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The Ebola virus ribonucleoprotein complex: A novel VP30–L interaction identified

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Abstract

The ribonucleoprotein (RNP) complex of Ebola virus (EBOV) is known to be a multiprotein/RNA structure, however, knowledge is rather limited regarding the actual protein–protein interactions involved in its formation. Here we show that singularly expressed VP35 and VP30 are present throughout the cytoplasm, while NP forms prominent cytoplasmic inclusions and L forms smaller perinuclear inclusions. We could demonstrate the existence of NP–VP35, NP–VP30 and VP35–L interactions, similar to those described for Marburg virus (MARV) based on the redistribution of protein partners into NP and L inclusion bodies. Significantly, a novel VP30–L interaction was also identified and found to form as part of an NP–VP30–L bridge structure, similar to that formed by VP35. The identification of these interactions allows a preliminary model of the EBOV RNP complex structure to be proposed, and may provide insight into filovirus transcriptional regulation.

Keywords

Ebola virus; Polymerase; VP30; Protein–protein interaction; Ribonucleoprotein complex

1. Introduction

The most recent classification divides *Filoviridae* into two genera, *Marburgvirus* and *Ebolavirus*. While the genus *Marburgvirus* consists of a single species, *Lake Victoria marburgvirus* (MARV), the genus *Ebolavirus* (EBOV) is subdivided into four species, *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Cote d'Ivoire ebolavirus* (CIEBOV), and

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Reston ebolavirus (REBOV) (Feldmann et al., 2004). A putative fifth species has also been postulated, which is the cause of a recent outbreak in Uganda (Towner et al., 2008). Apart from the obvious phylogenetic division between MARV and EBOV based on nucleotide sequence, they are further distinguished by their general lack of antigenic cross-reactivity and differences in their genome organization. However, most viral processes and the functions of the viral proteins are presumed to be identical between MARV and EBOV (Sanchez et al., 2007).

Of the seven structural proteins, four of these, together with the viral RNA, make up the ribonucleoprotein (RNP) complex (Elliott et al., 1985; Mühlberger et al., 1998, 1999; Mühlberger, 2004; Sanchez et al., 2007). Within this RNP complex the nucleoprotein (NP) functions in RNA encapsidation, virion protein (VP) 35 acts as an RNA-dependent RNA polymerase cofactor, VP30 is important structurally as a minor nucleoprotein, as well as acting as an EBOV-specific transcriptional activator, and L functions as the RNA-dependent RNA polymerase (Elliott et al., 1985; Mühlberger et al., 1998, 1999; Mühlberger, 2004; Sanchez et al., 2007). These four proteins also represent the minimal necessary factors for the transcription and replication of the EBOV genome, although VP30 has been shown to be dispensable for replication alone (Mühlberger et al., 1999). Interestingly, despite the presence of MARV VP30 in the RNP complex, it is not required for either the transcription or replication of MARV minigenomes (Mühlberger et al., 1998). Although a mechanistic basis for this difference has not yet been established, current evidence suggests a role for VP30 in overcoming a hairpin structure overlapping the NP transcriptional start site in EBOV (Weik et al., 2002). However, it has been recently reported that VP30 is necessary for the rescue of MARV using an infectious clone system, independent of residues important for its transcriptional activator function in EBOV, suggesting that VP30 serves additional functions critical for transcription when in the context of a full-length genome (Enterlein et al., 2006).

It has been previously shown that within the RNP complex of MARV both NP and L interact with VP35, but do not directly interact with each other (Becker et al., 1998). Thus VP35 serves as a bridging molecule, which is believed to recruit L to the encapsidated RNA (Becker et al., 1998). Based on more recent data, it also appears that oligomerization of MARV VP35 is necessary for the interaction with L, but not for interaction with NP (Möller et al., 2005), suggesting that interaction of NP and VP35 also occurs separately from interaction between VP35 and L. In addition, it was shown that MARV VP30 interacts directly with NP, a process that is likely essential for its function as a minor nucleoprotein (Becker et al., 1998). For EBOV no such systematic attempt has been made to address the interactions existing within the RNP complex. However, it is apparent from various studies that interaction occurs between NP and VP30 (Modrof et al., 2002), as well as NP and VP35 (Huang et al., 2002; Watanabe et al., 2006). Information regarding the interactions of L within the RNP complex has not yet been reported, likely due to the absence of antibodies available to detect the polymerase. Therefore, it was the purpose of this study to develop the necessary resources to facilitate detection of each of the EBOV RNP components in order to identify the protein-protein interactions involved in formation of the RNP complex of EBOV, with a particular focus on interaction partners for the polymerase.

