# Supplementary Methods

## Longitudinal waning of mRNA vaccine-induced neutralizing antibodies against SARS-CoV-2 detected by an LFIA rapid test

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**Construction of the NeutraXpress™ cassette**

## 1. Protein reagents:

(1) His-tagged RBD (aa319-591 of Spike) (GenScript, Cat. No. T80302): The protein was expressed in HEK293 cells and purified by NTA-$Ni^{2+}$ affinity resin. Concentration: 2.15 mg/mL in phosphate-buffered saline (PBS, pH7.2), determined by Bradford protein assay with BSA as a standard (ThermoFisher, Cat. No. 23236). Purity: 90%, estimated by densitometric analysis of the Coomassie Blue-stained SDS-PAGE gel under non-reducing condition.

(2) His-tagged ACE2 (www.Heavybio.cn, Cat. No. HP811-A1): The protein was expressed in HEK293 cells and purified by NTA-$Ni^{2+}$ affinity resin.

(3) Anti-human IgG Fc antibody, mAb, mouse: From GenScript (Cat. No. 90401). Concentration: >2 mg/mL. Purity: >95%. The antibody is in the buffer of 50 mM Na-citrate, 150 mM NaCl, pH 7.0, containing 0.03% ProClin 300.

(4) Anti-human IgM antibody, mAb, mouse: From SouthernBiotech (Cat. No. V9020-01). Concentration: 0.5 mg/mL in Borate buffered saline, pH8.2.

(5) Chicken IgY (Cat. No. C0400315) and goat anti-chicken IgY (Cat. No. C020229) are from http://www.ablab.com.cn/.

(6) Normal human serum (MilliporeSigma, Cat. S1-100ML): The product was collected off the clot from healthy humans, and each donor unit was tested and found negative for HIV-1&2, HIV-Ag, HCV, HBsAg, and RPR by FDA approved methods.

## 2. Nitrocellulose membrane:

Sartorius UnitSart CN 140 backed membrane (No. 1UN14ER100025NT).

## 3. Procedures:

Making buffers:

2% Sodium citrate: Weigh 1 gram of sodium citrate tribasic dihydrate (Sigmaaldrich, Cat. No. S4641), dissolve thoroughly into 50 mL Millipore pure water. Freshly make this buffer each time before making the colloidal gold solution. Or, aliquot and freeze at -20°C. Do not store at 4°C.

0.2 M Borax solution: Weigh 7.62 g $NaB_4O_7 \cdot 10H_2O$ (Sigmaaldrich, Cat. No. SX0355), dissolve thoroughly into 100 mL Millipore pure water. Store at room temperature.

Re-suspension buffer: 0.02 M Borax solution with 1% BSA (Sigma, Cat. No. A5611) and 0.1% PEG 20000 (Sigma, Cat. No. 8170181000).

Dilution buffer: 0.02 M Borax solution with 1% BSA, 0.1% PEG 20000, 1% Tween-20 (Sigma, Cat. No. P1379), 10% sucrose (Sigma, Cat. No. S0389) and 5% trehalose (Sigma, Cat. No. 90210).

Making 0.02% colloidal gold solution:

(1) Boil 1000 mL Millipore pure water in a clean 1000 mL glass beaker. Keep boiling for 15 min.

(2) Discard the water from the above step, add 980 mL Millipore pure water into the same 1000 mL glass beaker, heat the water to boiling point. Cover the beaker with alum foil to prevent evaporation.

(3) Quickly add 20 mL Chloroauric acid (1%) into the above boiling water in step 2. Keep boiling.

(4) Quickly add 7.5 mL 4% Sodium citrate into the above boiling water in step 3. Keep boiling for 11 min, until the color of the solution change into burgundy and until there is no further change of the solution color. Stop heating but stir the solution for 60 min. After that, cool the solution to room temperature. Adjust the volume to 1000 mL and filter through 0.22 μm for future use.

