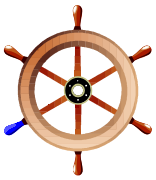

Mid-West Regional Microbiology, Brimouth

Health Protection Service

Influenza Reference Protocols

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Protocol 1: Safe Handling of Blood and Serum Samples

Unscreened fresh samples

Human blood and serum have a small but real chance of containing dangerous pathogens, such as hepatitis B virus and HIV-1. For this reason, unless the sample has been tested and found free of dangerous pathogens, or has been treated to inactivate pathogens, it must be handled under containment level 2. The Brimouth diagnostic laboratory is designed and operated at CL2.

- Handle unscreened samples using good microbiological practice.
- Label samples clearly labelled, store them safely and discard them by disinfection or autoclaving.
- Wear side-fastening laboratory coats fully fastened at all times.
- Segregate writing areas from those where unscreened samples are being manipulated.
- Do not eat, drink, apply make-up etc. in the laboratory
- Wash your hands after you remove your lab coat and before leaving the laboratory

Samples known to be contaminated with a dangerous pathogen

Influenza virus is a hazard group 2 (HG2) pathogen. HIV and HBV are HG3 pathogens. Do not handle samples known to contain HG3 pathogens in the diagnostic laboratory, but transfer them to the containment level 3 laboratory for manipulation.

Protocol 2: Safe handling of influenza virus

Influenza virus is a hazard group 2 pathogen and must be handled at all times in a containment level 2 laboratory. Unless the strain of virus being used is known to be particularly virulent (such as some avian strains) or has been concentrated, the risk of infection by the aerosol route should be acceptably small as long as CL2 procedures are followed (see above). In addition,

- Avoid generating aerosols by taking care not to produce bubbles during pipetting procedures.

Protocol 3: In vitro culture of influenza virus

Aseptically collected nasopharangeal swabs, and nasal and bronchial washes are suitable samples for the isolation of influenza virus from infected hosts. Samples will be received together with a record of the patient's details and the date and place of specimen collection. Inspect the sample to confirm it is in good condition and either process within 4h (storing on ice) or store long-term at –70˚C. To culture the virus, you will need: a 75cm2 tissue culture flask seeded with MDCK or MDBK cells at 80% confluence; phosphate-buffered saline; trypsin solution; antibiotic cocktail. Add 2ml PBS to the swab, wash it thoroughly and decant to a new, sterile container. In a tissue culture hood, aspirate the medium from the cells, wash once with PBS, add 2ml of the suspect sample, with 0.5ml antibiotics and 0.5ml trypsin. Mix and allow to interact with the cells for 30min on ice. Add 10ml of culture medium and place at 33˚C, 5%CO2 for up to seven days. Observe daily for signs of cytopathic effects. If no c.p.e. are seen after 7 days, transfer 2ml of the supernatant to a fresh flask of cells and culture for a further 7 days. Once c.p.e. are noted, take an aliquot for storage at –70˚C and test another aliquot for HA (see protocol 4). If HA >320/ml, proceed to identification by HI and other tests. If HA<320/ml, re-passage in fresh cells, as before.

Plaque Assay

NB: All material contaminated by virus must be sterilised in Trigene.

The day before

One confluent T75 of MDBK cells will produce just over 24 wells (4 6-well dishes) for assay. Trypsinise the flask and resuspend the cells in 50 ml MEM (10% FCS). Add 2 ml of resuspended cells to each well of a 6-well plate and incubate overnight.

Prepare (if you don't already have some) MEM with 0.5% FCS (for WSN) *or DMEM with no FCS (for other viruses).*

Dissolve 2 g low gelling temperature agarose in 100ml PBS by microwaving for short busts in an open bottle and swirling. Once all of the agarose has dissolved (no grains visible floating in liquid) cover the bottle and leave to set. (Can also do this on the day.)

Prepare Coomassie Blue solution: dissolve 2g Coomassie Brilliant Blue R in 250ml water, slowly add 75ml acetic acid, add 500ml ethanol, top up to 1000ml with water.

Day 1

Make 10-fold serial dilutions of the virus stock in 0.5% MEM. The volume is arbitrary, but 50 ul in 450 ul works well (can start at 10^{-2} using 5 ul in 495ul if

needed). Between each dilution vortex the tube and **change tips** (very important).