2. Materials and methods

2.1. Cells

Vero E6 (African green monkey kidney), 293T (human embryonic kidney) and Ad-293 (human embryonic kidney; Stratagene) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 (g/mL streptomycin and grown at 37 °C and 5% CO₂. *Escherichia coli* (*E. coli*) of the XL-1 Blue

strain were used for all routine cloning procedures, while BL-21 *E. coli* (GE Healthcare), deficient in the OmpT and Lon proteases, were used for the expression of recombinant REBOV proteins for antibody production.

2.2. Cloning of RNP complex sequences

The open reading frames (ORFs) for REBOV NP, VP35, VP30 and L were cloned into pCAGGS (Niwa et al., 1991) for eukaryotic expression and functionally validated as previously described (Groseth et al., 2005; Theriault et al., 2004). An HA tag (YPYDVPDYA) or Flag tag (DYKDDDDKK) was inserted into the L ORF of the pCAGGS-L construct at amino acid position 1703 as a molecular tag using a conventional PCR-based approach. In addition, the ORFs for VP35 and VP30, as well as peptide fragments from NP (amino acid 80–149 and 490–674) and L (amino acids 1–56 and 1643–1758) (Fig. 1A), were cloned into pGEX-6P (GE Healthcare) for bacterial expression as a fusion protein with the upstream glutathione-S-transferase (GST). Peptide fragments were selected based on an analysis of hydrophilic and surface exposed regions using the Kyte–Doolittle scale (Kyte and Doolittle, 1982).

2.3. Expression, purification and immunization with Ebola virus RNP complex proteins

Following isolation of recombinant plasmid DNAs encoding the GST fusion proteins, these vectors were re-transformed into *E. coli* strain BL-21 (GE Healthcare) for expression. Protein expression was induced using isopropyl β -D-1-thiogalactopyranoside (IPTG) according to the manufacturer's instructions (GE Healthcare) and the bacteria lysed using chicken egg white lysozyme (Sigma) and sonication. Purification was achieved using batch binding to glutathione-sepharose 4B according to the manufacturer's directions (GE Healthcare). Three 5–6 week old female BALB/c mice per antigen were injected with 0.1 mL of antigen emulsified with complete Freund's adjuvant at three intramuscular (i.m.) sites for a total of ~15 μ g fusion protein per animal. A booster immunization was prepared using incomplete Freund's adjuvant and administered 4 weeks later as a single 0.3 mL subcutaneous injection. Fourteen days later, immunized mice were bled and the serum from each group was pooled. These animal experiments were performed on animal use documents approved by the Canadian Science Centre for Human and Animal Health—Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

2.4. Detection of full-length REBOV proteins in eukaryotic cells by immunofluorescence assay

For analysis by immunofluorescence assay (IFA), Vero E6 cells were seeded for 50% confluence into 6-well plates containing cover-slips and transfected with 0.5 μ g of either pCAGGS-NP, VP35, VP30 or L using Fugene6 (Roche) according to the manufacturer's instructions using 6 μ L Fugene per μ g DNA. Samples were fixed after 48 h in 2% paraformaldehyde (PFA), and permeabilized in PBS containing 0.1% Triton X-100. Each coverslip was incubated with 20 μ L of primary antibody containing 1:100 to 1:1000 dilutions of each NP, VP35, VP30 or L antisera. These samples were incubated overnight at 4 °C prior to incubation with a secondary goat α -mouse Alexa 488 antibody (Molecular Probes; Table 1) for 1 h at room temperature. Finally, samples were fixed in 2% PFA and mounted for UV microscopy.

2.5. Analysis of RNP complex protein–protein interactions via subcellular relocalization

RNP complex component expression plasmids were transfected, as described in Section 2.4, into Vero E6 cells seeded into LABTEK 8-chamber slides (Nunc) in the following 2-plasmid combinations: NP + VP35, NP + VP30, VP35 + L, VP30 + L. As a control VP35 + VP30 were also transfected together. Subsequently, the localization of the proteins was

examined after 48 h as described above in Section 2.4, with the exception that 120 μ L of both the primary and secondary antibody were used in the optimal dilutions for IFA (Table 1).