(5) Quality check of the colloidal gold solution: Use the UV-spectrophotometer to scan from 450-600 nm, there should be absorption peaks at 510 nm and 550 nm.

Labeling RBD with colloidal gold:

Take 40 mL of 0.02% colloidal gold solution, add 480 μL 0.1 M $K_2CO_3$ to adjust pH to about 0.3-0.5 above the pI of the to-be-labeled protein, mix 5-10s, and rotate at room temperature for 3-5 min. In this case, the RBD-His protein has its pI of 8.25. So, adjust the pH of the colloidal gold solution to around 8.5. And then add 320 μL RBD-His protein (1.0-1.25 mg/mL) into the colloidal gold solution at 7.5-10 μg/mL, mix 5-10s and rotate at room temperature for 40 min.

At the end of incubation, add 1000 μL 20% BSA to 0.5% final concentration. Mix 5-10s and rotate at room temperature for 30 min. This is to block any unoccupied binding sites left on the gold particle.

At the end of incubation, spin at 10,000 rpm, 4°C for 40 min. Discard the colorless supernatant. Add 40 mL re-suspension solution to resuspend the dark purple pellet.

Labeling chicken IgY with colloidal gold:

Take 40 mL of 0.02% colloidal gold solution, add 320 μL 0.1 M $K_2CO_3$, mix 5-10s, and rotate at room temperature for 3-5 min. And then add 40 μL chicken IgY protein (10 mg/mL) into the colloidal gold solution at 10 μg/mL, mix 5-10s and rotate at room temperature for 40 min.

At the end of incubation, add 1000 μL 20% BSA to 0.5% final concentration. Mix 5-10s and rotate at room temperature for 30 min. At the end of incubation, spin at 10,000 rpm, 4°C for 40 min. Discard the colorless supernatant. Add 40 mL re-suspension solution to resuspend the dark purple pellet.

Preparing the conjugation pad:

Mix the solutions of gold nanoparticle (GNP)-labeled RBD and GNP-labeled chicken IgY at 40:1 ratio. Evenly dispense onto the conjugation pad (glass fiber #8965). Incubate at 37°C for 24 hrs to dry. Seal tight for future use.

Preparing the sample pad or RBC-filtering sample pad:

Soak the sample pad or RBC-filtering sample pad (V9/V8) in resuspension solution for 2-4

hrs. Then dry at 37°C for 24 hrs. As the application is for testing blood samples, sample pad with RBC filtering function is preferred.

Striping the C/T2/T1 lines:

Adhere the nitrocellulose (NC) membrane onto the PVC plate (JB-6). Dilute rabbit anti-chicken IgY antibody with the resuspension solution to 1.0 mg/mL, and stripe the antibody at 1 μL/cm at the C line position on the NC membrane. Dilute the mixed solution of anti-human IgG to the final concentration of 3.0 mg/mL and anti-human IgM to the final concentration of 1.2 mg/mL, and stripe the antibodies at 1 μL/cm at the T2 line position on the NC membrane. Dilute ACE2 protein to 0.2 mg/mL, and stripe the protein at 1 μL/cm at the T1 line position on the NC membrane.

Assembling the "big sheets":

Sequentially adhere RBC-filtering sample pad, conjugation pad, and absorption pad onto the NC membrane/PVC plate. Put the assembled "big sheets" at 37°C for 3 hrs with ventilation on. Seal tight for future use.

Cutting the "big sheets" and assembly the kit:

Use the automatic cutter to cut the big sheets into 4 mm wide strips, and assemble the strips into the cassettes. Make sure to exclude those strips with scratched NC membrane or any detached part of sample pad, conjugation pad, or absorption pad. Seal the cassettes in alum bags with desiccate pads.

**Expression of recombinant antibodies and Fc proteins in CHO cells**

The SARS-CoV-2 neutralizing human IgG1 antibody, REGN10933, was recombinantly expressed from Chinese hamster ovary (CHO) cells using Antagen's pDirect CHO system. The variable region genes (VH and VL) of REGN10933 were synthesized (Twist Biosciences, San Francisco, CA) and pieced together with the constant regions of human IgG1 by PCR. DNA sequencing (Genewiz, South Plainfield, NJ) confirmed that all the constructs were 100% correct.