For most stocks 6 dilutions will be sufficient (10^{-1} – 10^{-6}); for high-titre viruses it may be necessary to dilute to 10^{-8} and for concentrated stocks to 10^{-9}.

Aspirate medium from cells and wash twice with 1 ml PBS. Aspirate PBS and apply 400 ul of diluted virus to each well (tips can be re-used if working **from the most dilute sample up to the most concentrated**). Incubate for 30 min – 1 h with occasional rocking, during which time the virus will adsorb to the cells.

Melt agarose by short burst of microwaving in an open bottle, with swirling. Mix 1ml / well in a 1:1 ratio with 0.5% MEM *or DMEM* (ideally straight from the fridge).

Optionally, aspirate virus from cells, working from the most dilute solution (not all virus will have adsorbed in that time). Add 2 ml of agarose / MEM overlay, shake to cover, and leave on the bench to set for 15 min. Invert and return to incubator upside for 2-3 days.

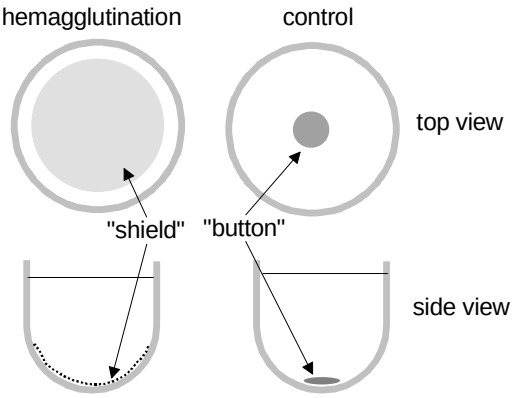
Day 3-4

After incubation plaques should be visible as clear spots in the monolayer when viewed with the desk lamp. To stain, remove the overlay by gently running a spatula around the edge (leaving on the bench to cool for c.10min can help) and add c.1ml Coomassie blue solution for c.30 min. You cannot over-stain. Rinse carefully with tap water and dry upside down.

Protocol 4: Titration of influenza virus by hemagglutination (CL2 if aerosols minimized)

Background

A convenient and rapid way to establish the concentration of virus in a sample is to make use of the ability of virions to cross-link cells that bear surface receptors for the virus. The receptor for influenza virus is n-acetyl neuraminic acid, a sialic acid, an acidic sugar found commonly on the ends of the oligosaccharides naturally occurring on glycoproteins and glycolipids of most vertebrate cells. Although not a natural target for infection *in vivo*, red blood cells are rich in sialic acid and the effect of their cross-linking by virus is easily seen. The viral protein that binds to cellular sialic acid receptors is called the *hemagglutinin* because of this effect.



If a sufficient concentration of influenza virions is mixed with a suspension of hen red blood cells, the agglutination reaction will develop over a few minutes and the aggregates will settle on the concave surface of microtitre well to form an extended layer (sometimes called a “shield”) which can easily be distinguished from the appearance of untreated red blood cells, which otherwise settle at the lowest point of the well to form a “button”.

If one makes a serial dilution of virus before mixing it with red blood cells, one will come to a point at which there are too few virions to produce hemagglutination. As long as procedures are followed exactly, the maximum dilution factor at which the sample still produces hemagglutination will be a reliable guide to the concentration of virus. By convention, the amount of virus in such a limiting dilution is known as one hemagglutination unit (1 HAU) and so the concentration of virus (in HAU/ml) in the original sample can be calculated by division of the dilution factor by the volume of diluted virus (in ml) added to the limiting well.

Materials for hemagglutination assay

96-well, round-bottomed microtitre plate
P200 Gilson Pipetman and tips
Phosphate-buffered saline
Virus sample to be assayed (keep on ice).
X% suspension of hen red blood cells in PBS
HA record sheet

Method

- For each virus to be titrated, add 50μl PBS to each of 11 wells of a microtitre plate
- Add 50μl virus to the first well (making a 1/2 dilution)