2.6. Detection of tagged REBOV L and confirmation of function in a minigenome assay

Plasmids encoding HA or Flag-tagged versions of L (0.5 μ g) were transfected using Eugene6 (Roche), as described in Section 2.4. After 48 h IFA was performed, according to the protocol in Section 2.4, using either a rabbit anti-HA serum (Sigma) or a rabbit anti-Flag serum (Sigma) at dilutions from 1:50 to 1:400, together with secondary goat anti-rabbit Cy3 conjugated antibody (Sigma) (Table 1). The functionality of these tagged L proteins was tested using a previously reported REBOV minigenome assay (Groseth et al., 2005). Briefly Ad-293 cells were transfected with 1 μ g pCAGGS-NP, 0.5 μ g pCAGGS-VP35, 0.3 μ g pCAGGS-VP30 and 1 μ g of either pCAGGS-L, pCAGGS-L-HA or pCAGGS-L-Flag. After 48 h cells were harvested and analyzed using the Fast-CAT System (Molecular Probes) as previously described (Groseth et al., 2005).

2.7. Confirmation of RNP complex protein–protein interactions via co-localization using two-colour co-immunofluorescence

RNP complex expression plasmids were co-transfected into Vero E6 cells seeded on glass coverslips, as in Section 2.4, in the following 2-plasmid combination: VP35 + L-HA, VP30 + L-HA, and as a control NP + L-HA. After 48 h the localization of the proteins was examined using the REBOV anti-NP, anti-VP35 and anti-VP30 antisera, as well as the rabbit anti-HA serum at the dilutions determined to be optimal (Table 1). We further analyzed tripartite complex formation by co-expression of the following plasmid combinations: NP + VP30 + L-HA and NP + VP35 + L-HA. As a negative control NP + L-HA were transfected together. As a negative control NP + L were transfected together. For this assay, RNP complex expression plasmids were co-transfected into Vero E6 cells seeded on glass coverslips, as in Section 2.4. After 48 h the localization of NP and L with relation to each other was examined using the anti-NP2 antiserum and a rabbit anti-HA serum (Table 1), as described above.

3. Results and discussion

3.1. Production and characterization of antibodies

The availability of immunological reagents for filoviruses was, and continues to be, limited, particularly for targets other than the glycoprotein (GP). In order to address this need, and allow detection of the RNP proteins of REBOV, antisera were raised against bacterially expressed GST fusion proteins, containing the VP35 ORF, the VP30 ORF or peptide sequences from NP or L (Fig. 1A). Since the NP and L proteins were too large to allow expression in their entirety as a GST fusion, peptides from each of these proteins were selected based on predictions of their hydrophobicity and surface exposure characteristics.

Expression of these GST fusion protein encoding plasmids in BL21 *E. coli* led to the production of high levels of each of the fusion proteins, which could be purified using affinity chromatography due to the presence of the GST moiety, an approach that yielded sufficiently pure protein preparations in quantities suitable for use in polyclonal antibody generation (data not shown). Once sera were obtained from immunized animals it was necessary to determine whether they were capable of detecting the relevant REBOV protein when expressed in their entirety from eukaryotic cells. The possibility of observing protein expression by IFA was of particular interest in order to make further observations related to the subcellular localization of the RNP components. For this purpose, RNA polymerase II-driven eukaryotic expression plasmids encoding each of the RNP complex proteins were

used. Following transfection into Vero E6 cells for IFA it was possible to observe staining with the anti-NP2, anti-VP35 and VP30 antisera (Fig. 1B), consistent with the utility of these antibody preparations in Western Blotting (data not shown). Unfortunately, the expression of L could not be directly confirmed in IFA using the L-peptide antisera generated in this study.

3.2. Ribonucleoprotein complex protein localization

Using these antibodies in IFA we could define the subcellular localization of singularly expressed REBOV NP, VP35 and VP30. Staining of Vero cells expressing these proteins indicated that NP is present in cytoplasmic inclusions, while both VP35 and VP30 were evenly dispersed throughout the cytoplasm (Fig. 1B). These localizations are consistent with those previously reported for the corresponding MARV proteins (Becker et al., 1998), as well as the assumption that these proteins serve largely analogous functions in both viruses.