The protein sequence of the heavy chain of REGN10933 IgG is as the following (VH in blue):

QVQLVESGGGLVKPGGSLRLSCAASGFTFSDYYMSWIRQAPGKGLEWVSYITYSGSTIYYADSVKGRFTISRDNAKS
SLYLQMNSLRAEDTAVYYCARDRGTTMVPFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP
EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPP
CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The protein sequence of the light chain of REGN10933 IgG is as the following (VL in blue):

DIQMTQSPSSLSASVGDRVTITCQASQDITNYLNWYQQKPGKAPKLLIYAASNLETGVPSRFSGSGSGTD
FTFTISGLQPEDIATYYCQQYDNLPLTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFY
PREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFN
RGEC

It has been reported that the anti-SARS antibody CR3022 also recognizes RBD of the Spike protein from SARS-CoV-2 with high affinity (Kd=6.3 nM). The variable region genes (VH and VL) of CR3022 were synthesized (Twist Biosciences) and pieced together with the constant regions of human IgG1 by PCR. DNA sequencing (Genewiz) confirmed that all the constructs were 100% correct.

The protein sequence of the heavy chain of CR3022 IgG is as the following (VH in blue):
QVQLVQSGTEVKKPGESLKISCKGSGYGFITYWIGWVRQMPGKGLEWMGIIYPGDSETRYSPSFQGQVTISADKSIN
TAYLQWSSLKASDTAIYYCAGGSGISTPMDVWGQGTMVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPE
PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPC
PAPELLGGPDVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
TVLHQDWLNGKEYKCKVSNKALPAPEEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

The protein sequence of the light chain of CR3022 IgG is as the following (VL in blue):
DIQLTQSPDSLAVSLGERATINCKSSQSVLYSSINKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDF
TLTISSLQAEDVAVYYCQQYYSTPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW
KVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

As a non-relevant control, anti-nucleocapsid (N) IgG1 antibody (clone# TJ21) was expressed by the same strategy with proprietary protein sequences. The hACE2-hIgG1 Fc fusion protein was also similarly expressed in CHO cells. Its protein sequence is the following (Fc in blue):

QSTIEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITEENVQNMNNAGDKWSAFLKEQSTLAQMYPLQEIQNLTVK
LQLQALQQNGSSVLSEDKSKRLNTILNTMSTIYSTGKVCNPDNPQECLLLEPGLNEIMANSLDYNERLWAWESWRSE
VGKQLRPLYEEYVVLKNEMARANHYEDYGDYWRGDYEVNGVDGYDYSRGQLIEDVEHTFEEIKPLYEHLHAYVRAKL
MNAYPSYISPIGCLPAHLLGDMWGRFWTNLYSLTVPFGQKPNIDVTDAMVDQAWDAQRIFKEAEKFFVSVGLPNMTQ
GFWENSMLTDPGNVQKAVCHPTAWDLGKGDFRILMCTKVTMDDFLTAHHEMGHIQYDMAYAAQPFLLRNGANEGFHE
AVGEIMSLSAATPKHLKSIGLLSPDFQEDNETEINFLLKQALTIVGTLPFTYMLEKWRWMVFKGEIPKDQWMKKWWE
MKREIVGVVEPVPHDETYCDPASLFHVSNDYSFIRYYTRTLYQFQFQEALCQAAKHEGPLHKCDISNSTEAGQKLFN
MLRLGKSEPWTLALENVVGAKNMNVRPLLNYFEPLFTWLKDQNKNSFVGWSTDWSPYADQSIKVRISLKSALGDKAY
EWNDNEMYLFRSSVAYAMRQYFLKVKNQMILFGEEDVRVANLKPRISFNFFVTAPKNVSDIIPRTEVEKAIRMSSGG
EPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR
EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLV
KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
K