- Using a fresh pipette tip, mix the contents of the first well and transfer 50μl to the second well. (N.B. Safety: do not produce bubbles during this operation, or potentially dangerous aerosols will be generated)
- In the same way, transfer 50μl from well 2 to well 3, and so on to well 10, producing a 2-fold dilution series.
- Discard 50 μl from well 10 to waste, leaving well 11 as a control.
- Thoroughly resuspend the hen erythrocytes (RBC).
- Starting with the control, and then moving from weaker to stronger virus concentrations, add 50μl of RBC to all 11 wells.
- Leave the plate on the bench until a button is formed in the control wells.
- Note the last cup showing hemagglutination

Records and calculations

Record the identities of the virus samples, and the appearance of each dilution on the hemagglutination mictrotitre plate, on an HA record sheet. Dummy data have been added to the table below for illustrative purposes:

Dilution / Sample I.D.	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Cont	Titre (HAU/ml)
P	S	S	S	S	S	S	S	B	B	B	B	2560[1]
Q	S	S	S	S	B	B	B	B	B	B	B	320

Notes:

1. Concentration of virus in sample is given as HA units (HAU) per ml (HAU/ml). For the example P above the final dilution to give a shield (positive result) was 1/128, but only 50ul were added per well, so the titre is calculated as the fold dilution/amount added in mls = 128/0.05 = 2560HAu/ml.
2. NB Standard antigen is provided for you to do the HI tests, so you do not need to dilute your virus samples.

Protocol 5: Titration of anti-HA antibodies by hemagglutination-inhibition (HI test)

Background

During infection by influenza virus, the host mounts a humoral immune response to the virus' envelope glycoproteins, HA and N. These antibodies, particularly against HA, are the main way by which the host develops immunity to re-infection, because they are able to block the virus from binding to its receptors on host cells. The sequence of HA and N, and consequently their susceptibility to particular antibody, changes by mutation between outbreaks, a process known as genetic *drift*. Occasionally, owing to a recombination event between different viruses, genetic *shift*, a more substantial change in HA and N sequence occurs. The change in virus antigens leads to the rapid spread of the virus in a population whose antibodies no longer bind to the viral glycoproteins, potentially producing a pandemic.

A key duty of public health laboratories is to characterize the HA and N serotypes of circulating viruses, in order to provide timely information should a new strain of virus appear, against which current vaccines might be ineffective. To do this, serum samples should be taken from infected and convalescent people and assayed for its ability to inhibit hemagglutination by standard strains of a range of serotypes.

A rising titre (more than three-fold increase in titre from acute sample to convalescent sample) of hemagglutination-inhibiting (HA) antibodies indicates recent infection with the corresponding serotype of virus. If only convalescent sera are available, conclusions about the recency of the infection must be cautious, though it is still useful information to know what viruses a person has been exposed to.

Materials

- 96-well, v-bottomed microtitre plate
- P200 Gilson Pipetman and tips
- Phosphate-buffered saline
- Antiserum to be assayed (keep on ice)
- Standard preparations of virus antigen at 160 HAU/ml (i.e., 8 HAU/50 µl) in PBS, e.g. H1N1 and H3N2
- 0.5% suspension of hen red blood cells in PBS
- HI record sheet

Method

- For each antiserum / standard antigen combination to be assayed, add 25µl of PBS to the first 10 wells of a row in your microtitre plate
- Add 25µl PBS to well 11 to act as the virus control

- Add 50μl of PBS to well 12 to act as a red blood cell control
- Add 25 μl of the serum sample to the first well
- Using a fresh pipette tip, mix the contents of the first well and transfer 25μl to the second well.
- In the same way, transfer 25μl from well 2 to well 3, and so on to well 10, producing a 2-fold dilution series.
- Discard 25 μl from well 10 to waste, leaving well 11 as a control.
- Add 25μl of standard antigen to wells 1 – 11 but not to well 12
- Thoroughly resuspend the hen erythrocytes (RBC).
- Starting with the RBC control, and then moving from weaker to stronger antiserum concentrations, add 50μl of RBC to all 12 wells.
- Leave the plate on the bench until a button is formed in the control wells.
- Note the last cup showing inhibition of hemagglutination

Records and calculations

Record the identities of the antigens and serum combinations, and the appearance of each dilution on the hemagglutination mictrotitre plate, on a standard HI record sheet. Dummy data have been entered into this example to illustrate the calculations.