A further observation indicated that interaction of VP35 or VP30 with NP during co-expression was sufficient to mediate relocalization of these proteins to cytoplasmic inclusions (Fig. 1C). Surprisingly, co-expression of VP35 and VP30 with L also showed redistribution of VP35 or VP30 into inclusion bodies. While an interaction between VP35 and L has been previously described for VP35 of MARV (Becker et al., 1998) the existence of an interaction between VP30 and L is first described here. Co-expression of VP35 with VP30 did not show any formation of inclusion bodies (Fig. 1C), indicating that the relocalization we observed is likely due to a specific influence of NP and L. These data also indicate that REBOV L itself is capable of inclusion body formation.

Interestingly, this observation is in conflict with previous data using an N-terminally flag-tagged form of MARV L (Becker et al., 1998), as well as a more recent study with an N-terminally flag-tagged ZEBOV L (Boehmann et al., 2005), both of which indicated that L is expressed diffusely in the cytoplasm. This may be due in part to the presence of a co-infecting vaccinia virus, which was used to facilitate protein expression in both of these earlier studies. Indeed, it was previously shown during expression of another filovirus protein, GP, that the presence of co-infecting MVA-T7 can affect protein processing and distribution (Sänger et al., 2001). Alternatively, for MARV this difference in L protein distribution could also suggest the existence of underlying differences in RNP complex assembly between the different filovirus genera. It was also considered that the formation of inclusion bodies by L in this study might occur as an artifact of over-expression from the pCAGGS plasmid. However, data using the REBOV minigenome system indicate that transcription/replication occur optimally after transfection of L expression plasmid amounts equal to or greater than those used in these experiments (Groseth et al., 2005), suggesting that the amount of L being expressed in these experiments is conducive to efficient protein function. Indeed, formation of inclusion bodies by L, while not previously reported for filoviruses, would be consistent with findings for Measles virus, where a polymerase tagged with GFP in the variable hinge domain was shown to accumulate in intracytoplasmic inclusion bodies (Duprex et al., 2002). Further, co-expression with the other RNP components, as part of a minigenome assay, showed that polymerase activity in Measles virus required the formation of large inclusion bodies in which L granules were present (Duprex et al., 2002).

Despite this intriguing parallel with the Measles virus system, it remained unclear from our data whether L alone would be capable of inclusion body formation, or if interaction with VP35 or VP30 would be necessary for L to exhibit this distribution. Unfortunately, as neither of the L peptide antisera generated in this study were able to detect the polymerase protein when expressed in its entirety, it was necessary to develop an alternative approach to detect L expression directly. For this purpose we attempted to identify variable regions in

the filovirus polymerase, since previous reports using Measles virus had indicated that even large inserts, such as GFP, were tolerated in the second “variable hinge domain” of the polymerase (Duprex et al., 2002). Based on an analysis comparing REBOV and ZEBOV we could identify a region of extensive variability, between amino acids 1648 and 1757 (Fig. 2A). Interestingly, in a sequence alignment, this amino acid position corresponds closely to the position identified as the paramyxovirus “variable hinge domain 2” (Duprex et al., 2002) and was, therefore, deemed an ideal choice for such an insertion in the REBOV polymerase. However, since the potential exists with the insertion of an exogenous sequence, such as a molecular tag, to significantly affect the protein function, we assessed the function of the variable hinge domain tagged polymerase proteins prior to their use in interaction studies. For this application we chose to use a previously described minigenome assay (Groseth et al., 2005), as such systems have been well described to model both viral transcription and replication by the RNP complex. Following the insertion of either an HA or a Flag tag in the center of the “variable hinge region” in REBOV L, no alteration in reporter output was observed in comparison to the wild-type REBOV L (Fig. 2B), indicating that insertions in this region are well tolerated with respect to protein function. Further, since the formation of the correct protein–protein interactions is critical for RNP complex function, these must also be maintained, making these tagged L proteins principally suitable for studying these interactions.

