which was recently defined as a more severe CRS patient endotype,¹ although it should be noted that the samples were preselected and these findings may not be generalizable to the entire population of patients with CRSwNP and asthma.

In conclusion, we developed a quantitative PCR assay for detecting total M1 prime transcripts and a novel, nonquantitative, nested and seminested PCR assay for detecting both mature and germline M1 prime mRNA transcripts of IgE. We validated the assay, establishing the presence of rare, membrane IgE-expressing B cells in tissues of patients with CRSwNP, consistent with previous publications providing evidence of IgE class switching in inflamed local nasal tissues. These findings not only strengthen the understanding of the pathology underlying CRSwNP and suggest that targeting these cells may be a novel therapeutic option for these patients but also provide a tool that can be used to further study the role of M1 prime-expressing B cells in human disease.

We thank the subjects and patients who participated in these studies. Editing and writing assistance was provided by Deborah Solymar (Genentech, Inc) and was funded by Genentech, Inc.

Heleen Scheerens, PhD^a
Ashley Smith, BA^a
Olga Li, BA^a
Lee Honigberg, PhD^a
Jeffrey M. Harris, MD, PhD^a
Gabriele Holtappels, BS^b
Claus Bachert, MD, PhD^{b,c}

From ^aGenentech, Inc, South San Francisco, Calif; ^bUpper Airways Research Laboratory, ENT Department, Ghent University Hospital, Ghent, Belgium; and ^cthe Division of ENT Diseases, CLINTEC, Karolinska Institute, Stockholm, Sweden. E-mail: scheerens.heleen@gene.com.

Genentech, Inc, supported this study and was involved in the collection, analysis, and interpretation of data, the writing of the report, and the decision to submit the article for publication.

Disclosure of potential conflict of interest: H. Scheerens, A. Smith, O. Li, L. Honigberg, and J. M. Harris are currently or were employees of Genentech, Inc, at the time this study was conducted and own Roche stock. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online October 22, 2018.
<https://doi.org/10.1016/j.jaci.2018.10.011>

ONLINE REPOSITORY

Letter to the Editor

Elevated IgE M1 prime transcripts in nasal tissues in patients with nasal polyps and asthma

Heleen Scheerens, PhD,^a Ashley Smith, BA,^a Olga Li, BA,^a Lee Honigberg, PhD,^a Jeffrey M. Harris, MD, PhD,^a Gabriele Holtappels, BS,^b Claus Bachert, MD, PhD^{b,c}

^aGenentech, Inc., One DNA Way, South San Francisco, CA 94080

^bUpper Airways Research Laboratory (URL), ENT Department, Ghent University Hospital, C. Heymanslaan 10, 9000 Ghent, Belgium

^cDivision of ENT Diseases, CLINTEC, Karolinska Institute, Stockholm, Sweden

Corresponding author

Heleen Scheerens

Genentech, Inc.

1 DNA Way

South San Francisco, CA 94080

Telephone: 650-225-6756

Email: scheerens.heleen@gene.com

METHODS

Subject samples and RNA isolation

Peripheral blood samples were obtained from healthy volunteer and allergic rhinitis subjects enrolled in quilizumab Phase 1a and 1b studies, respectively.^{E1, E2} Blood was collected in PAXgene RNA blood tubes (PreAnalytiX) and RNA was extracted using the PAXgene blood RNA kit (Qiagen) according to the manufacturer's protocol with DNase treatment to remove genomic DNA contamination. RNA was quantified using a NanoDrop 8000 (Thermo Fisher Scientific). B cells from blood of healthy volunteers were purified using RosetteSep™ (Stemcell Technologies). RNA from U266 (ATCC) cells, a B cell myeloma line that expresses membrane IgE, was used as a positive control to detect mature IgE transcripts.

Nasal polyp tissue (0.2–2.0 g) was obtained following surgical endoscopic removal of polyps in CRSwNP patients who were not using topical glucocorticosteroids for at least 2 weeks and oral glucocorticosteroids for at least one month before surgery. Six patients with CRSwNP and allergies without asthma, 6 patients with CRSwNP and asthma with elevated levels of IgE and SE-IgE, and 6 patients with allergic rhinitis with/without asthma or allergies were preselected. Patients with allergies were defined as those who had at least one positive reaction to skin prick testing with an in-house panel of the 12 most frequent inhalant allergens in our region. CRSwNP patients without allergies refers to patients with nasal polyps, but with negative skin prick testing (more than 50% of our patients with polyps). Biopsies were immediately snap-frozen for qPCR and IgE measurements (Table E1). RNA from nasal tissue samples was extracted by disrupting the nasal tissue, followed by homogenization and RNA isolation using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions.

