Norwegian University of Life Sciences

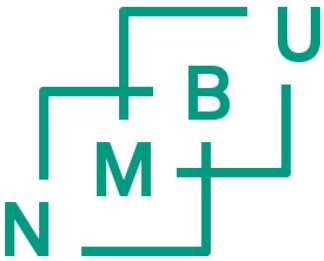
Master's Thesis 2022 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

IgG protection status for humoral immune response to MMR vaccine among the Norwegian population of children and method assessment of MMR Multi-Plex Immunoassay

Liva Kukule
Master of science, Biotechnology

IgG protection status for humoral immune response to MMR vaccine among the Norwegian population of children and method assessment of MMR Multi-Plex Immunoassay

Liva Kukule



Supervisors:

Johanna Eva Bodin

Gro Tunheim

Audun Aase

Siv Kjølsrud Bøhn

Master thesis

Faculty of Chemistry, Biotechnology and Food Science

The Norwegian University of Life Sciences

May 2022

IgG protection status for humoral immune response to MMR vaccine among the Norwegian population of children and method assessment of MMR Multi-Plex Immunoassay

https://nmbu.brage.unit.no

Acknowledgements

This master thesis was conducted from August 2021 to May 2022 at the Section for Immunology, within the Department of Method Development and Analytics at the Norwegian Institute of Public Health. This scientific project is a part of a master's degree in Biotechnology, belonging to the Faculty of Chemistry, Biotechnology and Food Science at the Norwegian University of Life Sciences.

First and foremost, I would like to express my gratitude to my main supervisors, senior scientists Gro Tunheim, Johanna Eva Bodin and Audun Aase at the Norwegian Institute of Public Health for giving me an opportunity to explore and increase my knowledge within the field of immunology as well as providing me with excellent academic feedback, support, and continuous positive encouragement throughout this master's project. Thank you for always being available for guidance and feedback in regards of laboratory work and academic writing. I am forever grateful for all the effort, time, valuable knowledge, and support that I have received from all of you. I would also like to express my gratitude to my internal supervisor Siv Kjølsrud Bøhn at the Norwegian University of Life Sciences for the continued positive encouragement and feedback throughout the writing process.

Secondly, I would like to thank the rest of the employees within the Section for Immunology for practical help in the laboratory, and valuable feedback. I am especially grateful to former laboratory technician Ida Johnsen for skilful training of the Multi-Plex Immunoassay, and senior engineer Tove Karin Herstad for guidance regarding Multi-Plex troubleshooting and help with the knowledge and performance of statistical analysis with Lin's Concordance Correlation Coefficient.

Lastly, I would like to thank my friends and loved ones for emotional support and words of encouragement throughout this process. A special thanks to my kind and supportive mother Zinta for always being proud of my achievements and believing in my goals.

Abstract

Background and aim: In order to prevent sudden outbreaks of serious disease, immune response to vaccines should be monitored to investigate the overall status of protection in a given population as well as identifying susceptible groups or individuals with a reduced or a negative response. The measles -mumps -rubella (MMR) vaccine is a live-attenuated combined vaccine used for the prevention of measles, mumps, and rubella. The main purpose of this study was to investigate the status of protection against measles, mumps, and rubella among the Norwegian population of children using IgG antibody concentrations as an indicator for humoral immune response to the MMR vaccine. An additional goal was to perform method assessment of MMR Multi-Plex Immunoassay by investigating intra-assay precision and inter-operator reproducibility of IgG measurements.

The study population and method: The study population were a part of The Norwegian Mother, Father and Child Cohort study (MoBa) including 306 children between the ages of 7 and 14. The collection of experimental material was performed by the Norwegian Environmental Biobank (NEB) during 2016-2017. The plasma IgG against measles, mumps, and rubella pathogen antigens were analysed using MMR Multi-Plex Immunoassay. Following IgG "protective levels" were set for each antigen: measles; >200 mIU/ml, mumps; >500 AU/ml, and rubella; >10 IU/ml, where concentrations below these thresholds were considered as "unprotected". The intra-assay precision assessment was performed by the investigation of CV% values, and inter-operator reproducibility assessment was accomplished with the help of scatter plots, Lin's Concordance Correlation Coefficient analysis, Bland-Altman plots, and re-analysis of plasma samples.

Results: The majority of IgG antibody concentrations were above 'protective level' for all three antigens (measles: 97.1%, mumps: 63.1%, rubella: 89.2%). Overall, 2.3% of the children had IgG antibody concentrations below 'protective level' for all the three antigens. Notably, the proportion of 'unprotected' children varied for the three pathogens with lowest protection rates for mumps (36.9% below protection limit) while only 2.9% and 10.8% were 'unprotected' for measles and rubella respectively. The intra-assay precision results showed high precision with low mean CV% values of duplicates for all the three antigens (measles: 3.9%, mumps: 3.0% and rubella: 3.5%). The assessment of inter-operator reproducibility showed an overall high agreement between operators for IgG levels measured against all three pathogens.

Conclusion: According to obtained results, the MMR Multi-Plex Immunoassay revealed a good intra-assay precision for the measurement of IgG levels against measles, mumps, and rubella. The results indicate an overall high degree of IgG antibody protection against measles, mumps, and rubella within the study population, which further indicates a substantial MMR vaccine effectiveness and heard immunity. However, a larger proportion of children were unprotected against mumps in comparison to measles and rubella indicating a possible need for booster vaccines or advances in vaccine technology against mumps.

Sammendrag

Bakgrunn og målsetting: For å hindre uventede utbrudd av alvorlig sykdom, burde immunrespons på vaksiner overvåkes for å undersøke den generelle beskyttelsesstatusen i en gitt populasjon, samt identifisere mottakelige grupper eller individer med redusert eller negativ respons. Vaksine mot meslinger - kusma - røde hunder (MMR) er en levende svekket, kombinert vaksine som brukes til forebygging av meslinger, kusma og røde hunder. Hovedformålet med denne studien var å undersøke status for antistoff beskyttelse mot meslinger, kusma og røde hunder blant norske barn ved å bruke IgG-antistoffkonsentrasjoner som en indikator på den humorale immunresponsen til MMR-vaksinen. Et tilleggsmål var å utføre kvalitetsvurdering av MMR Multi-Plex Immunoassay ved å undersøke intra-assay-presisjon og inter-operator reproduserbarhet av IgG-målingene.

Studiepopulasjonen og metode: Studiepopulasjonen var en del av den norske mor, far og barn -undersøkelsen (MoBa), og hadde en størrelse på 306 barn mellom 7 og 14 år. Innsamlingen av eksperimentelt materiale ble utført av Miljøbiobanken i perioden 2016-2017. IgG målinger i plasma mot meslinger, kusma og røde hunder ble analysert ved å bruke MMR Multi-Plex Immunoassay. Følgende grenser ble satt for IgG-beskyttelsesnivåer for hvert antigen: meslinger; >200 mIU/ml, kusma; >500 AU/ml, og røde hunder; >10 IE/ml, der konsentrasjoner under disse terskelverdiene ble ansett som «ubeskyttet». Presisjonsvurderingen innen analysen ble utført ved undersøkelse av CV%-verdier, og inter-operator reproduserbarhetsvurdering ble utført ved hjelp av spredningsplott, Lins konkordanskorrelasjonskoeffisientanalyse og Bland-Altman-plott.

Resultater: Flertallet av populasjonen hadde IgG-antistoffkonsentrasjoner over "beskyttende nivå" for alle tre antigener (meslinger: 97,1 %, kusma: 63,1 %, røde hunder: 89,2 %). Totalt sett hadde 2,3 % av barna IgG-antistoffkonsentrasjoner under terskelverdi for "beskyttende nivå" for meslinger, kusma og rubella. Det ble observert en stor variasjon blant andelen "ubeskyttede" barn blant de tre antigene, der laveste beskyttelsesgrad ble oppdaget for kusma (36,9 % under beskyttelsesgrensen), mens bare 2,9 % og 10,8 % var "ubeskyttet" for henholdsvis meslinger og røde hunder. Presisjonsresultatene for intraanalysen viste høy presisjon med lave gjennomsnittlige CV%-verdier av duplikater for alle tre antigener (meslinger: 3,9 %, kusma: 3,0 % og røde hunder: 3,5 %). Vurderingen av interoperator-

reproduserbarhet viste et generelt høy samsvar mellom operatører for IgG-konsentrasjonene for alle tre antigener.

Konklusjon: I henhold til de oppnådde resultatene, viste MMR Multi-Plex Immunoassay en god intra-assay presisjon for de målte IgG-nivåene mot meslinger, kusma og røde hunder. Resultatene indikerte også en høy grad av antistoffbeskyttelse mot meslinger, kusma og røde hunder i studiepopulasjonen, noe som indikerer en betydelig MMR-vaksineeffektivitet og flokkimmunitet. Derimot, var en større andel av barna ubeskyttet mot kusma sammenlignet med meslinger og røde hunder, som indikerer et mulig behov for boostervaksiner eller fremskritt innen vaksineteknologi mot kusma.

Table of Contents

List of figures

List of tables

Abbreviations

AU	Arbitrary unit
BA	Bland-Altman
BSA	Bovine serum albumin
CDC	Centres for Disease Control and Prevention
CRS	Congenital rubella syndrome
CV	Coefficient of Variation
DNA	Deoxyribonucleic acid
EDAC	1-Ethyl-3-(3- dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	ELISA units
FI	Fluorescence intensity
GMT's	Geometric mean titres
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPA	Isopropyl alcohol
IU	International unit
MCV	Measles-containing vaccine

MeV	Measles virus
MMR	Measles, mumps, and rubella
MoBa	The Norwegian Mother, Father and Child Cohort Study
MuV	Mumps virus
MSIS	The Norwegian Surveillance System for Communicable Diseases
NEB	Norwegian Environmental Biobank
NIPH	The Norwegian Institute of Public Health
PAMPs	Pathogen-associated molecular patterns
PCBs	Polychlorinated biphenyls
PBS	Phosphate-buffered saline
PRRs	Pattern-recognition receptors
RNA	Ribonucleic acid
REK	Regional Committees for Medical and Health Research Ethics
R_0	Basic reproductive rate
RT	Room temperature
RuV	Rubella virus
SYSVAK	The Norwegian Immunisation Register
WHO	World Health Organization

1 Introduction

1.1 Elements of the immune system

As a result of evolution, many microorganisms have evolved alongside humans and animals. Some of these microorganisms can cause infectious diseases able to wipe out millions of lives. Microorganisms are also able to reproduce and evolve at a much faster pace compared to their hosts. Therefore, a proper defence system is a crucial part of survival. To fight off different pathogens, evolution has equipped the human body with an immune system, an effective defence mechanism with a large number of cellular and physical components. Together, all the components involved in the immune system form a systematic network able to fight off foreign intruders, as well as obtaining specific immunity against a particular pathogen (Parham, 2014).

1.1.1 An introduction to innate and adaptive immunity

The immune system is primarily divided into two parts, innate immune system, and adaptive immune system. The innate immune system is more or less unspecific, quick, and able to fight off infections within a few days. Such infections are often generated by cuts, bites, wounds, abrasions or other irritations to the skin, mouth, or eyes. The outside surface of the body is considered to be the first line of defence in the innate immune response and comprises the skin and mucosal linings. These provide an effective chemical and mechanical barriers that prevent pathogens from entering internal tissues and organs (Parham, 2014). When the physical and chemical barriers are breached, the second line of defence is activated through pathogen recognition. Nonepithelial cells sense the presence of pathogens with the help of protein receptors which have the ability to recognize microbe-associated molecules. Such microbial molecules occur in repeating patterns and are commonly known as pathogen-associated molecular patterns (PAMPs), present in various microbial molecules. Protein receptors which recognize PAMPs are called pattern recognition receptors (PRRs) which are either located as transmembrane proteins on the surface of host cells or intracellularly for the detection of intracellular pathogens. When an infectious agents enters the body, it activates the PRRs creating an inflammatory response at the site of infection mediated by signal molecules called cytokines (Bruce *et al.*, 2014). Numerous effector cells contribute to the innate immune system, including macrophages, mast cells, natural killer (NK) cells, dendritic cells, monocytes, neutrophils, basophils, and eosinophils. In addition, an array of 20 different soluble proteins called the complement system, function to destroy extracellular pathogens. This particular

system is complementary to the antibody response of the adaptive immune system. When the different effector cells and mechanisms of the innate defence system are unable to clear an infection, the adaptive immune response is informed and activated (Gair and Molnar, 2015b), with the help of dendritic cells (Bruce *et al.*, 2014)

In contrast to innate immune system the adaptive defence system develops over time as a result of exposure to various infectious agents. The adaptive immune system is depended on a class of white blood cells called lymphocytes, which are separated into two major classes, B lymphocytes (B cells) and T lymphocytes (T cells). B cells produce and secrete antibodies able to bind specific pathogens. T cells can either directly kill pathogen-infected cells or produce various signal proteins that stimulate other host cells to help fight an infection. During the development of T-and B-cells, particular DNA sequences are rearranged in various combinations so that the cells can produce a limitless variety of receptors and antibodies (Bruce *et al.*, 2014). After the pathogen is eliminated, long-lived memory B-cells are generated. These cells are highly specific, and will have a strong response to the next encounter with the same pathogen and will generate new effector cells (Mirzaei, 2020). The adaptive immune system is also known as acquired immunity, and is separated into two mechanisms, cell-mediated immune response, and humoral immune response also known as antibody response. The cell-mediated response is carried out by T cells, while the humoral response is controlled by activated B cells and secreted antibodies. Upon antigen activation and with help from T cells, B cells will differentiate to plasma cells that produce huge amounts of antibodies of the same specificity that caused the activation. T cells can be divided into three major groups, cytotoxic, helper and suppressor T cells. Cytotoxic T cells kill virus-infected host cells, helper T cells help activate both the humoral and cell-mediated immune responses, and suppressor T cells deactivate other T cells and B cells in order to control the intensity of the immune response. The T-and B cell response work together to fight the infection and constitute the immunological memory (Gair and Molnar, 2015a).

1.1.2 Humoral immunity

The humoral immune response is thus mediated by B cells and antibody producing plasma cells with assistance from helper T cells (Janeway *et al.*, 2001). Immunoglobulins or antibodies are glycoproteins which constitute about 20% of the protein in the blood plasma. There are five types of immunoglobulins produced in humans, IgM, IgG, IgA, IgE and IgD, each with their own molecular structure, location, and main function (Angel *et al.*, 2022). Antibodies defend the body against extracellular pathogens. Antibodies have the ability to directly bind to viruses

, bacteria and microbial toxins, thus preventing host cells from viral entry and potential damage (Bruce *et al.*, 2014). Antibodies contribute to immunity in three different ways, neutralization, opsonization and complement activation (**Figure 1.1**). A virus or a bacterium has to bind to specific molecules on the surface of target cells in order to enter the cells. Neutralization is the process when an antibody binds to specific pathogens, thus preventing it from entering cells. In other word, the antibodies neutralize the pathogen. This same principle applies for when a toxin is prevented from entering the cells. During opsonization, binding of antibodies to a specific pathogen promotes the process of phagocytosis, the uptake and digestion of a pathogen by phagocytic cells. When a pathogen becomes coated with antibodies, the complex is recognized by antibody receptors of phagocytic cells. These cells bind to the antibodies coating the pathogen, thus promoting phagocytosis. Alternatively, the process of antibody binding to the pathogen surface can activate proteins of the complement system. This results in the binding of proteins to the surface of the pathogen, thus enhancing opsonization and bacterial lysis. The type of effectors mechanism that is generate depends on the antibody class (Janeway *et al.*, 2001).

To produce antibodies, B cells must first be activated. When an antigen binds to a B-cell antigen receptor (BCR), it is internalized and processed into peptides which activate armed helper T cells. Signals from both the T cell and the bound antigen induce the B cell to proliferate and differentiate into antibody producing plasma cells (Janeway *et al.*, 2001) (**Figure 1.1**). The process of B cell proliferation, differentiation and antibody production provides an explanation for immunological memory, a prolonged or even lifelong immunity to a certain pathogen as a result of initial exposure through either infection or vaccine induced response. Due to vaccination, many people are able to acquire immunological memory without serious disease (Bruce *et al.*, 2014).