Surprisingly, while both L-Flag and L-HA appear to be functional in the context of a REBOV minigenome assay, indicating the expression of a functional protein, only L-HA could be detected by IFA, suggesting that the Flag tag may be somehow obscured within the structure of the polymerase (Fig. 2C). However, detection using an anti-HA antibody allowed us to achieve direct observation of the polymerase during eukaryotic expression. Importantly, cells expressing L-HA showed expression in small punctuate inclusions concentrated in the perinuclear region (Fig. 2C), consistent with the sites to which VP35 and VP30 were being relocated following co-expression with an untagged version of the polymerase (Figs. 1C and 2C). This indicates that, as expected, tagging of the EBOV L in its “variable hinge domain” does not affect protein localization, as well as that L alone is able to form these characteristic inclusion bodies, independent of the expression of any other RNP components. Further, the success of this tagging approach in both Measles virus (Duprex et al., 2002) and EBOV may also indicate that it could be much more widely applied for the detection of L proteins from various members of *Mononegavirales*.

3.3. Co-localization of RNP components with L following co-transfection

With the ability to detect L directly, and particularly in light of the suggestion of a novel interaction between VP30 and L, we were further interested to confirm these results using two-colour co-IFA to demonstrate co-localization between each of the RNP components and L. In these experiments it was observed that NP is incapable of interacting directly with L (Fig. 3A); however, both VP35 and VP30 do interact with L (Fig. 3A). Further, it has been shown for MARV, that NP–VP35–L forms a tripartite complex with VP35 serving as a bridge for NP and L, which do not interact when expressed alone (Becker et al., 1998). As REBOV NP and L were also unable to interact directly when co-expressed, as seen by the absence of co-localization in their staining patterns, this provided us with the opportunity to examine whether a tripartite NP–VP35–L complex is also being formed within the EBOV RNP complex, as well as to determine whether VP30 might also be participating in a similar interaction. Indeed, when NP, VP35 and L are expressed together, co-localization of NP and L in large inclusion bodies can be observed (Fig. 3B). Similarly, simultaneous expression of NP, VP30 and L resulted in co-localization of NP and L by IFA (Fig. 3B). Unfortunately, attempts to confirm these interactions by co-immunoprecipitation studies have so far been hampered by difficulties in detecting L-HA in Western Blot (data not shown), possibly as a

result of a comparatively low expression level and/or difficulties in transfer of the polymerase owing to its large size. As a result it may be necessary to consider the use of two-hybrid systems as an alternative for future studies.

However, taken together the data indicate that, in EBOV, VP30 is also capable of participating in a bridging interaction between NP and L, although this does not exclude that distinct NP–VP30 and VP30–L interaction may also occur. Indeed, it is possible that these two RNP arrangements may exist at different times in the virus life cycle, and may further reflect the dual role of VP30 as both a structural RNP component and a transcriptional activator. Further, in light of the role of VP30 as an EBOV-specific transcriptional activation factor (Mühlberger et al., 1999; Weik et al., 2002), and the lack of any evidence for interaction between MARV VP30 and L (Becker et al., 1998), the identification of a direct VP30–L interaction, and/or the existence of NP–VP30–L bridge structures, may have considerable mechanistic significance for the regulation of transcription by the different filovirus RNP complexes.

In summary, we propose that the structure of the RNP complex can be represented as shown in Fig. 4 based on the findings of this study. As a basis for RNP formation in this model, NP is shown as an oligomer (Watanabe et al., 2006) in association with the viral RNA. Elaboration of the RNP model then involves the formation of two distinct bridging structures between NP and L. The first is formed by VP35 and, based on analogy with MARV, this interaction can be assumed to involve VP35 homo-oligomers (Möller et al., 2005, with the formation of trimers in particular having been shown for EBOV (Reid et al., 2005). Further interaction between NP and L is then mediated by the formation of an NP–VP30–L bridge, as indicated. It is, thus far, unclear if a particular oligomerization state would be involved in this novel interaction; however, formation of both VP30 dimers and hexamers by EBOV has been demonstrated (Hartlieb et al., 2007) and so it remains possible that either of these forms of VP30 could be involved during interaction with NP and L. The availability of a model of the EBOV RNP complex should now provide a useful starting point for further consideration of RNP structure and function, even though such RNP complexes are dynamic structures that almost certainly exist in various different forms throughout the virus lifecycle.

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Fig. 1.