Dilution of serum		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Antigen cont	RBC Cont	Titre (HIU/ ml)
Antigen I.D.	serum I.D.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	
H1N1	Xa	B	S	S	S	S	S	S	S	S	S	S	B	80
H3N2	Xa	B	B	S	S	S	S	S	S	S	S	S	B	160
H1N1	Xc	B	B	B	B	B	B	B	B	B	S	S	B	20480
H3N2	Xc	B	B	S	S	S	S	S	S	S	S	S	B	160

In the above example, “X” was a particular patient, “Xa” and “Xc” were their acute and convalescent sera, respectively. “B” indicates “button” (inhibition of hemagglutination) and “S” indicates “shield” (indicating hemagglutination).

The HI titre is given as hemagglutination inhibition units/ml or HIU/ml. It is calculated by divided the last dilution to give inhibition by the volume (in mls) of serum used in the well (which for this assay performed by HPA standards is 25ul. So for Xc sample the titre is calculated as the greatest dilution inhibiting hemagglutination (512 fold dilution) divided by 0.025ml (=25ul), =512/0.025 = 20480 HIU/ml).

In this case, patient X had a low and constant titre of HI antibody against H3N2 virus, but a strongly rising titre of HI antibody against H1N1 virus, strongly indicating contemporary infection with an H1N1 strain of influenza.

Protocol 6: Characterization of virus HA serotype by HI

Background

Although detecting antibodies in the serum of patients is useful and can indicate that a person has recently been infected, it circumstantial evidence. To inconclusively demonstrate a contemporary infection and determine its serotype, virus should be isolated from throat swabs taken during the acute phase and tested by HI using antibodies of known serotype reactivity.

If the lab has been able to isolate and grow virus from an acute case, one is then able to undertake tests that make a definitive diagnosis possible. The viral genome can be sequenced commercially following RT-PCR (Protocol 9), to provide very detailed information about the phylogenetic relationship of the isolate with circulating viruses. At a lower level of resolution, we can establish the identity of the virus cheaply and quickly in house. The HA titre of the virus must first be determined by protocol 4 and then this protocol is used to establish whether the hemagglutinin of the virus is inhibited by standard antisera to known HA types. A standard antigen preparation should be run in parallel with your test virus. Plan your assay carefully. The background to hemagglutination-inhibition assays is given in protocol 5.

Materials

- 96-well, round-bottomed microtitre plate
- P200 Gilson Pipetman and tips
- Phosphate-buffered saline
- Standard typing antisera (e.g. sheep anti-H1N1, or sheep anti H3N2)
- Test virus at 4 HAU/ml in PBS,
- X% suspension of hen red blood cells in PBS
- HI record sheet

Method

- For each combination of standard antiserum and test virus or standard antigen to be assayed, add 25μl of PBS to the first 10 wells of a row in your microtitre plate
- Add 25μl PBS to well 11 to act as the serum control
- Add 50μl of PBS to well 12 to act as a red blood cell control
- Add 25 μl of the standard antiserum to the first well
- Using a fresh pipette tip, mix the contents of the first well and transfer 25μl to the second well.
- In the same way, transfer 25μl from well 2 to well 3, and so on to well 10, producing a 2-fold dilution series.

- Discard 25 μl from well 10 to waste, leaving well 11 as a control.
- Add 25μl of the test virus **or** standard antigen at 4 HAU/ml to wells 1 – 10 but not to wells 11 or 12
- Thoroughly resuspend the hen erythrocytes (RBC).
- Starting with the RBC control, and then moving from weaker to stronger antiserum concentrations, add 50μl of RBC to all 12 wells.
- Leave the plate on the bench until a button is formed in the control wells.
- Note the last cup showing inhibition of hemagglutination

Records and calculations.

Use The HI record sheets and perform the calculations as described in protocol 5

Protocol 7: ELISA assay for anti-Neuraminidase antibodies

This in-house assay detects the presence of **antibodies** to influenza neuraminidase antigens present in the **serum** of patients who have made an immune response to influenza virus. It is an "indirect" ELISA assay (see http://en.wikipedia.org/wiki/ELISA).

This test **discriminates** between responses to different neuraminidase types (e.g. N1 vs N2).

Because many people in the population have previous experience of influenza infection, it is normal to find anti-N antibodies in patient samples.