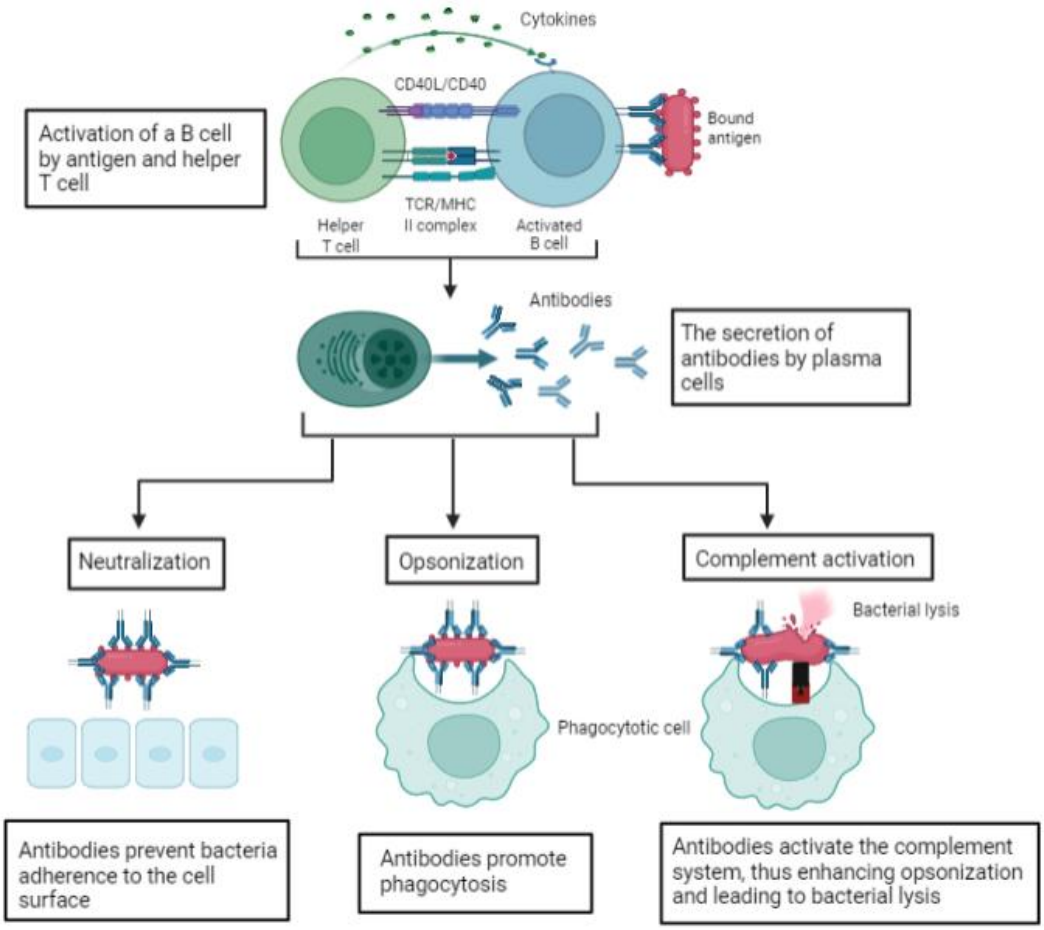


Figure 1.1 Overview of B- cell activation and the three mechanisms of antibody defence (neutralization, opsonization, complement activation) involved in the humoral immune response*. B-cell activation is induced when an antigen binds to a B-cell antigen receptor, which leads to internalization and peptide processing. The antigenic peptide activates a helper T -cell, which together with signals from the bound antigen induces the B-cell to proliferate and becomes an antibody secreting plasma cell. These antibodies are specific and are able to induce protection in three ways; neutralization, opsonization and complement activation. The illustration is inspired by (Janeway et al., 2001) and created with BioRender.com.*

Antibody response can be divided into two parts, primary and secondary response. The primary immune response refers to the very first encounter with a specific antigen. The majority of antibody-producing plasma cells made in the primary response are short-lived. The secondary response occurs in response to a second or subsequent exposure to the same antigen. B cell memory consist of memory B cells and descendant long-lived antibody-producing plasma cells. When an infection has been terminated by the primary response, elevated numbers of high-affinity pathogen-specific antibodies are present in the blood, tissues, lymph, and mucosal surfaces. The antibodies reside in the bone marrow or in the tissue beneath mucosal surfaces and are maintained at a high level for multiple months after the infection has been terminated. During this period, antibodies will hinder the same pathogen from causing an illness through

protective immunity. When the pathogen is cleared, the antibody levels decrease over the course of a year, until they reach a low, steady-state level, which is maintained for life by a few long-lived plasma cells. During secondary response, memory cells respond more forcefully than the primary response. During the activation of secondary response by the same antigen, memory B cells produce IgG, IgA, or IgE antibodies that are much better at binding the pathogen compared to the antibodies made during primary response (Parham, 2014).

1.1.3 Immunoglobulin G

Immunoglobulin G (IgG) is the most common antibody in the blood, and consists of four different subclasses IgG1, IgG2, IgG3 and IgG4. IgG is mostly synthesized during the secondary antibody response and is the only immunoglobulin able to cross the placenta, providing new-borns with immunological protection against disease (Angel *et al.*, 2022). Additionally, IgG is also the major antibody of the secondary immune response, as well as having the longest half-life amongst the five Ig isotypes (Cruse and Lewis, 2010).

IgG resembles a Y-shaped molecule, composed of four polypeptide chains; two heavy (H) chains and two light (L) chains bound by disulphide bonds (**Figure 1.2**). The same binding principle applies between the heavy chains. Since the two heavy chains and the two light chains are identical, the antibody molecule has two identical antigen-binding sites. The two light chains are termed as lambda (λ) and kappa (κ), and an antibody has either two lambda chains or two kappa chains. The amino acid sequences of heavy and light chains provide antibodies with their important features. Each chain usually consists of a series of similar sequences. These are approximately 110 amino acids long, and each repeat corresponds to a distinct protein domain. The light chain is composed of two domains, while the heavy chain of IgG has four such structures. In addition, immunoglobulin heavy-and light chains are composed of constant (C) and variable (V) regions. The V domains (V_H and V_L) give antibodies their ability to bind specific antigens (Janeway, A *et al.*, 2001). The C domains (C_H and C_L) specify effector functions like binding to Fc receptors and complement activation (Schroeder and Cavacini, 2010). Additionally, an immunoglobulin molecule has two structural fragments, termed Fab fragment and Fc fragment. Fab stands for “Fragment antigen binding and corresponds to the two identical arms on the Y-shaped molecule, which contain the light chains paired with the V_H and C_H1 heavy chain domains. The Fc fragment stands for “Fragment crystallizable” and corresponds to the paired C_H2 and C_H3 domains. This part of the antibody molecule does not contain antigen-binding activity, but instead interacts with receptors on effector cells and activates molecules of the complement system (Janeway, A *et al.*, 2001). In the middle point of

the Y-shaped molecule, is another structure called the hinge region, which is a flexible stretch of amino acids linking the two heavy chains together. The hinge region is rich in cysteine and proline, and has no distinct resemblance to any other region on the antibody molecule (Adlersberg, 1997).

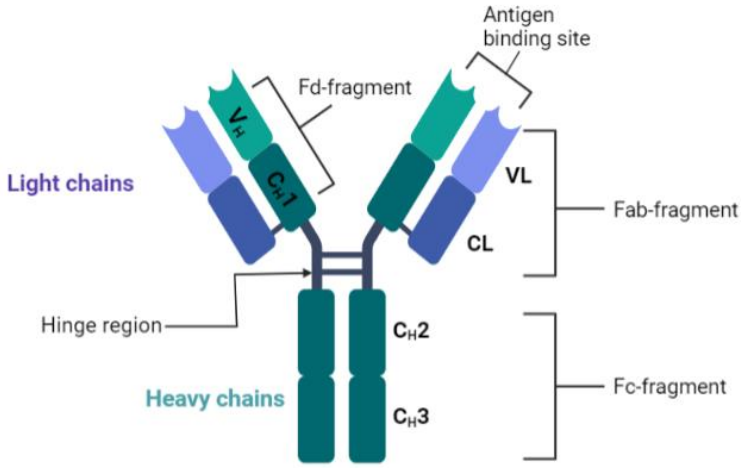


Figure 1.2 The structure of Immunoglobulin G (IgG)*. The molecule consists of two identical light chains (L: Purple) and two heavy chains (H: green) bound together by two disulphide bridges (hinge region). The bottom part of the IgG structure includes the Fc -fragment (fragment crystallizable) and the upper part illustrates the location of the Fab-fragment (antigen binding fragment), Fd-fragment (heavy chain of the Fab), and antigen-binding site. The illustration is inspired by (Dianova, 2003) and created with BioRender.com.*

1.2 Vaccines

1.2.1 General principle

The development of vaccines is considered to be one of the greatest achievements in medicine, and immunization has played an essential role in the protection against infectious diseases. As a result of vaccine introduction, many diseases are on the verge of eradication (Galiza and Heath, 2021). The World Health Organization (WHO) has estimated that about 2-3 million lives each year are saved due to current vaccination programs (Pollard and Bijker, 2021).

In general terms, vaccines take advantage of the immune system by artificially inducing an immune response resulting in immunological memory without the development of serious disease. In order to achieve this, a vaccine usually contains components either derived from the pathogen itself or representing it. The essential component of most vaccines is one or multiple protein antigens, however polysaccharide antigens are also used, especially to prevent bacterial infections. Vaccines can be classified as live or non-live vaccines. Live vaccines usually contain attenuated strains of a virus and are therefore referred to as live-attenuated such as the MMR

vaccine. Non-live vaccines contain components of a virus, or whole dead viruses. However, there are various other immunization methods developed over time such as DNA/RNA vaccines and viral vector vaccines. Live-attenuated vaccines are often produced so that the vaccine content is sufficient enough to generate a strong immune response, but not too strong to cause disease. Non-live vaccines are usually combined with an adjuvant, which helps the vaccine induce a much stronger immune response. Vaccines can also contain other components like emulsifiers, stabilizers, and preservatives. The majority of vaccines induce protection through humoral immune response, meaning that the production of antibodies plays an essential role in immunization. However, most vaccines also generate a T cell response, due to their role in B cell development and antibody production. Other aspects involving T cell protection during vaccination is still poorly described due to their diversity (Pollard and Bijker, 2021).

1.2.2 Live-attenuated vaccines

Live-attenuated vaccines contain a living virus or another type of pathogen, which has been attenuated or weakened in the lab during vaccine development. This is done to reduce the pathogenicity of a virus, and thus prevent the development of serious disease. To accomplish this, the virus is passed through several foreign hosts such as embryonated eggs, tissue cultures, or multiple generations of live animals. The process of serial passage allows the virus to mutate so it doesn't cause disease in humans but is effective enough to generate a sufficient immune response. After the pathogen undergoes serial passage, the virus is administered to the natural host. However, live-attenuated vaccines cannot be administered to patients who have a damaged or weakened immune system. Such vaccines are also considered to be the closest to a natural infection, thus generating a strong immune response with mostly one or two doses. Examples of live-attenuated vaccines include MMR, influenza, yellow fever and cowpox (Yadav, Yadav and Khurana, 2014).

1.2.3 Immunization through vaccination

In order to induce an immune response, a vaccine is injected directly into the muscle tissue, where dendritic cells are activated through adjuvant binding to pattern recognition receptors (PRRs) (Pollard and Bijker, 2021) (**Figure 1.3**). The antigen itself is also taken up by dendritic cells, digested and the fragments are displayed on the cell surface though MHC class II molecules (Yadav, Yadav and Khurana, 2014). After activation, dendritic cells are transported to the draining lymph node. Inside the lymph nodes, the protein antigen is presented to T helper cells through MHC class II molecules and the T helper cells are activated through their T cell

receptor (TCR) (Pollard and Bijker, 2021). There is also a second part of the activation signal, where dendritic cells display co-stimulatory molecules and MHC-antigen complexes on their cell surface. This drives T-cell activation and expansion, and activated T cells secrete molecules with the ability to activate other immune cells (Yadav, Yadav and Khurana, 2014). The T cells generate B cell development with the help of B cell receptor (BCR) through soluble antigen signalling. This process is called, T cell-dependent B cell development, and is responsible for the increase of antibody affinity and the diversity of isotype production. This induces the maturation of the antibody response, which results in the production of short-lived plasma cells and memory B cells. The short-lived plasma cells secrete high levels of antibodies specific for the vaccine antigen. This leads to a significant rise of antibodies in serum. Memory B cells provide immunological memory. A small population of long-lived plasma cells are generated and located to the bone marrow, where they continue antibody production for many years. During immunization, $CD8^+$ effector T cells and $CD8^+$ memory T cells are also generated (Pollard and Bijker, 2021).

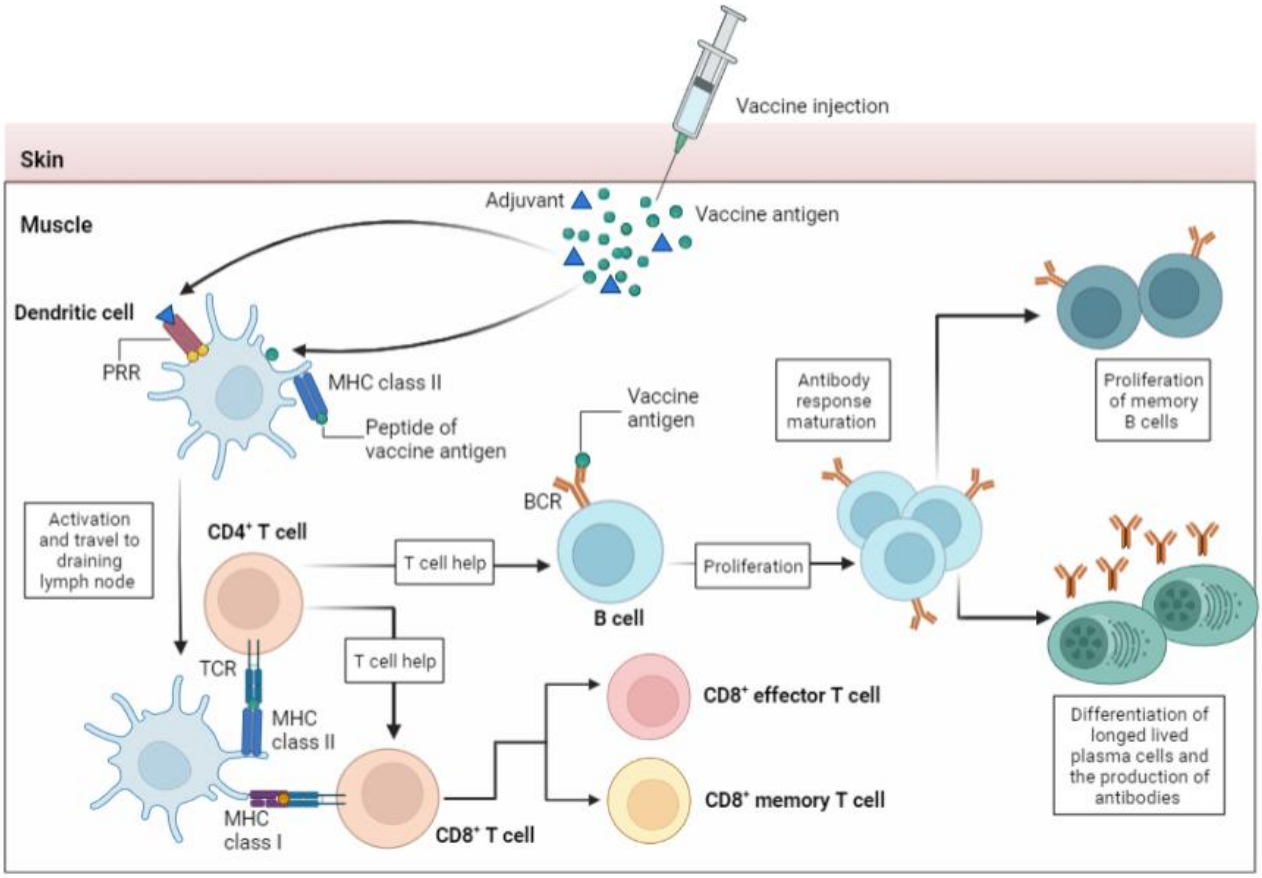


Figure 1.3 Cellular and molecular defence mechanisms involved in vaccine induced immune response. *A vaccine containing an inactivated antigen and adjuvants is directly injected into the muscle, thus leading to the activation of dendritic cells. Through a series of steps, dendritic cells induce T-cell activation and expansion, which in turn leads to T-cell dependent B-cell activation. As a result of this process, maturation of the antibody response occurs, including production of short-lived plasma cells and memory B cells. The illustration is inspired by (Pollard and Bijker, 2021) and created with BioRender.com.*

1.2.4 Childhood immunization programme in Norway

Vaccination has proved to be one of the most effective ways to prevent outbreaks of infectious diseases. This has led to the development of routine-based vaccination programmes in multiple countries. To achieve elimination of a specific pathogen, the vaccination coverage within a population has to be 80-95% (Steens *et al.*, 2020). In Norway, a childhood immunisation programme is offered to all children and adolescents under the age of 20. The programme consists of multiple vaccines against at least twelve different diseases: rotavirus disease, diphtheria, tetanus, whooping cough, poliomyelitis, haemophilus influenzae type b, hepatitis B, pneumococcal disease, human papillomavirus (HPV) and measles, mumps, rubella (Berild *et al.*, 2021) (**Table 1.1**). After each complete vaccination, the status is registered in a central database called the Norwegian Immunisation Register (SYVAK) (Steens *et al.*, 2020). In addition, Norwegian Surveillance System for Communicable Diseases (MSIS) is used a notification tool in the surveillance of infectious diseases amongst people in Norway. This is done through continues collection, analysis and reporting of incidence connected to infectious diseases (NIPH, 2017).