The antibody heavy and light chain genes were cloned into pDirect7.0 vector (Antagen), and ACE2-Fc gene was cloned into pDirect4.2 (Antagen). The plasmids were electroporated with Neon Electroporation System (LifeTech, 1600V, 10 ms, 3 pulses) into CHO-K1 cells. After electroporation, CHO cells were selected with Zeocin (InVivogen, 400 µg/mL) in 5%FBS-DMEM for 2 weeks. Stable resistant clones were pooled together and grew in HyCell serum-free medium (GE Health Sciences) in shaking culture on an orbital shaker (150 rpm) at 37°C for 2-3 weeks.

For IgG and Fc fusion protein purification, the 0.45 µm filter cleared supernatants were passed onto Protein A columns (GenScript, Piscataway, NJ), and eluted with 100 mM pH2.5 Glycin-HCl buffer, immediately neutralized by pH8.8 Tris-HCl to neutral pH7.0. SDS-PAGE protein gel showed that the purified proteins were >95% pure. The purified proteins were aliquoted and frozen at -20°C for future use.

**Procedure to perform the test and troubleshooting**

Please refer to the Instructions for Use (IFU) at www.antagendiagnostics.com for detailed procedure to perform the test. Here are the brief steps:

**Step 1:** Remove the test cassette from the sealed pouch and place on a clean and level surface.

**Step 2:** Mark the specimen ID, dates of vaccination and test date on the cassette.

**Step 3:** Use the dropper to transfer 1 drop of whole blood (≈25 μL, to the first bulge of the provided plastic dropper) or serum or plasma (≈15 μL) to the upper part of one of the sample wells of the cassette (e.g., Well 2). Hold the diluent vial vertically and add 1 drop of diluent to the lower part of the specimen well. Wait for 1 min.

**Step 4:** Then, hold the vial vertically, add 2 drops of diluent to the well with specimen and 3 drops of diluent to the opposite control well (e.g., Well 1). Start the timer.

**Step 5:** Wait for the colored line(s) to appear. Read at 30 min till the background clears (esp. for T1 which needs longer time). Take photos. Discard of the test cassette per biosafety requirements.

**Some key points for accurately obtaining the results:**

The procedure is designed to have the blood and one drop of diluent adequately soak the sample pad and the conjugation pad for 1 min, but without starting the capillary movement. This will give NAbs in the blood sufficient time to react with GNP-RBD. Otherwise, as the distance from conjugate pad to T1 line is quite short, if one directly adds all three drops of diluent altogether to the blood sample in Well 2, the capillary movement will start right away, and the front wave of the liquid will quickly passing T1 line, without sufficient time for NAbs to displace the RBD:ACE2 interaction. So, after 1 min, adding 2 drops diluent to Well 2 and 3 drops of diluent to Well 1 will then kick off the capillary movement together in equivalent volumes. The subtle difference in liquid volumes between Well 1 and Well 2 does not really matter, as long as both wells start the capillary movement roughly at the same time. This is also the beauty of our design compared to others, to eliminate intrinsic technical variations.

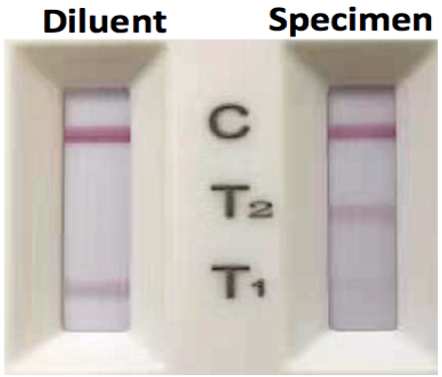
Pictures can be taken under good illumination without flashing light on at 30 min after the capillary movement starts. The percentage inhibition by NAb is derived from comparing the intensities of T1 lines of specimen wells with those of diluent only wells. The line intensities and differences can be estimated by naked eyes. Alternatively, this can also be achieved by analyzing the photo images with ImageJ software, as illustrated by the review paper [1].