A **rising titre** of anti-N antibody between **acute** and **convalescent** serum samples of the same patient, indicate a very recent infection with influenza.

Procedure

1. Coat a 96-well microtitre assay plate (Costar, see figure below) with neuraminidase antigen, as follows
    a. Thaw one ampoule each of stock N1 and N2 antigen (100 µg/mL) and invert to mix.
    b. Dilute each antigen to 1 µg/mL in carbonate binding buffer
    c. Add 20 µL of N1 antigen to each well of a column 1 (8 wells, for serum control), and do the same for column 3 (for the first serum sample), column 5 (for the second…) and so on.
    d. Add 20 µL of N2 antigen to each well of a column 2 (for serum control), and do the same for column 4 (for the first serum sample), column 6 (for the second…) and so on.
    e. Label the columns with "N1", "N2", as appropriate
    f. Seal the plate with a self-adhesive sheet, and place in the refrigerator for at least 8 hours, for the antigen to bind to the wells.
2. Warm the plate to room temperature and carefully remove the adhesive sheet, ensuring no drops of antigen contaminate adjoining wells.
3. Wash the plate six times with PBS-tween to remove excess antigen
4. Add 100 µL of albumin solution to each well, in order to block remaining non-specific sites.
5. Cover with adhesive sheet and leave at room temperature for 2 hours
6. In the mean time, warm the test serum samples and the control serum to room temperature. Mix thoroughly
7. Prepare a two-fold dilution series (1/8 to 1/1024) of each serum sample as follows:
    a. For each serum sample, set out eight, 5 mL dilution tubes
    b. Into the first tube of each set, add 2.1 mL PBS BSA
    c. Into tubes 2-8 of each set, add 1.20 mL PBS BSA

   d. Into tube 1, add 300 µL of the respective serum sample (1/8 dilution), and mix thoroughly
   e. Transfer 1.2 mL of the 1/8 dilution from tube 1 to tube 2 and mix thoroughly (1/16 dilution)
   f. Repeat for tubes 3-8
8. Remove the adhesive sheet and wash as in step 3
9. Add 70 µL of 1/1024-diluted control serum (dilution tube 8) to wells H1 and H2
10. Add 70 µL of 1/512-diluted control serum (dilution tube 7) to wells G1 and G2, and so on for the less diluted samples up to 1/8 in A1 and A2
11. Add 70 µL of 1/1024-diluted test serum 1 (dilution tube 8) to wells H3 and H4
12. Add 70 µL of 1/512-diluted test serum 1(dilution tube 7) to wells G3 and G4, and so on for the less diluted samples up to 1/8 in A3 and A4
13. Repeat steps 11 and 12, and so on, for test serum 2 (columns 5 and 6), and so on.
14. Seal the plate with adhesive sheet and incubate at room temperature for 1 hour.
15. In the mean time, prepare a 1/1000 dilution of detection antibody-alkaline phosphatase conjugate. You need 5 mL per plate.
16. Unseal the plate and wash as in step 3. Do not allow the plate to dry from this stage on.
17. Using a multi-channel pipettor, add 50 µL diluted detection antibody to each well.
18. Seal the plate with adhesive sheet and incubate at room temperature for 1 hour.
19. In the mean time, prepare the alkaline phosphatase substrate (5 mL per plate)
20. Unseal the plate and wash as in step 3. Do not allow the plate to dry.
21. Add 50 µL substrate solution per well. Gently tap the plate to ensure the wells are evenly filled.
22. Incubate at room temperature for up to 30 min, but inspect regularly.
23. Once a distinct yellow colour appears in any well, determine the optical absorbance using the ELISA plate reader. Repeat the measurement as the colour develops.
24. Count any test sample in which the reading is > 2-fold higher than that of the corresponding control serum as positive.
25. For any test serum showing positive wells against a given antigen, declare the titre to be that of the highest positive dilution. (I.e. if test serum 1 shows positive wells in A3, B3, C3 but negative in D3, E3, F3, and so on, the greatest dilution showing a positive result is 1/32 (well C3), and column 3 corresponds to antigen N1. Therefore the result is N1: 1/32.)
26. For any test serum that fails to show a positive well against a given antigen, declare it negative.