Table 1.1 Overview of The Childhood Immunization Programme in Norway. The information in the table includes age for when each vaccine is administered, disease- and vaccine type *(NIPH, 2021).*

Age	Disease	Vaccine type
6 weeks	Rotavirus	Rotarix
6-8 weeks	**Six valent vaccine:** diphtheria, tetanus, whooping cough, poliomyelitis, haemophilus influenza type B, hepatitis B	Hexyon/Infanrix Hexa
3 months	Rotavirus	Rotarix
	Six valent vaccine: diphtheria, tetanus, whooping cough, poliomyelitis, haemophilus influenza type B, hepatitis B (DTP-IPV-Hib-HepB)	Hexyon/Infanrix Hexa
	Pneumococcal disease (PKV)	Prevenar 13
5 months	**Six valent vaccine:** diphtheria, tetanus, whooping cough, poliomyelitis, haemophilus influenza type B, hepatitis B (DTP-IPV-Hib-HepB)	Hexyon/Infanrix Hexa
	Pneumococcal disease (PKV)	Prevenar 13

12 months	**Six valent vaccine:** diphtheria, tetanus, whooping cough, poliomyelitis, haemophilus influenza type B, hepatitis B (DTP-IPV-Hib-HepB)	Hexyon/Infanrix Hexa
	Pneumococcal disease (PKV)	Prevenar 13
15 months	Measles, mumps, and rubella (MMR)	M-M-RVaxPro/Priorix
2nd grade (7 years)	Diphtheria, tetanus, whooping cough, poliomyelitis (DTP-IPV)*	Tetravac
6th grade (11 years)	Measles, mumps, and rubella (MMR)	M-M-RVaxPro/Priorix
7th grade (12 years)	Human papillomavirus (HPV) - two doses	Cervarix
10th grade (15 years)	Diphtheria, tetanus, whooping cough, poliomyelitis (DTP-IPV)**	Boostrix polio
Children in defined risk groups * **	Tuberculosis (BCG) - one dose	AJVaccines
* High dose combination vaccine for basic vaccination ** Low dose combination vaccine for booster vaccination *** BCG-vaccine is recommended to children with a father or a mother from countries with high occurrence of tuberculosis		

1.3 Measles virus

1.3.1 Virology: molecular and biological features

Measles virus (MeV) is one of the most known infectious pathogens and occurs naturally only in humans. On a structural basis, MeV is spherical and surrounded with an envelope (**Figure 1.4**). The genome is composed of a non-segmented, single-stranded, negative -sense RNA, and the virus is a member of the *Morbillivirus* genus from the *Paramyxoviridae* family (WHO, 2020b). The viral genome encodes at least eight proteins (Maldonado and Shetty, 2018). Two are non-structural and six are structural proteins (WHO, 2020b). The glycoproteins on the surface of the viral envelope, in addition to the hemagglutinin (H) and fusion (F) proteins are the main targets for the development of neutralizing antibodies against the disease. Other proteins include the matrix (M) protein important for virus assembly. The viral structure has also multiple internal and non-structural proteins. Nucleoprotein (NP), large (L) protein and the polymerase phosphoprotein (P) are proteins forming the nucleocapsid, while the non-structural proteins such as C and V regulate the cellular response to infection (Maldonado and Shetty, 2018). Even though RNA viruses have generally high mutations rates, MeV is antigenically monotypic virus. This means that proteins on the surface of the viral structure responsible for

protective immunity, have retained their antigenic structure (Moss, Griffin and Feinstone, 2009).

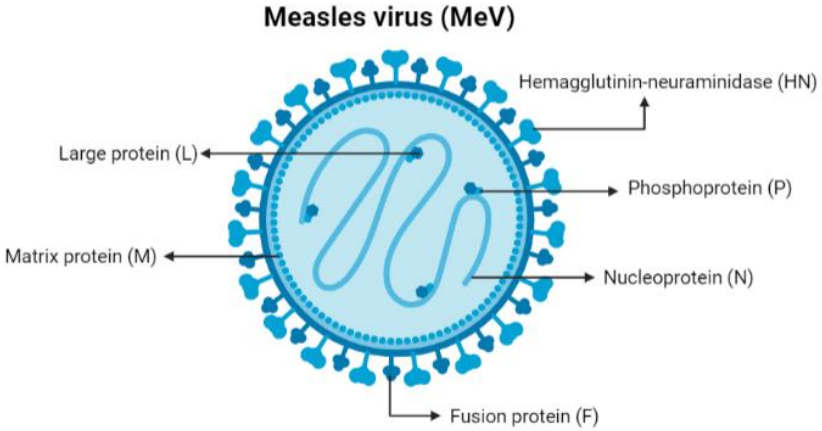


Figure 1.4 The structure and morphology of MeV*. The illustration is inspired by (Aref, Bailey and Fielding, 2016) and created with BioRender.com.*

The life cycle of MeV can be summarized in four stages, entry, dissemination, transmission, and immune suppression. The MeV is transmitted to the respiratory tract through respiratory droplets and aerosols generated through coughing by infected individuals. Upon entry, the virus usually targets myeloid cells located in the respiratory tract. During the second stage, the infection of T- and B cells leads to high levels of viral replication in all lymphoid tissues. This event is characterized as cell-associated viraemia. During transmission, infected lymphocytes transmit the virus to epithelial cells located in the respiratory mucosa. The infected epithelial cells produce new viral particles, which are excreted into the respiratory mucus. The infection of epithelial cells generates a cough reflex, which transmits droplets containing the newly produced viral particles to other hosts. Infection and depletion of T-and B cells causes damage to the immune system and leads to immune suppression. This results in an increased susceptibility to other infections (WHO, 2020b).

1.3.2 Pathogenesis and clinical features of MeV

The disease course can be divided into four phases, the incubation phase (1), the prodromal phase (2), the rash phase (3) and recovery phase (3). The incubation period of MeV usually lasts 8-12 days after the initial exposure and is asymptomatic. The prodromal phase begins upon the onset of the first symptoms such as fever, cough, runny nose, and red eyes (Jenson and Leach, T, 2012). During this period, patients are usually very contagious (WHO, 2020b). It is at the height of the fever, that the rash usually appears, marking the beginning of the rash phase. After about 4-5 days the rash begins to fade, and the recovery phase begins (Jenson and Leach, T, 2012).

1.3.3 Epidemiology

Today, due to vaccination the case fatality and the severity of MeV are usually dependent on the general health status of a population as well as health-care infrastructure. Some groups of individuals are more at risk than others. For instance, children with vitamin A deficiency or individuals suffering from HIV are at a much higher risk of developing severe or even fatal disease. In countries with poor resources, malnutrition and exposure to other infectious diseases, the case fatality ratio often rises to 5%. In contrast, countries with rich resources have a case fatality ratio of 0.01-0.1% (WHO, 2020b).

During the last fifteen years before vaccination was introduced in Norway, 20 000-30 000 cases of measles and 5-10 deaths were reported annually. After the introduction of measles vaccine, the incidence has steadily declined. Today, measles is no longer considered endemic in Norway but can occur as sporadic cases due to people infected abroad. This can sometimes lead to minor outbreaks in local environments with "pockets" of unvaccinated children. Measles can also occur amongst refugees in asylum centres. In 2007 there was one measles outbreak in Norway among children in traveling families from England, where 18 cases were registered in several parts of the country. In 2011, another outbreak occurred in several districts in Oslo amongst unvaccinated children (NIPH, 2010a).

When it comes to the rest of the world, the incidence of measles in some countries are higher compared to Norway. Although the situation in Europe improved after the introduction of vaccine in the 1960s, measles still remains endemic in several countries. For instance, in 2017 about 14 451 cases were reported in the EU/EEA region and 30 of these cases were registered as deaths from which the majority occurred in Romania. Most of the cases in 2017 were reported from Italy, Greece, Romania, and Germany. Several outbreaks in these countries occurred in population groups with low vaccination coverage (NIPH, 2010a). According to WHO, global measles immunization coverage in 2020 among one-year old children was 84%. In Europe the immunization coverage were somewhat higher with 94% for the same age group (WHO, 2021a). According to statistics, measles vaccination coverage in Norway for two-year olds in 2020 was 96.7% (NIPH, 2020a) and the total number of measles outbreaks that same year was 4 (MSIS, 2022a). In Europe, the total number of measles cases in 2020 was 12 205, reported by 37 countries. The majority of these cases (10 717) occurred in countries such as Uzbekistan, Kazakhstan, Russian Federation, Romania, Kyrgyzstan, and Turkey (WHO, 2021c).

1.4 Mumps virus

1.4.1 Virology: molecular and biological features

Mumps virus (MuV) is an enveloped virus, and the genome consists of a non-segmented negative sense RNA, and is a member of the *Paramyxoviridae* family (Rubin *et al.*, 2015), *Rubulavirinae* subfamily, and *Orthorubulavirus* genus. Mumps have only one virus serotype, however 12 genotypes have been described and termed as A-N with the exclusion of the letters E and M. Genotype G has been reported in various countries, including Norway. The structure of the mumps virion is usually spherical (**Figure 1.5**). The genome of the virus includes seven genes encoding nine proteins (WHO, 2020a), and the virion consists of a helical ribonucleocapsid core surrounded by a lipid envelope derived from host cells (Brgles *et al.*, 2016). Structural proteins such as nucleoprotein (NP), phosphoprotein (P) and large (L) protein together with RNA form the ribonucleoprotein complex. The host-cell derived envelope contains a matrix (M) protein, two surface glycoproteins, the haemagglutinin -neuraminidase (HN) and fusion (F, haemolysin) protein, as well as a short hydrophobic (SH) membrane-associated protein. The SH-protein modulates the host immune response and interferes with apoptosis. Non-structural proteins include protein V and protein I (WHO, 2020a).

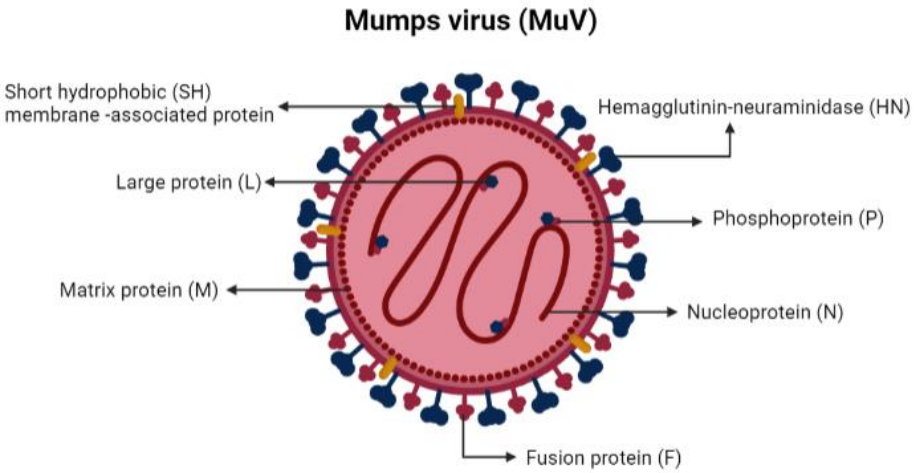


Figure 1.5 The structure and morphology of MuV. *The illustration is inspired by (Expasy, 2009) and created with BioRender.com*

MuV is usually transmitted through the respiratory route by oral contact or inhalation with infected droplets or secretions such as saliva (Rubin *et al.*, 2015). To enter host cells MuV utilizes the flexible glycan receptor, which is a common cell receptor recognized by multiple viruses (Kubota *et al.*, 2019). Following infection of the upper respiratory mucosa, the virus also spreads to regional lymph nodes, which results in viraemia (Rubin *et al.*, 2015). (Expasy, 2009)

1.4.2 Pathogenesis and clinical features of MuV

Mumps is described as an acute viral illness often characterized by swelling of the parotid or other salivary glands. Compared to measles, mumps is less infectious and is transmitted through direct contact with saliva or respiratory droplets from an infected individual. The average incubation period for the virus is 16-18 days but can range from 12-25 days. MuV replicates in the salivary glands, as well as local lymph nodes, with a secondary viraemia occurring late in the incubation period. In most cases, only the salivary glands are affected, however the MuV can also disturb the pancreas, spleen, liver, kidneys, genital organs, and the central nervous system. Clinical features and symptoms of the MuV is often dependent on age and varies from asymptomatic or nonspecific respiratory symptoms to complications. The disease will typically appear as parotitis, or swelling of often salivary glands, which last about 2 days, but there are cases where the symptom has lasted longer than 10 days. Prodromal symptoms are nonspecific including low -grade fever, headache, myalgia, anorexia and malaise (WHO, 2020a).

1.4.3 Epidemiology

Before the development of vaccination programs, MuV was a very serious disease, causing mortality worldwide. Before vaccine development, mumps had a high morbidity of 40 -726 cases per 100 000 population per year, and the virus was circulating the population with a periodic spike of 2-5 years. The majority of incidence was among children aged 5-7 years old in various countries globally. The infections often occurred in crowded population places such as kindergartens, prisons, and boarding schools. As a result of vaccines, the widespread of mumps disease has been significantly reduces, as well as the number of serious complications. The epidemiological patterns of the MuV varies, and is often influenced by vaccine coverage, age for vaccination and the number of vaccine doses. If the vaccine coverage rate is not sufficient high enough., it can cause the disease to shift to older age groups where complications have a much higher rate (Su, Chang and Chen, 2020).

Before the introduction of vaccines, mumps was very common in Norway with outbreaks in schools and military camps. After the vaccine became a part of the childhood immunization program in 1983, the number of cases has dropped. Today, cases of mumps are rare, but small outbreaks can occur. In 2006, there was a local outbreak in Buskerud with 13 reported cases. In recent years, reported cases have increases both nationally and globally. In Norway, a certain increase of mumps incidents has been observed amongst adults. In 2015, there was an outbreak in student communities several places in the country. In total 143 cases were confirmed, and most of the sick students were vaccinated. However, this was due to a special variant of MuV

that is not well covered by the MMR vaccine. The same variant has also led to small outbreaks in the Netherlands and the US (NIPH, 2010b).

In 2017 almost 14 000 cases of mumps were reported in the EU/EEA region. Most cases per 100 000 inhabitants were reported from countries like Czech Republic, Spain, Ireland, and Poland (NIPH, 2010b). According to WHO, the number of reported cases of mumps in 2020 globally was 268 924, from which 11 487 were registered in Europe (WHO, 2021b). The total number of registered mumps cases in Norway during 2020 were 9 (MSIS, 2022b). Mumps vaccination coverage in Norway during 2020 for two- nine- and 16-year-old children were 96.7%, 97.5% and 94.9% respectively. However, the immunization coverage for Europe and the rest of the world during that same year is somewhat undefined (NIPH, 2020b).

1.5 Rubella virus

1.5.1 Virology: molecular and biological features

Rubella virus (RuV) has a spherical structure which ranges from 40-80 nm in size and is a member of the genus *Rubivirus* in the family *Togaviridae.* The virus has an electron-dense core surrounded by a lipoprotein envelope (Parkman, 1996). The viral genome consists of a single-stranded RNA, and encodes three structural proteins E1, E2 and C (**Figure 1.6**). The protein termed as C is an internal nucleocapsid protein. The viral core is surrounded by an envelope consisting of lipoprotein monolayer, which contains the two glycoproteins termed as E1 and E2 (WHO, 2008). The genome of RuV also encodes two non-structural proteins such as p150 and p90. The gene encoding these proteins are translated as a single polyprotein termed as p200, which later undergoes a single cleavage, producing p150 and p90 (Liang, Yao and Gillam, 2000).

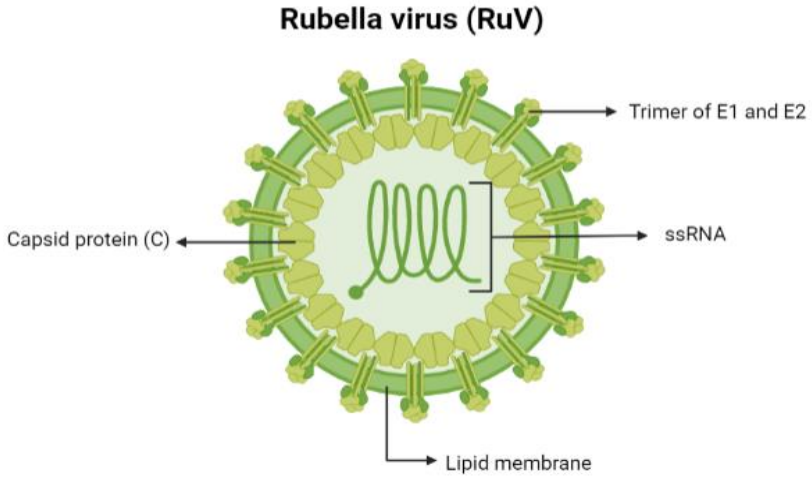


Figure 1.6 The structure and morphology of RuV*. The illustration is inspired by (Racaniello, 2016) and made with BioRender.com.*

RuV usually enters its target cells through clathrin-mediated endocytosis. E1 glycoprotein mediates the fusion of the viral and endosome membranes, and the process occurs in a low pH- and calcium dependent reaction. Replication of the viral RNA occurs in the host cell cytoplasm. The newly produced nucleocapsid core (NC), E1 and E2 assemble and form new virions inside the Golgi complex. The new virions are transported through the secretory pathway and released at the plasma membrane, thus spreading the virus to other cells (Das and Kielian, 2021). (Racaniello, 2016)

1.5.2 Pathogenesis and clinical features of RuV

The disease caused by the RuV is commonly known as German measles, which is a mild self-limited rash illness that in most cases occurs during childhood (WHO, 2008). The virus is transmitted from person to person through respiratory aerosols. Some studies suggest that the infection can also be induced by aerosol presentation to the nasopharyngeal mucosa. The first sites of virus replication is the upper respiratory tract and nasopharyngeal lymphoid tissue (Lee and Bowden, 2000). The average incubation period is 14 days but may range between 12-21 days. During the first week of exposure, there are usually no visible symptoms, however during the second week symptoms like fever, malaise and mild coryza may appear leading to prodromal illness. At the end of the incubation period, a rash appears on the neck and face. Rubella disease is often mild, resulting in a very few complications, however the most important and serious consequence of RuV is congenital rubella infection (CRS). In pregnant women, the period of viremia during a primary rubella infection can infect the placenta and the foetus. The consequences of the infection during pregnancy can be detrimental and includes spontaneous abortion, stillbirth and birth of an CRS infected child (WHO, 2008).

1.5.3 Epidemiology

During the period before vaccines, rubella was an acute viral disease infecting children and young adults all over the world. However, due to the introduction of vaccines, the number of cases has drastically been reduced in numerous countries. In European countries the basic reproductive rate or R_0 for rubella has been estimated to 3-8, while in crowded developing countries the R_0 can be as high as 12. It is also known that the RuV has a much lower R_0 compared to measles, thus making RuV much easier to eradicate (Lambert *et al.*, 2015). Today, limited sporadic outbreaks of rubella continue to occur each year, especially where susceptible individuals come in close contact. The incident of infection displays a seasonal pattern, and

increase in winter, peaks in spring and decreases drastically in summer and fall. Many rubella cases which occur now, are usually reported in unvaccinated young adults (Parkman, 1996).