The applicable local ethics committee or institutional review boards approved all studies. All subjects gave their written informed consent before collection of material.

M1 prime quantitative RT-PCR

cDNA was synthesized from 500 ng of RNA using random hexamers and SuperScript III (VILO cDNA synthesis kit, Invitrogen). Thermal cycling conditions: 25°C, 10 min; 42°C, 2 h; 85°C, 5 min. RNA samples without reverse transcriptase added were included as a negative control for each sample.

A quantitative real-time RT-PCR assay (TaqMan) was developed to measure M1 prime-containing transcripts (Figure E1). The TaqMan primers and probe sequence were: forward primer, 5'- CAGCGAGCGGTGTCTGT -3'; reverse primer, 5'- GTGGCAGAGCACCTATCC -3'; and TaqMan probe, FAM-CCAGCCCGGGATTT-NFQ. The expression of the housekeeping gene beta glucuronidase (GusB; Applied Biosystems, assay ID HS99999908_m1) was simultaneously analyzed as an endogenous control. Real-time PCR was performed using standard cycling conditions with 50 cycles on a 7900HT instrument (Applied Biosystems).

A nested and semi-nested, non-quantitative PCR approach was used to amplify an approximately 1.4 kb PCR fragment for germline membrane IgE (Figure 1B) and an approximately 1.35 kb PCR fragment for mature membrane IgE (Figure 1C). cDNA or first round PCR products were amplified using PicoMaxx master mix (Agilent Technologies) and 40 cycles of amplification. The primer sequences for the first round were: germline forward primer: 5' AGGCTCCACTGCCCCGGCACAGAAAT 3'; mature forward primer^{E3}: 5' ACCCTGGTCACCGTCTCCTCA 3'; germline reverse primer: 5' ACGCTGAGCAGGAAGAGTG 3'; and mature reverse primer: 5' AAGAGTGCGGCGAAGATG 3'. The primer sequences for the second round were: germline forward primer: 5' CTGTCCAGGAACCCGACAG 3'; mature forward primer: 5' ACCCTGGTCACCGTCTCCTCA 3'; germline reverse primer: GTGGCAGAGCACCTATCC; and mature reverse primer: GTGGCAGAGCACCTATCC. The forward primers were designed

75 to match either the sequence for the I ϵ region (germline) or the variable region (mature) and the
76 reverse primers were designed to match the sequence for the IgE membrane region. PCR
77 products were visualized using E-gel EX gels and imaged with the E-Gel Imager System (Life
78 Technologies). PCR bands were purified and confirmed by Sanger sequencing for all samples
79 in Figure 2B, except for BOF1219.

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Table E1. Raw threshold cycle (Ct) and Δ Ct quantitative RT-PCR values for M1 prime and GusB transcripts measured in peripheral blood samples of healthy volunteers and allergic rhinitis patients.

Healthy volunteers			Allergic rhinitis patients		
Ct value M1 prime	Ct value GusB	Δ Ct M1 prime – GusB	Ct value M1 prime	Ct value GusB	Δ Ct M1 prime – GusB
33.42	22.74	10.69	34.90	23.00	11.90
33.63	22.89	10.74	34.96	23.07	11.90
33.63	22.93	10.70	35.04	23.23	11.81
34.13	22.96	11.17	35.12	23.43	11.69
34.19	23.14	11.05	35.57	23.54	12.03
34.24	23.18	11.06	35.78	23.69	12.10
34.51	23.20	11.31	35.81	23.76	12.05
34.55	23.26	11.29	35.85	23.80	12.05
34.60	23.31	11.29	36.31	23.84	12.46
34.63	23.31	11.31	36.99	23.92	13.07
34.71	23.33	11.38	40.03	24.31	15.73
35.08	23.36	11.72	41.67	24.39	17.28
35.26	23.39	11.87	33.80	22.59	11.22
35.27	23.39	11.87	34.04	22.62	11.42
35.30	23.40	11.89	34.47	22.68	11.79
35.80	23.41	12.39	35.08	22.69	12.39
36.41	23.44	12.97	35.30	22.94	12.37
36.51	23.45	13.06	35.43	23.30	12.12
36.93	23.51	13.42	35.79	23.31	12.48
37.02	23.54	13.48	36.00	23.32	12.68
37.39	23.56	13.83	37.81	23.34	14.47
43.32	23.57	19.74			
33.44	23.59	9.85			
33.66	23.59	10.06			
34.14	23.60	10.54			
34.29	23.62	10.67			
34.53	23.65	10.88			
34.65	23.68	10.97			
34.78	23.73	11.05			
35.07	23.78	11.29			
35.19	23.97	11.22			
35.29	24.00	11.29			
35.46	24.02	11.44			
35.53	24.06	11.47			
35.59	24.12	11.47			
35.77	24.25	11.53			
36.46	24.27	12.19			
36.61	24.46	12.15			
36.64	24.49	12.15			