Protocol 8: QuickVue test for Influenza A

The QuickVue Influenza A+B test allows for the rapid, qualitative detection of influenza type A and type B **antigens** directly from **nasal swab**, nasopharyngeal swab,nasal wash and/or nasal aspirate specimens.

The test is intended as an aid in the rapid differential diagnosis of acute influenza type A and type B virus infection.

For more details, see
http://www.quidel.com/products/product_detail.php?prod=101&group=1&cat=

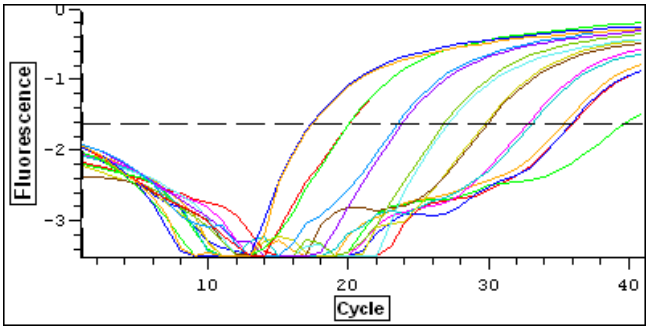
Time to Result	10 minutes or less
Specimen Types:	Nasal swab, nasal wash / nasal aspirate
Storage	24 months at room temperature *
Internal Procedural Controls:	Yes positive and negative
External Controls:	Yes Pos (A), Pos (B), and Neg
Sensitivity:	A-94%, B 70% - Nasal Swab A-83%, B 62% - Nasopharyngeal swab A-77%, B 82% - Nasal aspirate/nasal wash
Specificity	A-90%, B 97% - Nasal Swab A-89%, B 98% - Nasopharyngeal swab A-99%, B 99% - Nasal aspirate/nasal wash
Positive Predictive Value	A-62%, B 82% - Nasal Swab A-67%, B 80% - Nasopharyngeal swab A-91%, B 90% - Nasal aspirate/nasal wash
Negative Predictive Value	A-99%, B 94% - Nasal Swab A-95%, B 95% - Nasopharyngeal swab A-96%, B 97% - Nasal aspirate/nasal wash
Overall Accuracy:	A-91%, B 93% - Nasal Swab A-88%, B 94% - Nasopharyngeal Swab A-95%, B 96% - Nasal aspirate/nasal wa

Protocol 9: Real Time RT-PCR for Influenza vRNA typing

Real-time reverse transcriptase PCR is a highly sensitive method for quantifying the amount of specific RNAs in a sample. Clinical samples containing active influenza virions, such as nasal swabs from acute cases contain sufficient virion RNA (vRNA) that it can be detected by this method.

Specificity is conferred by the complementarity of the sequences of DNA oligonucleotides used to prime reverse transcription (of the vRNA into complementary DNA), to prime PCR-based amplification of cDNA, and to detect amplification products.

Sensitivity and quantification is conferred by the detection in real-time (i.e. during the PCR process) of the liberation of a fluorochrome from the labelled detection oligonucleotide. The number of PCR cycles taken for the fluorescence signal to break through the detection threshold is directly related to the logarithm of the abundance of the specific viral RNA (see figure below).



At Brimouth HPS, we don't do this assay in-house, but make use of the service offered by Brimouth Molecular Diagnostics plc in the adjoining PFI building to do this for us. They offer the following primer sets, and you must specify which set is used with which sample when you submit them (note: each combination of primer set and sample costs two credits, but any number can be run simultaneously, within the 6h turn-round period).

qRT-PCR primer sets available for influenza at BMD:

1. Influenza A (**one** primer set; to matrix-encoding vRNA; gives positive reaction for all flu A but not flu B or C)
2. Influenza A Haemagglutinin vRNA (**four** primer sets reporting H1, H3, H5 and H7 serotypes)
3. Influenza A Neuraminidase vRNA (**two** primer sets reporting N1 and N2)

See the following web sites for more information of real-time PCR:

- Real Time PCR Tutorial by Dr Margaret Hunt, University of South Carolina, http://www.microbiologybook.org/pcr/pcr-home.htm
- Wikipedia account of real-time PCR http://en.wikipedia.org/wiki/Real-time_polymerase_chain_reaction