In Norway, rubella was mainly a childhood disease, and before the introduction of rubella vaccine, appeared in epidemics every 4-5 years. The last major outbreak of RuV occurred in 1978-1979, and the last minor outbreak was connected to an international school in Sogn and Fjordane during 1995-1996. Today, the occurrence of the disease in Norway is very rare compared to both measles and mumps, and diagnosed cases are usually due to infection acquired abroad. The transmission of rubella infection from mother to child is also very rare, where the last case was reported in 2002. Rubella is per today considered to be eliminated in Norway, even though some cases can occur due to travel (NIPH, 2010c).

In Europe as a result of a higher vaccination coverage, the total cases of rubella declined by >99% from 234.9 cases per 1 million population in 2005 to 0.67 cases per 1 million population by 2019. In Europe, during 2005-2019, estimated regional immunization coverage with the first dose of rubella -containing vaccine was 93%-95% (O'Connor *et al.*, 2021). During that same time period no cases of rubella were reported in Norway (MSIS, 2022c). In addition, vaccine coverage for children in 2020 were high with numbers such as 96.7%, 97.8% and 94.9 % for two-nine and 16-year olds in that exact order (NIPH, 2020c).

1.6 Immunological response to immunization

1.6.1 Measles

A measles-containing vaccine (MCV) is able to induce both a cellular and humoral immune response, which are similar to a natural MeV infection. With the help of a live-attenuated vaccine, the immune system is stimulated to generate a response (WHO, 2020b). A live attenuated vaccine contains a weakened version of a living virus, so that the vaccine does not cause serious disease (Plitnick, 2013). As a result of immunization with a MCV, IgG antibodies will usually appear 12-15 days after vaccination, and peak at 21-28 days. In addition, IgM antibodies appear in the blood, and IgA are dominant in mucosal secretions. The production of IgM usually signifies a primary response to MCV. The production of IgG antibodies provides protection against viral attachment to the cell surface. This type of humoral protection mediated by IgG can persist for decades following immunization. Measles vaccination also activates long-lived T cell responses. However, both cellular and humoral responses due to immunization are lower in magnitude compared to a natural MeV infection (WHO, 2020b).

1.6.2 Mumps

Following vaccination against mumps, over 90% of infants and children develop detectable antibodies against the pathogen. However, the level of antibodies produced is somewhat influenced by the type of vaccine combinations. Studies which examined the presence of antibodies following a second dose of the vaccine, found that in most of the children vaccinated with one dose, a secondary immune response was induced due to revaccination. These individuals usually have a detectable IgM response against mumps. According to studies, vaccination with live attenuated mumps vaccine also leads to a lymphocyte proliferation response in most, but not all children with anti-mumps antibodies in their sera. The lymphocyte responses to mumps, as a result of a second dose of MMR, are also higher than measles and rubella responses. However, responses returned to baseline five weeks after the second vaccine dose. Mumps specific lymphocyte responses can also be detected up to 10 – 21 years after vaccination and is equivalent in all age groups. There have been reported cases where mumps specific memory CD4+ T cells have been detected in the bone marrow of healthy adults (WHO, 2020a).

1.6.3 Rubella

All licensed rubella vaccines lead to a good antibody response; however, titres are 1/4 - 1/8 lower compared to natural infection. Studies have shown that after vaccination rubella-specific IgG, IgA, and IgM as well as nasopharyngeal IgG and IgA are detectable. Rubella IgG antibodies usually persists for a long time, with a gradual decline over years. Rubella-specific IgM can be detected between 3 – 8 weeks after immunization, and low levels can still persist up to three years following vaccination. IgA in serum may also persists for at least 7 – 9 years after immunization, and after 6 weeks post rubella vaccine, nasopharyngeal IgA is present in nasal washings. Rubella IgA and IgG has also been detected in urine as a result of vaccination. Generally speaking, for the majority of vaccines, rubella immunity persists for life. Even though, the level of antibodies decreases over time, immunological memory persists, where a secondary response will be activated upon exposure to RuV. When it comes to cell-mediated immunity, studies carried out in the 1970s and 1980s showed that the detection of lymphocytes is difficult, because the cellular response after immunization is lower compared to natural infection (WHO, 2008).

1.7 MMR vaccine and disease statistics in Norway

1.7.1 Background information

The measles -mumps -rubella (MMR) vaccine is a live-attenuated combined vaccine used for the prevention of measles, mumps, and rubella (Bailey and Sapra, 2022). The MMR vaccine gives prolonged protection and was introduced into the developed world in the late 1960s. As a result, the incidence of MMR in these countries were reduced by 98%. (Mak and Saunders, 2006).A combination vaccine includes two or more vaccines to reduce the number of shots while also giving protection against multiple pathogens. The vaccines that could be given individually are combined into one shot such as the MMR vaccine (CDC, 2014). The MMR vaccine contains attenuated strains of measles, mumps and rubella and various forms of the vaccine are available (DeStefano and Shimabukuro, 2019). To date, only the genotype A mumps strains Jeryl Lynn (MMRII (SPMSD)), M-M-RVAXPro (MSD) and RIT 4385 (Priorix (GSK)) has been used in vaccines in Norway (Veneti *et al.*, 2018). The GSK-MMR (Priorix) vaccine contains the Schwarz measles, the RIT 4385 mumps (derived from the Jeryl Lynn mumps strain) and the Wistar RA 27/3 rubella strains (Wellington and Goa, 2003). The first dose of the vaccine is usually offered to all children at the age of 15 months, and the second dose at the age of 11-12 years (Veneti *et al.*, 2018).

1.7.2 MMR vaccine effectiveness and coverage

According to a report published by NIPH in 2021, the effectiveness of the MMR vaccine varies among the three antigens. In regard to MuV the vaccine is expected to induce 90% protection after one dose, but to obtain long-term protection two doses are usually required. However, the MMR vaccine does not provide an individual with a life-long humoral immune protection due to the diversity of MuV subtypes. It has also been reported that the duration of protection against MuV is in general shorter than for MeV and RuV. In contrast, the MMR vaccine provides a 95% protection against MeV after the first dose, and the second vaccine shot is primarily recommended for the immunization of the remaining individuals which for various reasons did not obtain a valid antibody response after the first dose. Similar response strength is expected for RuV, where the vaccine provides over 95% protection as a result of the first dose. Rubella is also less contagious than measles, and therefore has a slightly higher level of protection after the first MMR dose. Consequently, the second dose of the MMR vaccine in the Childhood Immunization Programme is mainly offered to increase protection against measles and mumps (NIPH, 2021b).

The MMR vaccine coverage among the Norwegian population of children is relatively high for all the three antigens according to data from SYSVAK. **Figure 1.7** illustrates MMR vaccine

coverage among two-and nine-year olds during 2002-2017, where both age groups have a high degree of vaccination coverage (two-year olds; 87%-96.8%, nine-year olds; 94%-96.8%). The data also suggest that the number of unvaccinated individuals for the same age-group and time period is low (unvaccinated; 3.2%-13.0%) and therefore a high level of vaccine induced immune protection is expected for the Norwegian population of children (NIPH, 2020d).

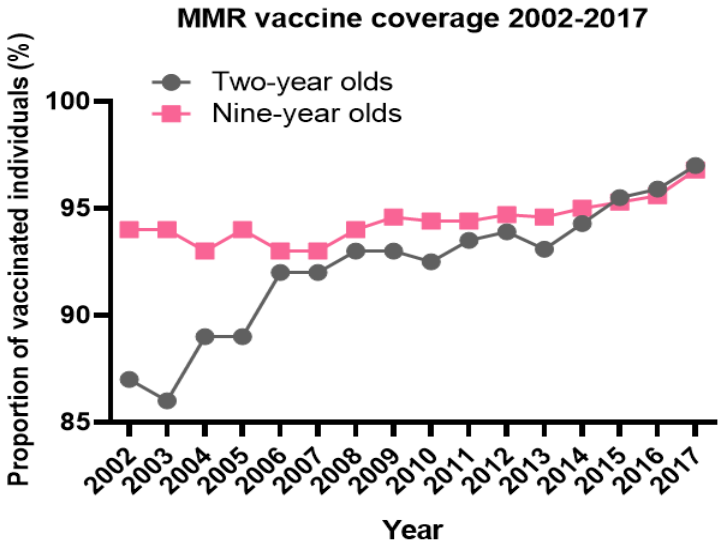


Figure 1.7 Statistics over MMR vaccine coverage in Norway during 2002-2017 for two- (grey) and nine-year olds (pink). *The illustration was created in GraphPad Prism, and statistical data obtained from (NIPH, 2020d).*

1.7.3 MMR disease incidence

After the introduction of immunization, the occurrence of measles, mumps and rubella have drastically been reduced in Norway and globally. However, sporadic cases of the mention infectious diseases have still occurred among the Norwegian population from time to time. The number of cases reported to MSIS each year varies between the three antigens. **Figure 1.8** demonstrates the MMR disease incidence in Norway among children ages 0-19 during 2002-2017. According to data from MSIS, cases of measles and mumps were still occurring within the population of children during the specified time period and age, while the number of rubella cases are non-existent. Consequently, infrequent flourishing of measles and mumps is to be expected among the Norwegian population of children (NIPH, 2022).

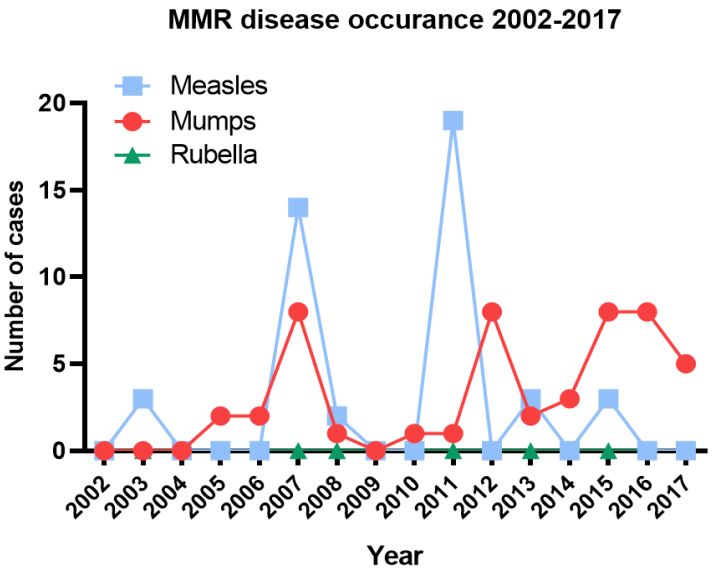


Figure 1.8 Statistical overview of MMR (measles: light blue, mumps: red, rubella: green) disease occurrence among the Norwegian population of children aged 0-19 during 2002-2017. *The illustration was created in GraphPad Prism and statistical data obtained from (NIPH, 2022).*

2 Aim of the study

This master thesis was conducted at the Norwegian institute of public health (NIPH), within the department of Method Development and Analytics. The current thesis is a part of a sub-project called NON-PROTECTED within a larger study cohort, The Norwegian Mother, Father and Child Cohort study (MoBa), which aims to investigate different causes of disease among mothers and their children. As a result of vaccination among the general population, there has been a significant reduction in deaths related to infectious diseases, however a few cases of childhood diseases are still being reported. To prevent sudden outbreaks of serious disease, immune response to vaccines should be monitored to investigate the overall status of protection in a given population as well as identifying susceptible groups or individuals with a reduced or a negative response. The most common way to quantify vaccine response is by measuring antibody levels in plasma or serum for one or several infectious diseases. The major goal of this master's thesis is to investigate the overall status for humoral immune response to MMR vaccine among the Norwegian population of children and perform quality assessment of MMR Multi-Plex Immunoassay.

The main objectives of this master thesis:

I. Investigate the overall status for humoral immune response to MMR vaccine among 306 Norwegian children aged 7-14 years by determining the concentration of IgG antibody levels in plasma against measles, mumps, and rubella.

II. Determine the proportion of individuals with low or reduced IgG antibody protection against measles, mumps, or rubella, and discuss possible predictors for lowered vaccine response based on current research and literature.

III. Investigate the precision and reproducibility of MMR Multi-Plex Immunoassay by performing an intra-assay-and inter-operator assessment of the method.

3 Materials and methods

3.1 Study population

The current study population was composed of 306 children between the age of 7 and 14 years old, and the collection of experimental material was performed by the Norwegian Environmental Biobank (NEB) in the period between 2016 and 2017. The children from who the samples were collected were a part of a larger study cohort, The Norwegian Mother, Father and Child Cohort study (MoBa), which started their patient recruitment in June 1999. Pregnant women as well as fathers of the children were welcome to participate in the study. In 2008 more than 100 000 pregnancies were obtained, where different samples and health-related data were collected since the 17th week of pregnancy, including biological material and questionnaire data (Schreuder and Alsaker, 2014).

NEB is an important research tool for monitoring the exposure and effects of environmental toxicants on the Norwegian public health. Their main purpose is to collect and store blood and urine samples from people living in Norway, where the biological material is frozen and stored over time and made available for future health surveillance and research. During a follow-up in 2016, 9000 mothers, fathers, and children from the MoBa study were invited to submit samples and answer questionnaires (NIPH, 2021a). A modest proportion of the samples (306 individuals) collected from these children have been used for analysis in the current thesis, which is a part of a sub-project called NON-PROTECTED, where the main purpose is study the effects of environmental toxicants (PFAS) on vaccine response, either by direct immunotoxicity or indirectly by affecting the diversity of the gut microbiota. (NIPH, 2018).

To access biological material from NEB, an application (application number: ES596401) was written and submitted by NIPH describing the main objectives and motivation of the NON-PROTECTED project (project number: 275903) in order to get ethical approval for analysing the samples. The application was submitted and approved by The Regional committees for medical and health research ethics (REK) (Torp, Synnøve, 2019).

3.1.1 Sample composition, preparation, and storage

The biological material used for the analysis was composed of plasma, extracted, and prepared from the blood collected of the 306 children. Speaking in general terms, blood contains platelets, plasma, and different cell types such as erythrocytes (red blood cells) and leukocytes

(white blood cells: lymphocytes, granulocytes, and monocytes). Plasma is characterized as the clear, liquid part of whole blood with a light-yellow appearance and contains 91-92% water and 8-9% macromolecules and salts. This includes coagulants such as fibrinogen, proteins, electrolytes, and immunoglobulins. The extraction of plasma from the blood is usually performed with the help of centrifugation (Mathew, Sankar and Varacallo, 2022). Whole-blood is collected in EDTA (anticoagulant) tubes (Rønningen *et al.*, 2006), which are spun down to mechanically separate the tube content in three phases: erythrocytes (bottom layer), leukocytes and platelets (thin middle layer) and plasma (upper layer) (Piao, Park and Jo, 2017). The plasma is usually immediately separated from the rest of the tube contents at the hospital laboratory before the samples are shipped overnight to the Biobank for aliquotation and further processing. The finished sample material is stored at -80°C (Rønningen *et al.*, 2006). The whole process of collecting, preparing, and storing of the plasma samples was performed by NEB, and the finished study material was accessed by NIPH.

3.2 Multi-Plex Immunoassay

3.2.1 General principle

An immunoassay can be described as a biochemical test, which measures the concentration or the presence of a particular macromolecule in a solution such as antibodies. During an immunoassay analysis, the detected macromolecule is often referred to as the analyte. Common formats for immunoassays include capture sandwich assay, competitive (antibody) assay and indirect serological assay. The Multi-Plex Immunoassay in this project focuses on the indirect serological method (Angeloni *et al.*, 2016), which requires a surface-bound antigen, primary antibody, and an enzyme-labelled, anti-species immunoglobulin conjugate (**Figure 3.1**). Instead of a plane surface, Multi-Plex utilizes magnetic beads coated with antigens, proteins, or polysaccharides. The addition of a secondary antibody conjugated to the fluorochrome phycoerythrin (PE), makes it possible to quantify the amount of specific antibody present in a particular sample. The intensity of the fluorescence signal is directly proportional to amount of antibodies present in the sample (Aryal, 2021).

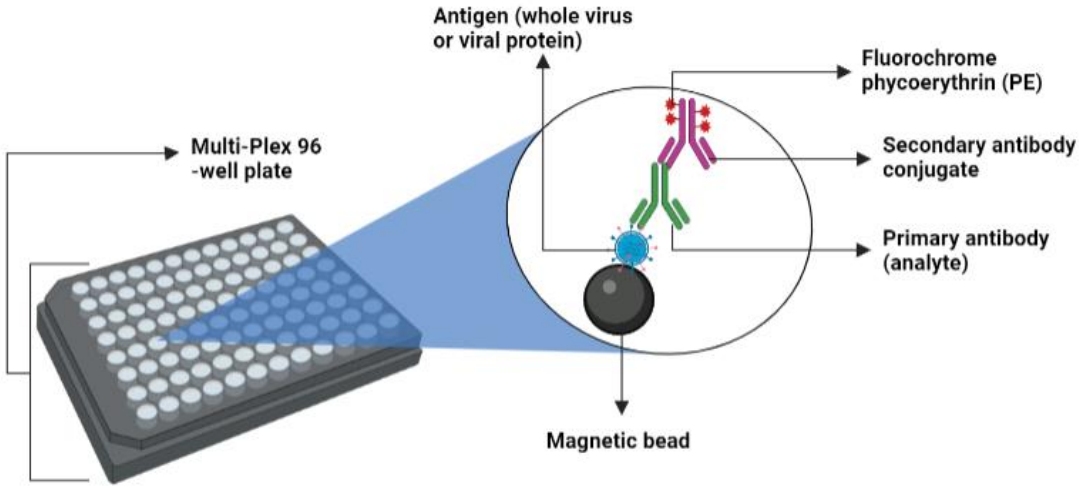


Figure 3.1 Simplified representation of the principle behind Multi-Plex Immunoassay*. The figure illustrates the setup for the indirect serological method used for the detection of specific antibodies (green) within a single sample volume. The method requires an antigen-bound magnetic bead, primary antibody or the analyte of choice (green) and a fluorochrome phycoerythrin (PE) conjugated secondary antibody (pink). These assay components are added to each well on the Multi-Plex 96-well plate in the mentioned order, and the plate is analysed. The illustration is inspired by (Aryal, 2021) and created with BioRender.com.*

The Multi-Plex Immunoassay utilizes xMAP Technology in order to quantify antibody levels in a given sample. xMAP Technology enables a high number of analysis performed within a single sample volume and uses coloured beads to perform biological assays. Protein or nucleic acid labelled microscopic beads are colour coded into multiple spectral colour sets (Angeloni *et al.*, 2016). The colour spectrum is unique for each set of microscopic beads. The beads are normally internally dyed with two different fluorophores, and the ratio between the two dyes define the each bead set (Reslova *et al.*, 2017). One excitation wavelength allows the observation of two distinct emission wavelengths (**Figure 3.2**). This produces sets of 100 unique microscopic beads as a result of a 10x10 dye matrix. Each bead region making up the matrix is assigned a particular number (Angeloni *et al.*, 2016).