Detection of RNP complex proteins using antisera generated against individual REBOV RNP components. (A) Location of antibody targets. Proteins and peptides against which antisera were raised are indicated as boxes and labeled above the portion of the viral genome by which they are encoded. (B) Detection of REBOV RNP protein expression from pCAGGS constructs by IFA. pCAGGS constructs expressing NP, VP35 and VP30 were transfected into Vero E6 cells and expression detected using antisera directed against the NP2 peptide, VP35 or VP30, respectively. (C) Relocalization of REBOV (R) proteins during co-expression. Vero E6 cells were co-transfected with expression plasmids for NP + VP35 (35), NP + VP30 (30), L + VP35, L + VP30 or VP35 + VP30, as indicated. Expression and localization of VP35 or VP30 in these combinations was detected with antisera directed against REBOV VP35 or VP30, respectively, and compared to the localization patterns of these proteins when singularly expressed.

Fig. 2.

HA tagging of REBOV L for detection. (A) Schematic representation of the strategy for generating a tagged REBOV L construct. The relative position of the "variable hinge domain" within the L open reading frame is indicated in gray. The position at which the HA and Flag tags were inserted is further indicated, as is the tag sequence. (B) Detection of polymerase activity of HA and Flag-tagged L proteins using a REBOV minigenome assay. To determine if either the HA or Flag epitope tag had a negative impact on polymerase activity, Ad-293 cells were transfected with REBOV minigenome components, including 0.25 μ g minigenome, 1 μ g pCAGGS-NP, 0.5 μ g pCAGGS VP35, 0.3 μ g VP30 and 1 μ g of L (wild-type), L-HA or L-Flag. Lysates were analyzed using the FastCAT system (Molecular Probes) and separated by thin-layer chromatography prior to visualization under UV. (C) Detection of L-Flag and L-HA by IFA. To detect tagged L proteins by IFA, 0.5 μ g of pCAGGS-L-HA or pCAGGS-L-Flag were transfected into Vero E6 cells. After 48 h staining could be detected using an anti-HA serum but not an anti-flag serum.

Fig. 3.

Detection of RNP complex protein interactions by co-immunofluorescence (A) Co-immunofluorescence for interactions of NP, VP35 and VP30 with L. To assess interaction of L with other RNP components directly, two-colour co-IFA was used. For this approach Vero E6 cells were transfected with 0.5 μ g of each of NP + L-HA, VP35 + L-HA or VP30 + L-HA. Merger of the images showed no interaction between NP and L-HA. However, both VP35 and VP30 interacted with L-HA, as demonstrated by the significant overlap in their staining patterns. (B) Detection of tripartite bridging interactions. In order to determine if VP30 is able to participate in bridging interactions between NP and L-HA, the expression vectors for these proteins were transfected in the following combinations: NP + VP35 + L-HA or NP + VP30 + L-HA. It was observed that both VP35 and VP30 were able to bridge NP and L-HA, as indicated by the co-localization in large inclusions positive for both NP and L-HA in the presence of either of these additional components. L in these assays represents HA-tagged L (L-HA); detection of L was achieved using anti-HA serum.

Fig. 4.

Proposed model for RNP structure. Based on the identification of various protein–protein interactions formed by EBOV RNP complex components in this study, the RNP structure shown is proposed. This model also incorporates available information regarding oligomerization of the various RNP complex components, although for VP30 the exact stoichiometry of the oligomers is currently unknown.

Table 1

Antibody working dilutions for detection by immunofluorescence.

Antibody target	Source	Supplier	Specificity	Evaluated methods	Optimal working dilution
Primary antibodies					
α -REBOV NP 2	Mouse	N/A	REBOV NP	IFA	1:400
α -REBOV VP30	Mouse	N/A	REBOV VP30	IFA	1:1000
α -REBOV VP35	Mouse	N/A	REBOV VP35	IFA	1:1000
α -HA	Rabbit	Sigma	HA tag	IFA	1:100
α -Flag	Rabbit	Sigma	Flag tag	IFA	1:100
Secondary antibodies					
α -Mouse IgG; Alexa 488	Goat	Molecular Probes	Mouse IgG	IFA	1:200
α -Rabbit IgG; Cy3	Goat	Sigma	Rabbit IgG	IFA	1:100