**Reference:**

1. **Wang JJ**, **Zhang N**, **Richardson SA**, **Wu JV**. Rapid lateral flow tests for the detection of SARS-CoV-2 neutralizing antibodies. *Expert Rev Mol Diagn*. 2021; **21**:363–370.DOI: 10.1080/14737159.2021.1913123.

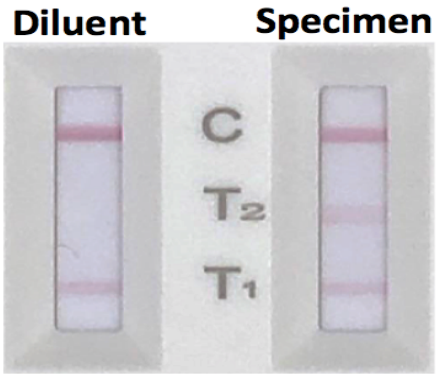
**Interpretation of LFIA test results**

# How to Read NeutraXpress™ Test Result




## Neutralizing Ab positive:

As NAb binds the gold-labeled RBD of the Spike protein, it prevents the RBD from binding the ACE2 at T1 line. Therefore, NAb positive sample will show the disappearance of or diminished T1 line as compared with that in the Diluent only control lane. Because the gold-labeled RBD-Nab no longer bind ACE2 at T1, they migrate to T2 line joining other non-NAb antibodies and are all captured by the anti-human IgG/M antibody there. High ratio of T2:T1 indicates strong NAb.




## Neutralizing Ab negative:

When there is no NAb in the sample, the gold-labeled RBD of the Spike protein will migrate and bind ACE2 at T1 line. Therefore, you will see a line comparable to that in the diluent only control lane on the left. The non-neutralizing Spike-binding antibodies will be captured by anti-human IgG/M antibody at T2 line. The subject has mounted immune response to Spike protein. However, his/her antibodies cannot block virus binding to ACE2, therefore, provide weak protection.


## Spike (RBD) Ab negative:

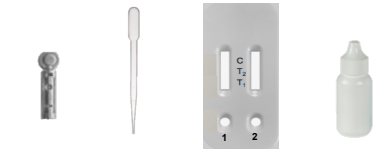
When sample shows no T2 line and the same T1 line as Diluent control, it means the subject has no RBD-binding Abs. The subject likely has weak or no immune response towards the virus, and therefore little protection. This also happens in fully vaccinated subjects, indicating the neutralizing Ab response has waned in the body. Please note, memory B cells and cellular immunity will persist to provide protection against severe illness and death.

# Instruction for NeutraXpress™ Test

**-- Fast Detection of COVID-19 Neutralizing Antibodies (NAb)**

Antagen Diagnostics

**1** Gather 4 components of the kit. Fill in the info: name initials, age, gender, vaccine type and dates, test date.

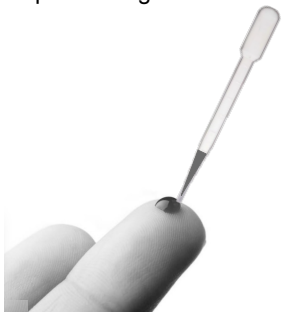



Lancet Dropper Test Cassette Diluent Vial

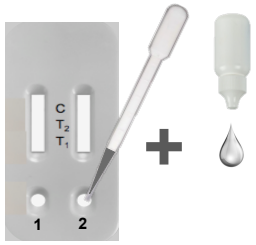
**2** Disinfect finger with alcohol pad and air dry the wiped area.

**3** Apply lancet to finger.

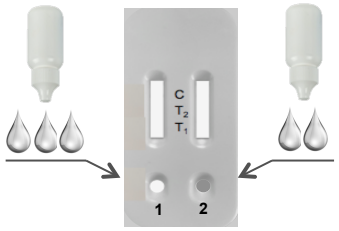
**4** Touch tip of dropper to blood droplet on finger.