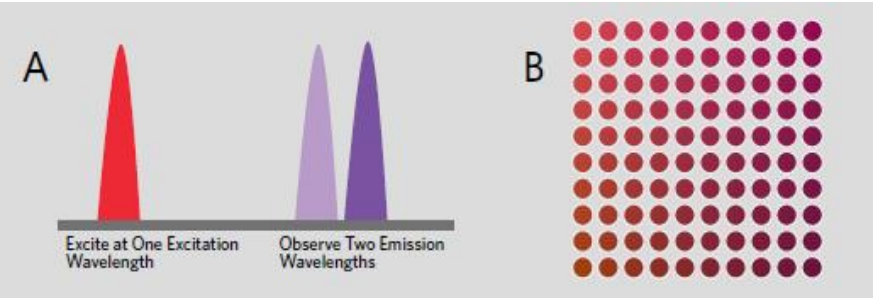


Figure 3.2 Schematic representation of xMAP Technology microscopic bead spectrum (B) as a result of wavelength excitation (one excitation) and emission (two emissions) (A) *(Angeloni et al., 2016).*

The detection of antigen-coupled beads in a solution is performed by two lasers, the red classification laser and the green one. The red laser (635 nm) excites the inner fluorescent dyes, which enables the laser to identify a specific microsphere set. The green laser (525-532 nm) on the other hand, recognizes the fluorescent reporter molecule bound to the analyte of interest on the surface of the magnetic bead (Reslova *et al.*, 2017). The instrument itself is able to detect multiple reading for each set of beads (Angeloni *et al.*, 2016). Advanced instruments such as Luminex 200 (Bio-Rad) is based on the principle of flow cytometry, where the microspheres and their bound analyte of interest are focused into a flowing stream of fluid. Each microscopic bead is illuminated by the lasers and analysed by the detector when the stream of fluid passes through the imaging cuvette (**Figure 3.3**). The Luminex 200 instrument reads a 96-well-plate, where, beads, samples and conjugate are added to the wells for analysis (Reslova *et al.*, 2017).

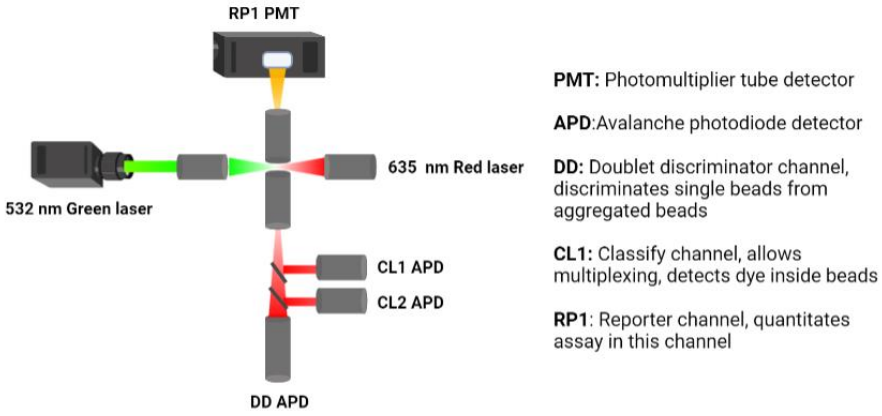


Figure 3.3 The design and set-up of the Multi-Plex array reader. *The reader (with four detectors) combines two lasers, the red "classify" laser (635 nm) and the green "reporter" laser (532 nm) with real-time digital signal processing for the detection of antigen-coupled beads. The instrument is based on the principle of flow cytometry, where the beads are sent into a stream of sheath fluid, and into the flow cell. Here each bead is individually analysed based on their colour and the strength of the fluorescence signal reported by the fluorochrome phycoerythrin (PE). The intensity of the detected signal is proportional to the concentration of analyte (primary antibody) present in sample. The lasers are focused to excite individual beads, and upon excitation the fluorescence signal travels through optical paths to individual detectors (PMT, DD APD, CL1 APD, CL2 APD). This illustration is created with BioRender.com and inspired by (Bio-Rad, 2013).*

3.2.2 MMR method

At the NIPH, a multiplex method for detecting IgG antibodies against MMR antigens has recently been established. The method itself is based on an already published paper by *Smits et al.,2012.* The surface utilized for antigen binding in this case is a magnetic microsphere, and the antigens of choice are MeV, MuV, and RuV. Additionally, the primary antibody or the analyte of interest is IgG and the conjugate of choice used in the procedure was Anti-human IgG R-Phycorythrin extracted from a goat. In order to perform the analysis, the procedure

requires three different magnetic beads (with specific bead regions) coupled to measles, mumps, and rubella through covalent bonding (Smits *et al.*, 2012).

Information about the materials chosen for the development of MMR Multi-Plex Immunoassay are listen in appendix A. The microspheres chosen for this established method were the Bio-Plex Pro™ Magnetic COOH Beads with the following bead regions; MeV (26), MuV (29) and RuV (46). The antigens chosen for the bead coupling procedure were Measles-PIP013 Edmonston strain (UV-inactivated native measles virus) (Bio-Rad) cultured in Vero cells, Mumps MBS318648 Enders strain (UV-inactivated Mumps Virus A) (MyBiosource) and Rubella antigen (virus strain HPV-77) Native Protein (Zinc finger and BTB-domain containing protein 5)(GenWay). In addition, specific controls and a standard was used to check the daily quality of the runs and create a standard dilution for the indication and measurement of IgG concentrations. Altogether five controls were used in the development of the procedure from which measles, mumps and rubella controls were commercial, while the positive and negative controls were made in-house. The standard chosen for the method was RUBI-1-94 which is an international WHO standard for rubella, was specifically calibrated for measles and mumps (Smits *et al.*, 2012).

3.2.3 Protein coupling chemistry

In order to use the microscopic beads for analysis, they must first be coupled to a capture antigen. This is done with the principle of covalently coupling using carbodiimide coupling chemistry. During the coupling process, a covalent bond is formed between the primary amines of the capture molecule (antigen) and the activated carboxyl groups on the surface of the beads (Angeloni *et al.*, 2016) (**Figure 3.4**).

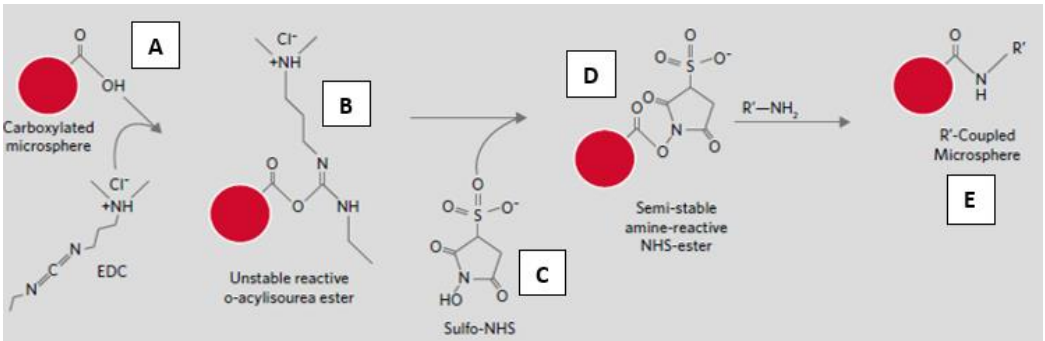


Figure 3.4 The principle for protein coupling chemistry between microscopic beads and capture antigens. *The figure illustrates the coupling process, where a covalent bond is formed between the primary amines of the capture molecule and the activated carboxyl groups on the surface of the beads (Angeloni et al., 2016).*

The carboxyl groups on the surface of the microbeads are first activated with EDAC (A). This is done in the presence of Sulfo-NHS in order to form a sulfo-NHS-ester intermediate (B, C and D). A covalent amide bond is then formed, as a result of a reaction between the intermediate and primary amine on the coupling molecule (E) (Angeloni *et al.*, 2016).

3.3 Laboratory procedure for the coupling of magnetic microspheres with MMR antigens

Information about the materials, instruments, laboratory equipment, software, templates, control values and standard curves used in the following experiments are all listen in **appendix A-F**. The different buffers used in the following procedure were obtained from the Bio-Plex Amine Coupling Kit (Bio-Rad).

3.3.1 Washing and preparing of uncoupled beads

Sulfo -NHS (ThermoFisherScientific) and EDAC (ThermoFisherScientific) were taken out of the freezer and stored with a desiccator at RT for one hour. Additionally, the Amine Coupling Kit (Bio-Rad) was brought from the 4°C refrigerator and kept at RT before use. The XMAP Reagent MagPlex Microspheres (Bio-Rad) were vortexed at medium-high speed for approximately 30 seconds and sonicated in an ultrasonic bath (Grant) for 20 seconds. For each bead coupling reaction, 200µl of the uncoupled beads (2.5×10^6 beads) were transferred to 2 ml Protein LoBind tubes (Eppendorf). The tubes were placed on DynaMag^{TM}-2 magnetic separator (ThermoFisherScientific) and oriented in such way where the bead pellets were facing the magnet. The tubes were left on the separator for 30-60 seconds and the supernatant were carefully removed and discarded from the bead pellet. The remaining bead pellet were washed with 100µl bead wash buffer, vortexed for 30 seconds and sonicated for 20 seconds.

3.3.2 Activation of uncoupled beads

The tubes containing the washed beads were placed on a magnetic separator for 30-60 seconds. The supernatant was carefully removed and discarded from the bead pellet. The beads were resuspended in 80µl of bead activation buffer, vortexed and sonicated for 30 seconds in that exact order. A 50 mg/ml S-NHS solution was prepared in bead activation buffer, followed by a preparation of 50 mg/ml solution of EDAC using the same buffer. Both solutions were vortexed. 10µl of each solution were added to the tubes and gently mixed by vortex. Parafilm and aluminium foil was used to wrap and cover the tubes, followed by a 20-minute incubation

period on a rotator at RT. The beads were placed on a magnetic separator for 30-60 seconds. The supernatant was then carefully removed and discarded from the bead pellet.

3.3.3 Antigen coupling of beads

Activated beads were washed with 200µl of PBS and vortexed at high speed for 10 seconds and placed on a magnetic separator for 30-60 seconds, before the supernatant was removed and discarded from the bead pellet. The beads were once again washed with 200µl of PBS, vortex at high speed for 10 seconds, and placed on a magnetic separator for 30-60 seconds. The supernatant was carefully removed and discarded from the bead pellet. Activated beads were resuspended with 100µl of PBS, vortexed at medium speed for 30 seconds, and sonicated for 20 seconds. The correct amount of measles (Bio-Rad), mumps (MyBioSource), and rubella (GenWay) antigens was added to the tubes, where the total volume was brought up to 500 µl with corresponding coupling buffer (**Table 3.1**). The tubes were mixed by vortex, wrapped, and covered with parafilm and aluminium foil and then incubated on a rotator for 2 hours at RT. Following incubation, the tubes were placed on a magnetic separator for 30-60 seconds. The supernatant was carefully removed and discarded from the bead pellet. The tubes were then removed from the magnet, and the coupled beads were washed with 500µl of PBS, vortexed for 30 seconds and sonicated for 10 seconds.

Table 3.1: The quantity of MMR antigens (µL) and PBS (µl) added to Protein LoBind tubes for the bead-coupling procedure.

Type of antigen added (µl)	Amount of coupling buffer/PBS added (µl)
Measles 4.4µl	395.6µl
Mumps 10µl	390µl
Rubella 6µl	394µl

3.3.4 Blocking of unspecific binding

The samples containing the washed antigen-coupled beads were placed on a magnetic separator for 30-60 seconds. The supernatant was then carefully removed and discarded from the bead pellet before resuspension with 250µl of blocking buffer and vortexed at medium speed for 15 seconds. The tubes were wrapped with parafilm, covered with aluminium foil, and incubated on a rotator for 30 minutes at RT.

3.3.5 Bead storage

Protein LoBind tubes containing the antigen-coupled beads with blocked unspecific binding were placed on a magnetic separator for 30-60 seconds prior to washing. The supernatant was carefully removed and discarded from the bead pellet. The beads were then washed with 500µl of storage buffer and vortexed at medium speed for 20 seconds. The tubes were then placed on a magnetic separator for 30-60 seconds, before the supernatant was carefully removed and discarded from the bead pellet. The beads were thereafter resuspended in 300µl of storage buffer, labelled with coupling date, antigen type, and then wrapped in aluminium foil and stored at 4°C in the dark.

3.3.6 Testing of coupled beads

Prior to the use of newly coupled magnetic beads, their analysis performance was compared with previous beads ("older beads") in an assay to verify the quality of the coupling procedure. This was done in the exact same manner as the Multi-Plex procedure, with the same standards and controls, but no plasma samples. Two assays were set up on the Bio-Plex pro flat bottom 96-wells plate (Bio-Rad) Assay 1 was assigned to old beads, and occupied the first part of the plate, while Assay 2 was assigned to the new beads on the second part of the plate (**Appendix D, Figure D.1)**. Two bead solutions were prepared, one for the old beads and one for the newly coupled beads. Two 15ml tubes were marked and filled with 1455µl PBS each, and for the testing only 15µl of each bead region were added (15µl x3). Otherwise, the dilution of standards and control samples and the rest of the preparation procedure was the same as a regular Multi-Plex run described in more detail in the next paragraph. The device was calibrated, and the plate was analysed using Multi-Assay Protocol in Bio-Plex Manager Software version 6.2 (Bio-Rad). After the run, the IgG antibody concentrations with their respective FI-values for the control samples were registered in separate Excel-sheet, and the obtained concentrations were compared between the two bead-couplings. Additionally, the standard curve for each antigen (**Appendix F, Figure F.1-F.3**) was also quality checked. The testing of newly coupled beads was performed the day after coupling to ensure the reliability and performance of the new beads. Any deviations from assigned control values were evaluated for re-testing and re-coupling.

3.4 Laboratory procedure for MMR Multi-Plex Immunoassay analysis

Information about the materials, instruments, laboratory equipment, software, templates, control values and standard curves used in the following experiments are all listen in **appendix A-F.**

3.4.1 1x PBS buffer composition

12.5ml of 20x Waaler PBS was diluted with 237.5ml of Milli -Q-water using a measuring cylinder. The finished buffer was labelled and stored at 4°C until use.

3.4.2 Preparation of dilution buffer

1.5g of BSA (Sigma Aldrich) was weighed out in a 50 ml tube, and 50 ml of freshly prepared 1x PBS buffer was added to the mixture. The contents of the tube were mixed carefully to avoid formation of bubbles. When all of the BSA was dissolved, and the liquid had a clear appearance, 0.1% 50µl of Tween-20 (Sigma Aldrich) was added to the tube and the mixture was vortexed for a few seconds.

3.4.3 Dilution of the standard, controls, and blood plasma samples

Before the procedure, all required reagents, plasma samples, controls, and standards were stored at RT for 1 hour, and all necessary tubes were labelled with their corresponding content and dilution factor (1:200, 1:1000) and organized in a rack according to MMR Multi-Plex Immunoassay template (**Appendix D, Figure D.2**). Additionally, a second rack with tubes for the controls and plasma samples were labelled similarly, but with a different dilution factor (1:40).

For the serial dilution, 375µl of the freshly prepared dilution buffer was added to the seven last tubes, while the first one was left empty. For the controls, blanks, and plasma samples 400µl of dilution buffer were added to the tubes labelled with 1:200 and 1:1000 dilution factor. Anti-rubella standard RUBI- 1-94 was vortexed for 30 seconds, and 5µl of the standard was pipetted into the first, empty tube followed by the addition of 495µl of dilution buffer. The tube was vortexed for a few seconds, before 125µl of the mixture was pipetted up and down eight times and transferred to the second tube. The same exact procedure was done for the rest of the tubes, creating a 3-fold serial dilution (**Figure 3.5**).

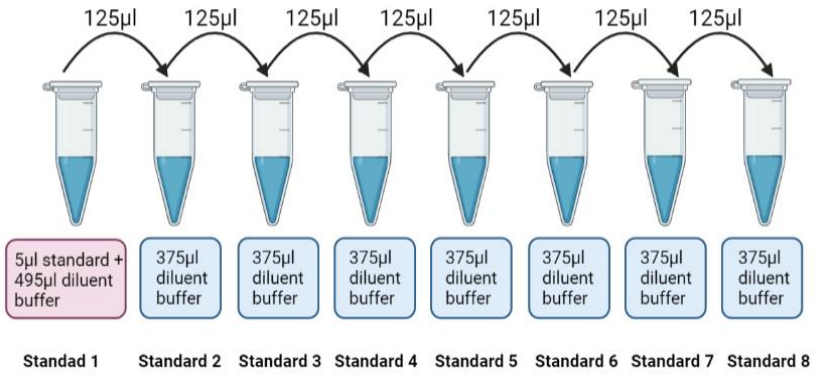


Figure 3.5 Schematic representation of the principle behind 3-fold serial dilution procedure for the standard samples (1-8). *The illustration was created with BioRender.com.*

Internal controls and plasma samples from the study population were prepared with dilution factors 1:40, 1:200 and 1:1000. **Table 3.2** shows an overview of the dilution procedure, including the amount of buffer and sample added to the tubes labelled with their corresponding dilution factors. Tubes labelled with dilution factor 1:40 were prepared first, where each control and plasma tube were vortexed for 30 seconds, before 7.5µl of the content was pipetted into an empty tube, mixed with 293µl of dilution buffer and vortexed for a few seconds. The mixture from the 1:40 tubes were then further diluted into 1:200 and 1:1000 tubes containing 400µl of dilution buffer. 100µl of the diluted sample from the 1:40 tube was pipetted into the 1:200 tube, mixed up and down eight times with a pipette, before another set of 100µl from the 1:200 tube was transferred to the 1:1000 tube. This procedure was performed for each control-and plasma sample.