95 **Table E2.** Mucosal tissue samples from patients with allergic rhinitis, CRSwNP, and CRSwNP
 96 and asthma. Patients with allergies were defined as having at least one positive reaction to
 97 standardized skin prick testing with an in-house panel of the 12 most frequent regional inhalant
 98 allergens. Non-allergic patients were defined as those negative for skin prick testing reactions.
 99 Tissue total IgE and *S. aureus* enterotoxin-specific IgE (SE-IgE) antibodies were determined by
 100 Immunocap (Thermo Fisher Scientific, Phadia).

Sample ID	Disease	Inhalant allergies	Asthma	Total IgE U/g tissue	SE-IgE UA/g tissue	Ct value M1 prime	Ct value GusB	Δ Ct M1 prime – GusB
BOF690	AR	Yes	No	BDL	BDL	UND	22.42	UND
BOF682R	AR	Yes	No	282	BDL	36.74	23.00	13.73
BOF801R	AR	Yes	No	105	BDL	36.65	21.96	14.69
BOF802R	AR	Yes	No	335	BDL	37.23	21.86	15.36
BOF928	AR	Yes	Yes	38	BDL	35.33	21.74	13.59
BOF730	AR	Yes	No	30	BDL	UND	22.11	UND
BOF947	CRSwNP	Yes	No	370	BDL	46.58	40.20	UND
BOF1240	CRSwNP	Yes	No	270	BDL	32.63	21.02	11.61
BOF1252	CRSwNP	Yes	No	264	BDL	33.47	21.28	12.19
BOF1107	CRSwNP	Yes	No	126	BDL	32.12	21.74	10.38
BOF937	CRSwNP	Yes	No	107	BDL	33.74	22.32	11.42
BOF1005	CRSwNP	Yes	No	180	BDL	38.45	22.63	15.83
BOF782	CRSwNP	No	Yes	1012	12.5	31.67	22.61	9.06
BOF804	CRSwNP	Yes	Yes	1584	14.6	30.15	22.31	7.84
BOF935	CRSwNP	No	Yes	712	10.6	30.35	22.93	7.42
BOF1399	CRSwNP	Yes	Yes	3982	4.8	29.83	21.43	8.40
BOF1219	CRSwNP	No	Yes	951	11.9	30.75	23.25	7.50
BOF952	CRSwNP	No	Yes	1787	6.4	30.55	21.99	8.56

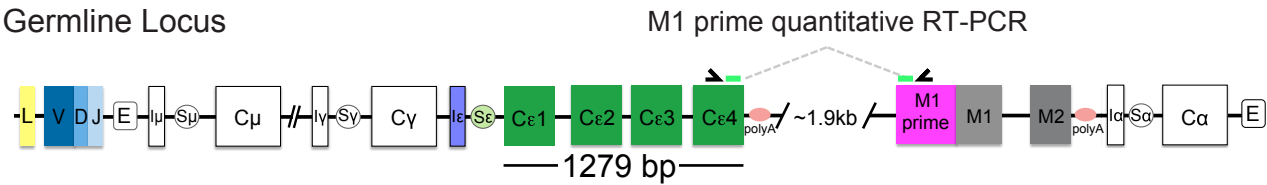
AR, allergic rhinitis; BDL, below detection limit; CRSwNP, chronic rhinosinusitis with nasal polyps; Ct, threshold cycle; GusB, beta glucuronidase; UA, allergen-specific unit; UND, undetermined.

102 **Figure legends**

103

104 **Figure E1.** The M1 prime quantitative RT-PCR assay is specific to membrane IgE. The primers
105 in this assay were designed to distinguish between membrane and secreted IgE transcripts. The
106 sequence of the IgE locus was too long to design a qPCR assay that spans the VDJ and
107 membrane regions; therefore, this qPCR assay does not distinguish between M1 prime
108 germline and mature transcripts.

Germline Locus



Possible Transcripts