**5** To Well #2, add 1 drop of blood and 1 drop of diluent. Wait for 1 min.

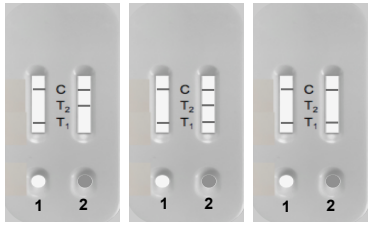



**6** Add diluent 2 drops to Well #2, and 3 drops to Well #1. Set the timer for 30 min.




**7** At 30 min, read the result.


**8** Read the results in both lanes. Take photos to record.



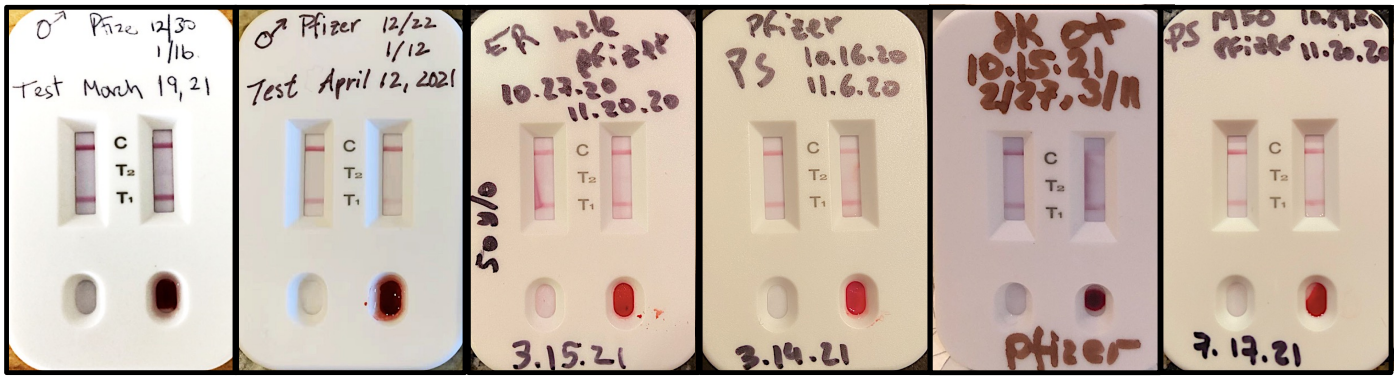

| | | | |
|---|---|---|---|
| Spike binding Ab | + | + | - |
| Spike NAb | + | - | - |

Read the complete instruction before performing the test. Failure to follow the manufacturer's instructions or modifications to the test instructions may result in test error. Ensure ALL components are at room temperature (15–30°C) when running the test.

**Supplementary Figure 1**

**A**

Different subjects, Pfizer

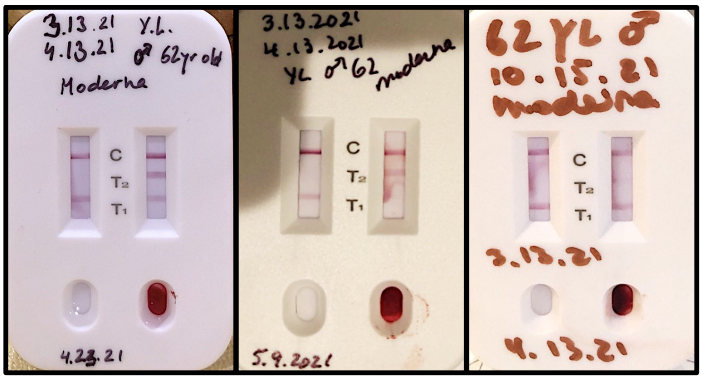



62 90 114 127 218 239

Days after 2nd dose of vaccine

**B**

Male, 62 yr longitudinal Moderna




10 26 185

**C**

Male, 86 yr longitudinal Moderna


17 34 130

Days after 2nd dose of vaccine

**Supplementary Figure 2**