Tabell 3.2 Overview of the dilution procedure for control-and plasma samples, including dilution factor (1:40, 1:200 and 1:1000), and volume of added buffer (µl) and plasma (µl).

Dilution factor	Volume of dilution buffer (µl)	Volume of plasma/diluted sample (µl)
1:40	293µl	7.5µl plasma
1:200	400µl	100µl from 1:40 dilution
1:1000	400µl	100µl from 1:200 dilution

3.4.4 Mixing of bead solution

2910µl of PBS was pipetted into an empty 15 ml tube and the coupled beads were taken out of the 4°C storage fridge. The tubes were carefully vortexed for 30 seconds, before 30µl from each antigen region (30µl x3) were added to the PBS creating a mixture of beads. The finished 15 ml tube was wrapped in aluminium foil to protect the bead solution from light.

3.4.5 Plate preparation

The tube containing the prepared bead solution was vortexed for a few seconds and added to an empty tray for better pipetting access. The tray was cowered with aluminium foil to protect the beads from the light. The Bio-Plex pro flat bottom 96-wells plate (Bio-Rad) was labelled and fixed the right way, so that well A1 was oriented on the upper left side and 25µl of the bead solution was added to each well. Tubes containing all the samples, standards and controls were vortexed for a few seconds, before 25µl of each tube content were added to the plate according to MMR Bio-Multiplex IgG Immunoassay template. The plate was covered with sealing tape (ThermoFisherScientific) and aluminium foil and incubated on a shaker (Heidolph) for 45

minutes and 850 rpm at RT. After incubation, both the aluminium foil and sealing tape were removed, and the plate was washed in the Bio-Plex Pro Wash Station (Bio-Rad) using Rinse 2:MAGx3 program, where 30 ml was chosen as volume. Anti-human IgG-Phycoerythrin conjugate (Sigma Aldrich) was taken out of the refrigerator and vortexed for a few seconds before 30µl of the solution was added to a tube containing 6 ml of PBS. The tube containing the diluted conjugate was vortexed for a few seconds and poured into a plastic container for optimal pipetting access. 50µl of the diluted conjugate was added to each well on the washed plate using a multichannel pipette. The plate was then covered with a new sealing tape and aluminium foil, and incubated on a plate shaker for 30 minutes and 850 rpm at RT.

3.4.6 Start-up and calibration

After incubation, the Bio-Plex pro flat bottom 96-wells plate was washed once again using Rinse 2:Magx3 program, and 125µl of PBS were added to each well. The plate was covered in sealing tape and aluminium foil and incubated on a plate shaker for 1 minute and 1100 rpm at RT. After incubation, Bio-Plex Manager Software (Bio-Rad) was used to open and activate a new protocol for the corresponding run. The sealing tape and aluminium foil was removed from the plate, which was then placed onto the loading tray on the device. In addition, a Bio-Plex Reservoir (Bio-Rad) was prepared, where the containers for sterile water and bleach (for cleaning) were filled up to the top. The reservoir was then carefully placed beside the plate onto the loading tray and the MMR IgG analysis were initiated through the Bio-Plex Manager Software.

During the 30-minute plate incubation period, the Bio-Plex 200 System LX10021034421 device was calibrated before the initial run. On the computer, Bio-Plex Manager Software 6.2 was opened and the function “Start up and Calibrate” was chosen from the task menu in the upper right corner. The following calibration steps were done according to the procedure on the screen. The Bio-Plex Calibration Kit (Bio-Rad) was taken out of the fridge and the tubes marked with Cal1 and Cal2 were vortexed for 30 seconds, before they were registered in the software. Approximately six drops of both Cal1 and Cal 2 were added to their corresponding wells on the Bio-Plex MCV Plate IV (Bio-Rad). Sterile water and 70% IPA were also added to the plate, where the containers were filled up to the top. The Bio-Plex MCV Plate IV was placed onto the tray and inserted into the device before the calibration was activated. After passed calibration, the calibration plate was taken out from the device, washed with water, and placed on paper towels to dry.

3.4.7 Plate reading

After the plate was run, the generated results were saved and exported as an Excel-file. The loading tray on the device was opened, the plate was thrown into the appropriate waste bin for biological material, and the Bio-Plex Reservoir (Bio-Rad) was washed with water and placed onto paper towels for drying.

3.5 Multi-Plex Immunoassay run validation

3.5.1 Assessment of IgG antibody measurement quality

The IgG antibody concentration results with their corresponding FI-values for controls, standards and plasma samples from each analysis were logged and verified.. A certain range had been set by NIPH for accepted FI- values and IgG antibody concentrations for the control samples (**Appendix E, Figure E.1-E.3).** After logging and checking the quality of the controls, the IgG concentration ratio of the samples were also tested. This was done by dividing the 1:200 concentration by 1:1000 concentration for each sample and checking if the ration between the two values lies within a certain range (0.7-1.3: determined by NIPH) as shown in the formula below:

$$ratio = \frac{1{:}200\ IgG\ concentration}{1{:}1000\ IgG\ concentration} \quad \text{(I)}$$

In theory, both dilution factors are supposed to give similar IgG concentrations, but extremely low or high IgG antibody concentrations usually deviate from this principle and can therefore give a significantly higher/lower ratio between the two dilutions. As a result of the detection of low IgG levels in a particular sample, the 1:200 concentration is selected as the more accurate measurement even though the ratio is outside the range. On the other hand, as a consequence of high IgG antibody levels are measured within a sample, the 1:1000 concentration is selected as the more accurate measurement if it isn't in the linear area of the standard curve for that given antigen. However, this does not apply for samples with moderate levels of IgG concentrations, meaning samples with concentrations which fall into the linear area of the standard curve (datapoint 3 to 7). If there have been no visible errors during the analysis, and the samples had regular IgG concentrations where the FI-values range between datapoint 3 and 7(linear area) on the standard curve, the 1:200 concentration was chosen as the appropriate measurement. During the analysis of the results samples with errors or very high differences in concentration ratios were logged and eventually re-analysed. The same principle was applied

for the control samples. If multiple controls lay outside their set range, a whole run had to be considered for re-analysis.

3.5.2 Procedure for inter-operator assessment

IgG concentrations from every sample were compared between two operators, in order to investigate the reliability and accuracy of the MMR Multiplex Immunoassay. This was done by registering the final results for each antigen in a separate Excel-file, where the IgG concentrations for every sample were compared and the difference between values were quantified as CV% (coefficient of variation). This was done by first calculating the average IgG value (**Formula II**) and standard deviation (**Formula III**) for each sample:

$$mean = \frac{(a_1 + a_2 + a_n)}{n} \quad \text{(II)}$$

$$s_x = \sqrt{\frac{\sum_{i=n}^{n} (x_i - \bar{x})^2}{n-1}} \quad \text{(III)}$$

The values from the standard deviation and mean were used to calculate CV% according to **Formula IV** below:

$$CV\ (\%) = \frac{Standard\ deviation}{mean} \times 100 \quad \text{(IV)}$$

An acceptable range for CV% between the operators was set to 25%. IgG concentrations with CV% values above the 25% limit were evaluated for re -analysis due to possible deviations or errors performed during the lab procedure. The statistical method for evaluation of the MMR Multi-Plex Immunoassay was based on a previously published paper by *Bårnes et.,al 2015.*

3.6 IgG antibody protective levels for measles, mumps, and rubella

Following IgG protective levels were determined for each antigen: measles; >200 mIU/ml, mumps; >500 AU/ml, and rubella; >10 IU/ml, where concentrations below these thresholds were considered as "unprotected". IgG protective levels for rubella were based on an established correlate of protection (WHO, 2008), while protective levels for measles (Argüelles *et al.*, 2006) and mumps (Plans *et al.*, 2015; Won *et al.*, 2021) were determined in accordance with literature and protocols from commercial methods published by others since no correlate of protection has been established for there two antigens. Therefore, IgG protective levels for mumps and measles should not be considered as absolute limits.

3.7 Statistical analysis

Bland-Altman (BA) plots were made using GraphPad Prism software version 8.0 to visualize the agreement between IgG antibody concentrations measured by the different operators. For the BA plot we first calculate the difference between the operators for each individual and the mean of the two operator measurements for each individual. Then the mean is plotted against the difference for all individuals. The upper limit of agreement was set to the mean difference + 1.96 * standard deviation, while the lower limit of agreement was calculated by the mean difference - 1.96 * standard deviation (Bland and Altman, 1986). A systematic bias between the measurements could be easily observed based on the mean differences of the methods. No pattern would indicate absence of systematic bias while a linear relationship would indicate a proportional bias where the operators do not *equally* agree through the range of measurements for example if one method overestimated for higher values while underestimating for lower values. Also it is possible to use the plots to see whether there is a *fixed* bias where one operator constantly estimates higher or lower values than the other (Ludbrook, 1997).

Additionally, Lin's Concordance Correlation Coefficient (CCC) with 95% CI were calculated to statistically quantify the agreement between the same IgG antibody measurements obtained by operator 1 and operator 2. Lin's CCC is a statistical index for how well a measurement, or a test (Y) reproduces a gold standard measurement or a test (X) (Zaiontz, 2022).

Lin's CCC between two variables x and y is defined as:

$$p_c = \frac{2\rho\sigma_x\sigma_y}{(\mu_x-\mu_y)^2+\sigma_x^2+\sigma_y^2} \quad \text{(V)}$$

where *p* represents the correlation coefficient between x and y. Values that are near +1 indicate a high level of concordance between the two measurements (x and y), values near -1 indicate a strong discordance, and values near 0 indicate no concordance at all. There is no clear agreement of how to interpret the values, however it is possible to use the same interpretation as for Pearson's correlation coefficient (less than .20: poor, greater than .80: excellent) (Zaiontz, 2022).

IgG antibody levels measured against measles, mumps, and rubella by operator 1 and operator 2 were plotted in a calculator setup in Microsoft Excel 2016, where values for Lin's r alongside 95% CI were automatically generated by the software. The obtained results were interpreted using the same interpretation as for Pearson's correlation coefficient.

4 Results

4.1 IgG antibody levels and MMR vaccine response

The measles -mumps -rubella (MMR) vaccine is a live-attenuated combined vaccine used for the prevention of measles, mumps, and rubella. According to data from SYSVAK, MMR vaccine coverage for the Norwegian population of children is substantial (2002-20017: two-year olds; 87%-96.8%, nine-year olds; 94%-96.8%), and therefore a high level of vaccine induced immune response is expected to be observed within the study population. However, according to disease incidence reported by MSIS during 2002-2017 (corresponds to the study population) cases of measles and mumps were still occurring within the Norwegian population of children aged 0-19 years. To prevent sudden outbreaks of serious disease, immune response to vaccines should be monitored to investigate the overall status of protection in a given population as well as identifying susceptible groups or individuals with a reduced or a negative response. Additionally, this type of knowledge can also generate indirect information about the rest of the mechanisms involved in the immune system, because the synthesis of antibodies requires the presence of immune cells such as B-and T cells. The IgG antibody is the most important antibody for the protection of infectious diseases. Therefore, one of the major goals of this master's thesis was to investigate IgG antibody concentrations in plasma against measles, mumps, and rubella as an indicator for humoral immune response to the MMR vaccine among the Norwegian population of children.

4.1.1 IgG antibody levels for measles, mumps, and rubella

IgG Bio-Plex Immunoassay was used to measure IgG antibody levels in plasma collected from children (n=306) aged 7-14 years. This was done in order to quantify and examine the status for humoral immune response to the MMR vaccine. IgG antibody concentration in each sample were measured for measles (mIU/ml), mumps (AU/ml), and rubella (IU/ml) and "protective levels" were determined categorizing individual results into two groups; "protected" (measles; >200 mIU/ml, mumps; >500 AU/ml, rubella; >10 IU/ml and "unprotected" (measles; <200 mIU/ml, mumps; <500 AU/ml, rubella; <10 IU/ml). However, the following terms: "protected", "unprotected" and "protective level" have to be used with cation when referred to vaccine effectiveness, because there is no established correlate of protection against mumps and measles so that the "protective level" in this master's thesis was set according to research from relevant articles. The protective IgG levels for rubella are an established correlate of protection

according to WHO (WHO, 2008). The results for IgG antibody concentrations were categorized according to each virus, where each data point represents the concentration values for every subject (**Figure 4.1**).

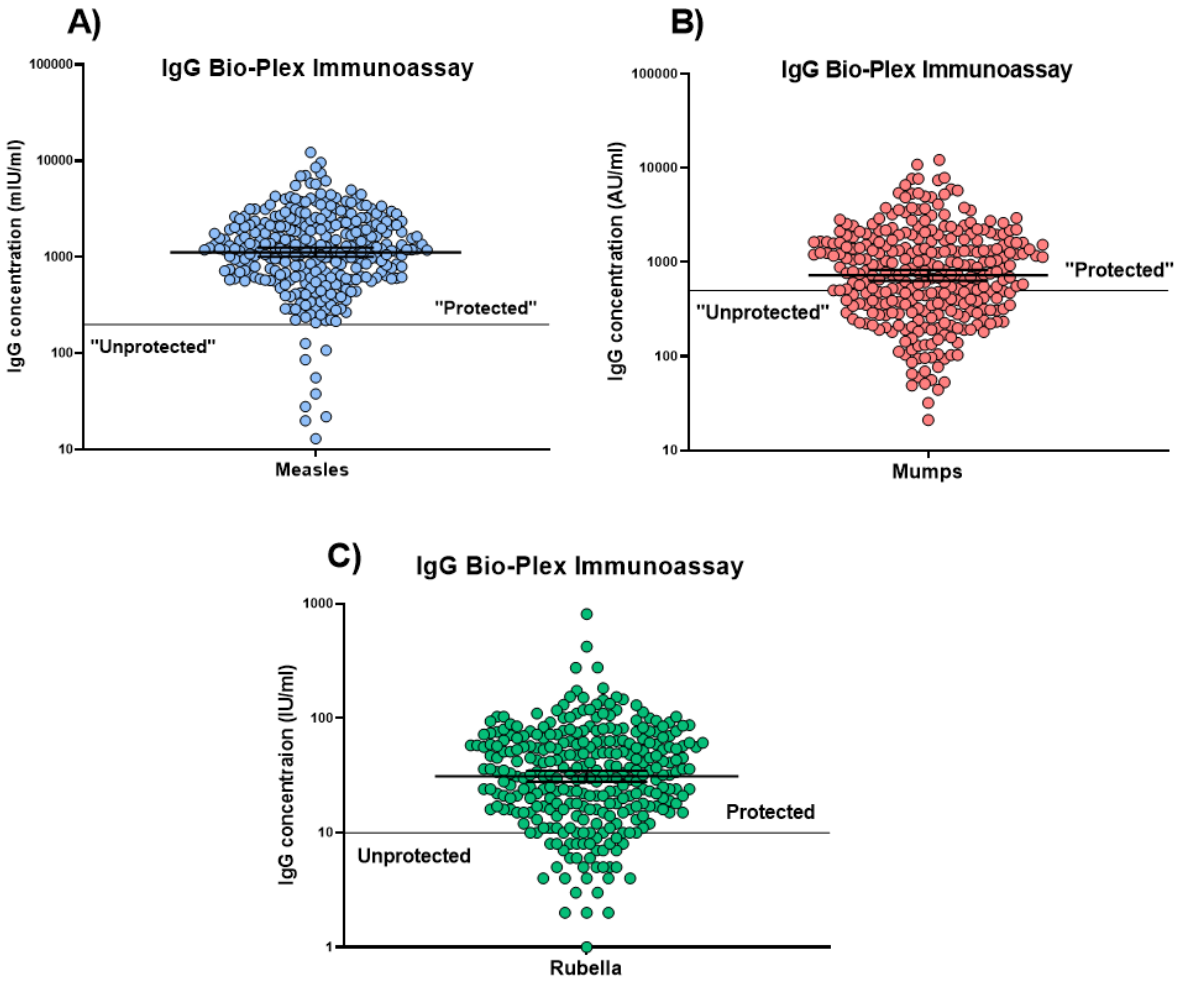


Figure 4.1 The results for IgG antibody concentrations measured with Bio-Plex Immunoassay. *The figure illustrates the distribution of IgG antibody concentrations for measles **(A)**, mumps **(B)** and rubella **(C)** within the following categories: "protected" (measles >200 mIU/ml; mumps >500 AU/ml; rubella >10 IU/ml) and "unprotected" (measles <200 mIU/ml; mumps <500 AU/ml; rubella <10 IU/ml. The lines located on the datapoints represent geometric mean (longer line) and 95% CI (short line).*

According to the results presented in **Figure 4.1**, the majority of IgG antibody concentrations were above "protective level" for all three antigens (measles: 297, mumps: 193, rubella: 273) and a slight proportion of the study population had IgG levels below "protective level" (measles: 8, mumps: 113, rubella: 33). However, the number of samples that fell into the three categories varied for each antigen type. Compared to measles, and rubella, a larger proportion of IgG antibody concentrations for mumps were "unprotected" (**Figure 4.1B**), while measles had the smallest proportion of IgG concentrations below "protective level" (**Figure 4.1A**) among the three antigens. Therefore, these samples were also selected for further investigation

in order to determine whether or not the same individuals were also "unprotected" against mumps and rubella. According to **Table 4.1** seven of the nine subjects isolated for further investigation, who had IgG antibody concentrations below "protective level" for measles were also "unprotected" against mumps and rubella. However, this was not the case for two of the nine subjects, where one individual was "protected" against mumps and rubella, but "unprotected" against measles, and a second individual (sample nr. 1120) was "protected" against rubella and "unprotected" against measles and mumps.

Table 4.1: A comparison of IgG antibody protection status (protected(+)/ unprotected (–)) for mumps and rubella against the "unprotected" measles samples.

IgG protection status ("protected"/"unprotected") *			
	Antigen		
Sample nr.	**Measles**	**Mumps**	**Rubella**
961	–	–	–
1017	–	+	+
1023	–	–	–
1032	–	–	–
1107	–	–	–
1120	–	–	+
1123	–	–	–
1196	–	–	–
1216	–	–	–

*Unprotected (-)

*Protected (+)

4.1.2 IgG antibody protective status

IgG antibody concentrations were sorted into two groups: below "protective level" and above "protective level" for measles, mumps and rubella, and the proportion of individuals falling within each category (%) was calculated and visualised (**Figure 4.2**). This was done in order to investigate the overall IgG antibody protection status for each antigen among the population of children. In addition, the same data were also categorized into four following groups: unprotected (lacks IgG protection against all 3 pathogens), protected against 1 pathogen,

protected against 2 pathogens, and protected against 3 pathogens, where the goal was to assess protective status for all the three antigens combined (**Figure 4.2D**).

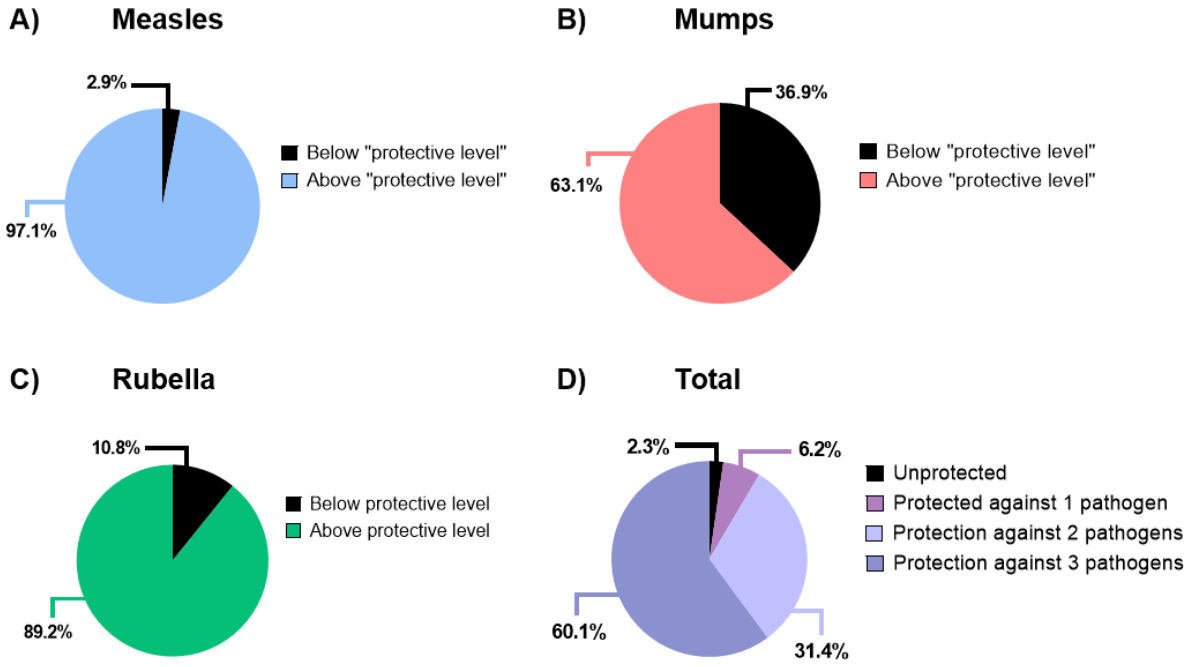


Figure 4.2 Statistical representation of IgG antibody protection by percentage (below "protective level", above "protective level") among the study population (n=306) against measles (A), mumps (B), and rubella (C). *In order to investigate the IgG antibody protective status for all three antigens combined, the results were categorized into four following groups: unprotected (lacks IgG antibody protection against all 3 pathogens) , protected against 1 pathogen, protected against 2 pathogens, and protected against 3 pathogens as shown in* ***D.***

According to the results illustrates in **Figure 4.2** the majority of subjects making up the study population (n=306) have a significantly high level of IgG antibody protection against measles, mumps, and rubella. When investigating each antigen individually, 97.1% of the subjects are "protected" against measles, and 2.9% seem to have a low or a reduced IgG antibody response (**Figure 4.2A**). In comparison, the proportion of IgG antibody "protection" among the population is somewhat lower against rubella and mumps, especially in the case of the latter. According to the results, 63.1% of children have IgG concentrations above "protective level" for mumps, which is the lowest percentage among the three antigens and a 36.9% are below "protective level" (**Figure 4.2B**). In the case of rubella, 89.2% have IgG concentrations above protective level and 5.2% have IgG concentrations below protective level (**Figure 4.2C**).

The IgG antibody protection status was also assessed for all three antigens combined, where the results suggested that 60.1% of the 306 subjects had detectable IgG antibody levels for all 3 pathogens, 31.4% for 2 pathogens, 6.2 % for 1 pathogen (**Figure 4.2D**), while the results for the rest of the study population (2.3%) reported a lack of IgG antibody response. Overall, the

children seem to have a high degree of MMR vaccine induced IgG antibody protection against measles, mumps, and rubella, and only a small proportion of the population have a lowered response.

4.2 Multi-Plex Immunoassay assessment

To investigate the overall quality of the Multi-Plex Immunoassay, the method was assessed based on its intra-assay precision and inter-operator reproducibility. The goal of the intra-assay assessment was to investigate how precise the IgG levels were measured within the same assay. In addition, IgG antibody concentrations for the same study population were measured by two different operators, thus giving the possibility to investigate the agreement for the measured IgG antibody values between the different operators as well as investigate the reproducibility of Multi-Plex Immunoassay.

4.2.1 Intra-assay precision

The plasma samples analysed with the Bio-Plex Immunoassay were run with two dilutions (1:200 and 1:1000) and duplicates on the 96-well plate. Therefore, a CV% (coefficient of variation) for each sample was calculated by the Bio-Plex Manager Software (Bio-Rad) and extracted for further investigation. The CV% values for the 306 duplicates were sorted in an ascending order and a mean for each antigen was calculated (**Table 4.2**). The CV% values within the range were sorted into four groups based on the following definition: CV%<10 (very good), CV%=10-20 (good), CV%=20-30 (acceptable), CV%>30 (not acceptable) (Ebrahimi, 2018) and the proportion of values falling within each group was calculated (**Table 4.2**).

Table 4.1 Intra-assay assessment of Multi-Plex Immunoassay represented by mean CV% values and CV% range of duplicates (n=306) for measles, mumps, and rubella antigens.

Parameter	Measles	Mumps	Rubella
Mean CV% of duplicates*	3.9	3.0	3.5
Range CV% of duplicates*	0.0-17.9	0.0-72.0	0.0-80.9
CV% <10	94.0%	96.7%	95.8%
CV% = 10-20	6.0%	2.8%	3.7%
CV% = 20-30	0.0%	0.2%	0.3%

CV% >30	0.0%	0.3%	0.2%

* n=306 duplicates

In accordance with **Table 4.2** the mean CV% values of duplicates for all the three antigens were <10 (measles: 3.9%, mumps: 3.0% and rubella: 3.5%) as well as all the individual CV% values (measles: 94.0%, mumps: 96.7%, rubella: 95.8%), meaning that the precision of the intra-assay measurement of IgG antibody levels was very good. However, a small percentage of the CV% values were between 20-30 (mumps:0.2%, rubella:0.3%) or above 30 (mumps: 0.3%, rubella: 0.2%). The samples with such high CV% values were termed as outliers, and further investigated considering IgG concentration (low or high) and dilution (1:200 and 1:1000). Two samples had CV% values above 30 for mumps (nr. 1149, dilution 1:200: 42.2 CV% and nr. 1249, dilution 1:200: 72.0 CV%) and one sample had CV% value above 30 for rubella (nr. 1249, dilution 1:200: 80.9 CV%). None of these samples had an unusually high IgG concentration, and the stated CV% values were applied to the 1:200 dilution, thus indicating a possible error occurring during pipetting of duplicates.

4.2.2 Inter-operator reproducibility

The measurements of IgG antibody levels for measles, mumps and rubella were compared between two operators to assess inter-operator reproducibility of the MMR Multi-Plex Immunoassay as this was a recently established method at NIPH. IgG antibody concentration for the same plasma samples (n=306) were measured by operator 1 (laboratory technician) and operator 2 (master student), a correlation plot was created for the IgG concentration values against measles, mumps, and rubella (**Figure 4.3**) and the obtained results were compared using Lin's Concordance Correlation Coefficient (CCC). For the same data, a Bland-Altman plot was created for each antigen to illustrate agreement for the measured IgG concentrations between operator 1 and operator 2 (**Figure 4.3**).

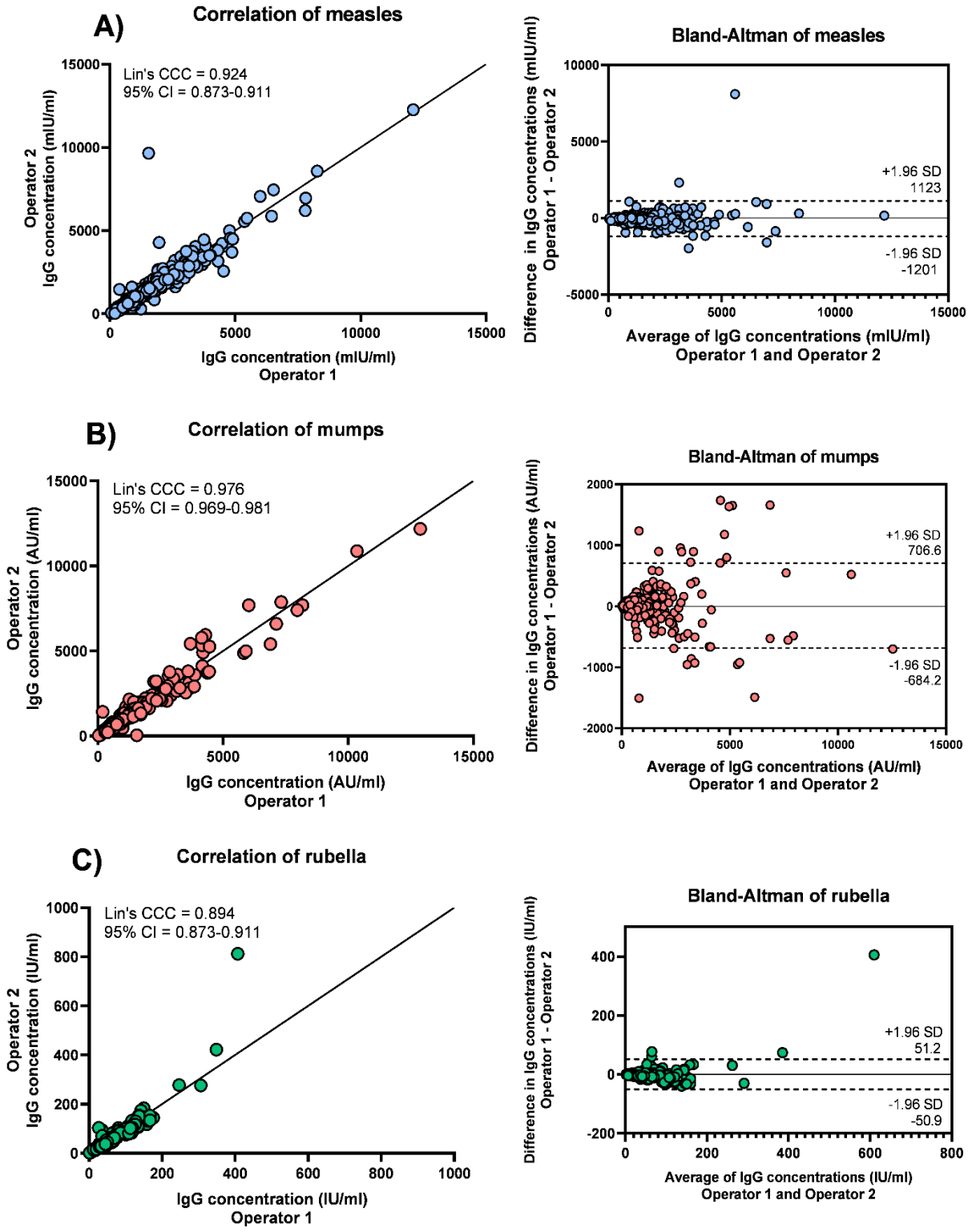


Figure 1.3 ***Inter-operator assessment of the Multi-Plex Immunoassay for the measurement of IgG antibody levels obtained by operator 1 and operator 2****. The figure demonstrates corelation plots (Lin's concordance coefficient and 95% CI) in accordance with Bland-Altman plots describing the relationship between the average (operator 1 and operator 2) and difference (operator 1 – operator 2) for the measurement of IgG antibody values against measles* ***(A),*** *mumps (****B****) and rubella (****C****) including 95% Limits of agreement (±1.96 SD: -1201 to 1123 for measles, -684.2 to 706.6 for mumps and -50.9 to 51.2 for rubella) The angled line in each correlation plot (****A, B*** *and* ***C****) represents the line of identity.*

The correlation plots for measles, mumps and rubella illustrated in **Figure 4.3** demonstrate a positive compliance between operator 1 and operator 2 for the measurement of IgG antibody levels. According to Lin's concordance correlation analysis expressed as Lin's CCC: 0.924, 95% CI=0.905-0.938 (measles); 0.976, 95% CI=0.969-0.981 (mumps); 0.894, 95% CI=0.873-0.911 (rubella), the concordance between the IgG concentration measures is excellent for all the three antigens. This is also further confirmed and illustrated with Bland-Altman plots (**Figure 4.3**), where the agreement between the IgG concentrations is good since the mean difference between the two operators is close to zero for most of the values. This observation applies to all the three antigens, especially measles and rubella, where the agreement is near perfect where only few of the measurements have random fluctuations representing possible outliers. However, for mumps there seem to be lesser agreement between the operators at higher concentrations (>5000) according to Bland-Altman plots. Otherwise, there is no systematic proportional bias.

4.2.3 Inter-operator assessment

After the inter-operator assessment of the whole study population, specific samples were selected for re-analysis due to a high CV% value for the IgG antibody values between operator 1 and operator 2. A CV% value was calculated for each IgG antibody measurement (n=306) and samples with a CV% higher than 25.% were selected for re-analysis (n=54). This was done to investigate the effects of re-analysis on overall measurement precision and reproducibility of the Multi-Plex Immunoassay. From the 54 samples selected for re-analysed, 34 had CV% values higher than 25% for measles, 18 for mumps and 15 for rubella including the overlap among the antigens. The IgG concentrations in the selected samples were measured for a second time, and the results as well as CV% values between the operators were compared with each other pre- and post-re-analysis (**Figure 4.4, 4.5 and 4.6**).

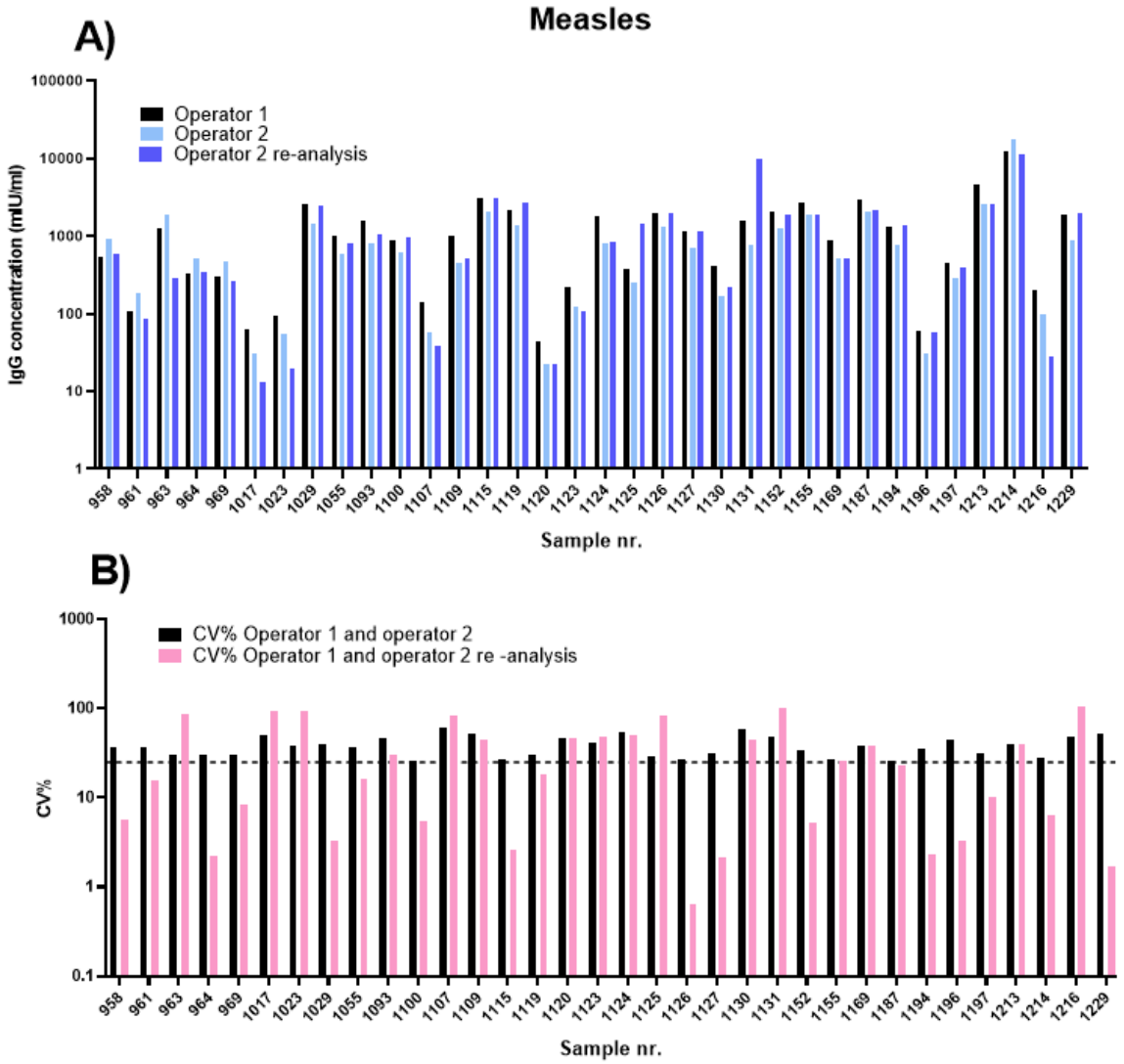


Figure 4.4 Schematic representation of CV% values and IgG antibody concentrations against measles measured by operator 1 (laboratory technician) and operator 2 (master student). A) *Comparison of IgG antibody concentrations (n=34) obtained during pre (light blue)-and post (dark blue) re-analysis.* ***B)*** *CV% values for measles pre (black)- and post (light pink)-re-analysis. The dotted line illustrated in figure* ***B)*** *represents the 25% CV% limit.*

The results showed in **Figure 4.4A** illustrate that the majority of IgG concentrations obtained by operator 2 during re-analysis were closer to IgG values obtained by operator 1 during the initial measurement. The same observation applies for the comparison of CV%, where most CV% values dropped below the 25% limit after re-analysis (**Figure 4.4B**). This indicates an increase in precision as a result of re-analysis, and the number of errors occurring during the laboratory procedure are reduced when problematic samples are analysed for the second time. Additionally, the results illustrate a visible compliance between the difference in IgG antibody levels among the operators and the CV% values for the same samples. The bigger the difference between IgG antibody values, the higher the CV% among the operators. Despite re-analysis

there were a few samples where both IgG levels and CV% values deviated even further from the data obtained by operator 1. In some cases, re-analysis performed by operator 2 lead to a drastic increase or decrease in the measured IgG levels compared to the initial measurement performed by the same operator. These particular findings demonstrate that it is impossible to exclude all possible errors during the laboratory procedure when performing analysis with Multi-Plex Immunoassay, therefore some deviation is supposed to be expected. However, this can also mean possible issues with the plasma samples themselves, and not necessarily the method.

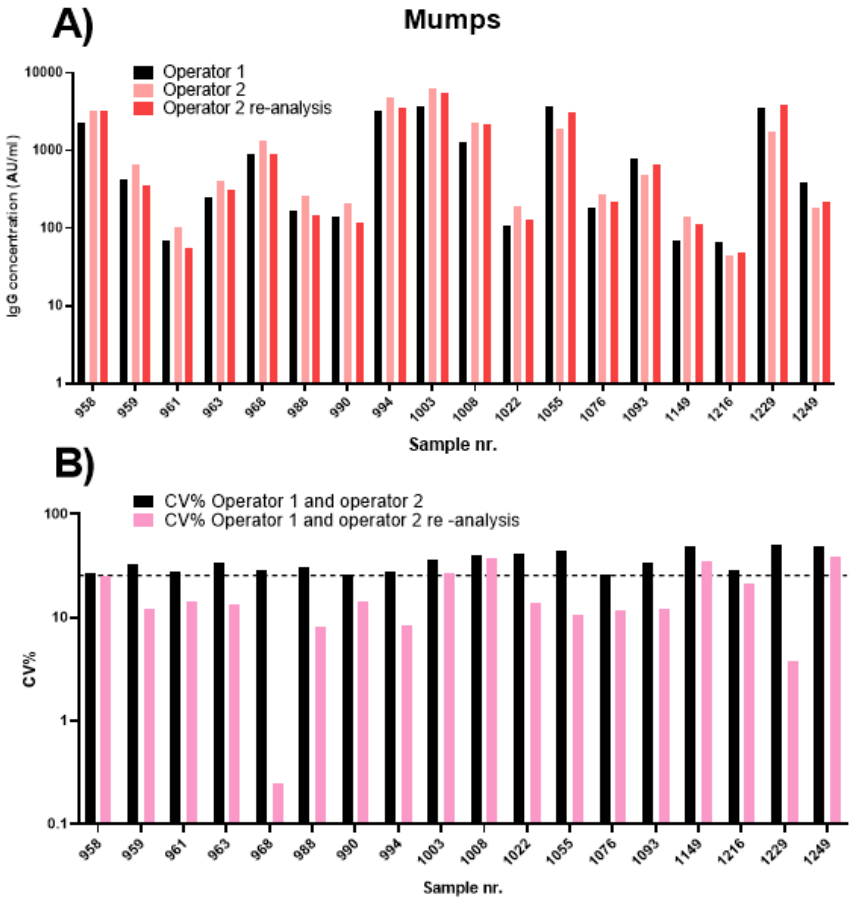


Figure 4.5 Schematic representation of CV% values and IgG antibody concentrations measured with Multi-Plex Immunoassay against mumps between operator 1 (laboratory technician) and operator 2 (master student). ***A)*** *Comparison of IgG antibody concentrations (n=15) obtained during pre (light red)- and post (dark red) – re-analysis.* ***B)*** *Comparison of CV% values for mumps between operator 1(black) and operator 2 pre (black)- and post (light pink) -re-analysis. The dotted line represents the 25.0% CV% limit.*

The same analysis was also performed for the mumps and rubella antigen, where CV% values and IgG antibody concentrations obtained by operator 1 and operator 2 during pre-and post-re-analysis were compared and visualised (mumps: **Figure 4.5**, rubella: **Figure 4.6**). According to

the results (**Figure 4.5A**), the new concentrations for all the samples obtained by operator 2 during re-analysis were closer to the values obtained by operator 1. The same observation applies for the comparison of CV%, where nearly all of the CV% values decreased below the 25.0% limit. The subtle increase or decrease in IgG concentrations during re-analysis brought the concentration values obtained by operator 2 closer to the initial values obtained by operator 1, thus also decreasing the CV% values between operator 1 and operator 2 re-analysis. The same exact observation applies to the results for rubella antigen (**Figure 4.6**) for both the IgG concentrations and CV% values. Overall, the observations obtained during operator 2 re-analysis for all the three antigens, implies that the level of measurement precision as well as reproducibility increases. However, measles seems to have the largest IgG concentration deviation among the two operators as well as the highest number of samples which had to be analysed for the second time due to high CV% values. These findings indicate that the IgG concentration measurements against measles performed by Multi-Plex Immunoassay might be more sensitive to errors during the lab procedure compared to mumps and rubella. However, this can also be influenced by the purity of the antigen coupled to the magnetic beads.

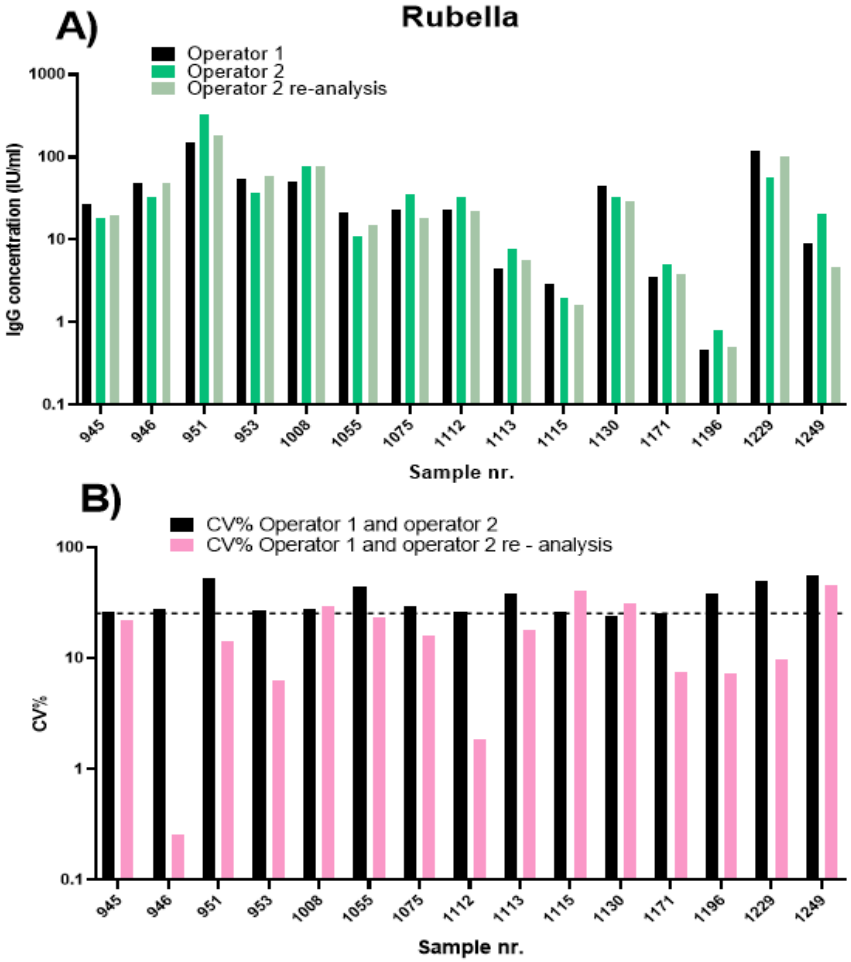


Figure 4.6 Schematic representation of CV% values and IgG antibody concentrations measured with Multi-Plex Immunoassay against rubella between operator 1 (laboratory technician) and operator 2 (master student). A) *Comparison of IgG concentrations (n=18) obtained during pre (light red)- and post (dark red) – re-analysis.* ***B)*** *Comparison of CV% values for mumps between operator 1(black) and operator 2 pre (black)- and post (light pink) -re-analysis. The dotted line represents the 25.0% CV% limit.*

After re-analysis and the evaluations of selected samples (n=54) the results for these specific subjects were replaced with IgG antibody concentrations obtained during the second round of analysis. A final mean of CV% values were calculated between operator 1 and operator 2 for measles, mumps, and rubella (**Table 4.3**). According to **Table 4.3**, the mean of CV% values for all the three antigens (measles: 12.7%, mumps: 11.3%, rubella: 10.2%) were fairly good, thus indicating a minor extent of variability in relation to the mean of IgG concentrations between the two operators is minimal and dispersion among the values is low.

Table 4.3 Final mean of CV% values between operator 1 and operator 2 calculated for IgG antibody concentrations (n=306) measured by Multi-Plex Immunoassay against measles, mumps, and rubella

Mean CV% between operator 1 and operator 2			
Parameter:	**Measles**	**Mumps**	**Rubella**
Mean CV% of samples*			
IgG	12.7%	11.3%	10.2%

*n=306

5 Discussion

The aim of this study was to investigate the levels of IgG antibody concentrations measured against measles, mumps, and rubella in a selection of plasma samples as an indicator for the humoral immune response to the MMR vaccine among children in Norway. The analysis was performed with Multi-Plex Immunoassay, using a recently established method for MMR antibody detection at NIPH based on an already published paper by *Smits et al.,2012*. Therefore, an additional goal of this thesis was to perform method quality assessment by analysing intra-assay precision and inter-operator reproducibility.

The results showed an overall high degree of IgG antibody protection against measles, mumps, and rubella among the Norwegian population of children, thus reporting a substantial MMR vaccine effectiveness. However, a small percentage (2.3%) of the children had no measurable IgG levels against any of the MMR viruses present in plasma, which implies that these individuals are not protected against measles, mumps, and rubella. The obtained results also seem to correspond well with vaccine coverage data reported by SYSVAK and disease occurrence data from MSIS. Furthermore, the IgG antibody protection among the children seems to be lower against mumps when compared to the antibody protection against measles and rubella. Therefore, a more thorough insight into research related to these findings is needed to reveal the cause and prevalence of these observations within the general public. To prevent sudden outbreaks of serious disease, immune response to vaccines should be monitored to investigate the overall status of protection in a given population as well as identifying susceptible groups or individuals with a reduced or a negative response.

According to intra-assay and inter-operator assessment of MMR Multi-Plex Immunoassay, both precision of the intra-assay IgG level measurement as well as reproducibility between the different operators were good. However, according to intra-operator assessment findings, lesser agreement between the operators was observed for mumps, and results also indicates that IgG levels measured against measles might be more sensitive to laboratory errors than for mumps and rubella. To rule out obvious reasons as well as optimizing the method, the obtained data should be reproduced to confirm the findings. This is important to reveal possible limitations and ensure the reliability and the accuracy of the MMR Multi-Plex Immunoassay.

5.1 Discussion of the method

5.1.1 MMR Bio-Plex Immunoassay development

The Multi-Plex Immunoassay is a biochemical test, which measures the concentration or the presence of a particular macromolecule in a solution. The method can be customized for the analyte of interest, thus giving a broad range of possibilities for the analysis of various biological samples. The immunological assay in this project focuses on the indirect serological method which requires a surface-bound antigen, primary antibody, and an enzyme-labelled, anti-species immunoglobulin conjugate to quantify the presence of particular antibody in a given sample. At the NIPH, a multiplex method for detecting IgG antibodies against MMR antigens has recently been established based on an already published paper by *Smits et al., 2012.* The method has a high rate of efficiency due to xMAP Technology which enables a simultaneous measurement of IgG antibody levels against three different antigens within a single assay well. However, several challenges have to be considered regarding method development of MMR Multi-Plex Immunoassay.

One of the first things to consider is the purity of the antigens used for the coupling procedure to the magnetic microspheres. This can influence the measurement accuracy of IgG antibody levels. For instance, the measles and mumps antigens chosen for this MMR method are based on viruses grown in cell culture that have been inactivated by gamma radiation or UV, respectively, instead of purified or recombinant viral proteins. This means that whole, inactivated viral structures were coupled to the magnetic beads instead of purified viral proteins. However, there could be a potential issue with this. Since the vaccine contains whole live-attenuated viruses, vaccinated individuals will be able to produce antibodies against all antigen epitopes. During the coupling procedure of beads to their respective antigens, the virus can become slightly denatured so that less reactive epitopes are available, and therefore fewer IgG molecules are able to bind to the antigen. Consequently, this may make the beads more vulnerable to variations during coupling and could be a problem for samples with higher antibody concentrations because not enough antigen is available for IgG binding.

Another issue to consider is cross-reactivity among the assay components and the different analytes. It is crucial that the antibodies chosen for the assay are very specific to ensure they don’t cross-react with each so that the detected signal only applies to the specific target. Additionally, when designing an in-house Multi-plex Immunoassay, it is also important to use appropriate controls during each run, which can be plasma or serum samples with known

concentrations of the target analyte. The negative (PBS or serum without detectable antibody levels) and positive controls (high concentration) should be run together with the regular samples in order to verify the assay. The measured values against the target analyte for every control should fall within a specific range corresponding to the expected levels for each run. If a particular control deviates from the set range, the quality of the run must be considered, and a possible re-analysis performed. This ensures a robust way to track the quality of the analysis by having comparable results from day- to-day while using the same assay for the analysis of different samples. Therefor the type of controls chosen for assay development is essential in the assessment of daily quality (Ando *et al.*, 2020).

During the development of the assay, two different dilutions were set as the default (1:200, 1:1000) for the preparation of the samples and controls. However, this particular setup can also lead to measurement issues regarding samples with significantly low or high IgG antibody concentrations. In the case of the latter, the 1:200 dilution will give an inaccurate reading due to the inadequate dilution procedure, and the concentration appears to be much higher than the actual value. Therefore, the 1:1000 dilution factor is also applied to solve this issue, where the concentration measured is chosen as the more accurate value for samples containing high antibody concentrations. The same principle applies for samples with extremely low IgG concentrations, where the 1:200 dilution factor gives a more accurate measurement compared to the 1:1000 dilution factor. However, it is necessary to perform an adequate dilution of the sample to eliminate possible interferences from other substances present in that specimen able to disturb the analysis. A possible way to optimize the method even more, is to add one or two more dilution factors in order to minimize measurement inaccuracy regarding significantly low or high IgG concentration samples. Nonetheless, this was not a significant issue for this particular project but may be an important point of consideration for the optimalization of method development in the future.

Other points to mention in regards of method development is its sensitivity to errors and sample quality. Since the laboratory procedure for the MMR Multi-Plex Immunoassay requires several dilution steps, good pipetting skills are required for the obtainment of accurate IgG antibody concentrations measurements. Significant errors during the pipetting of diluted samples, controls or standards can lead to inconsistent concentration measurements. Additionally, the quality of the plasma samples can also influence the results, especially if the samples are incorrectly or inconsistently handled, stored, or prepared before reaching its final destination. For instance, when collecting biological material for research purposes, the blood collection

should be scheduled at the same time of the day to reduce the impact of variations caused by daily fluctuations within the human body, which are often influenced by different external factors. The type of anticoagulants used for the plasma preparation can also influence the measurements of antibody levels (Braunschweig, 2017). Another possible error which can influence the overall quality of antibody analysis is the occurrence of haemolysis. During this process, an accelerated breakdown of erythrocyte (red blood cells) membranes occurs, and the internal contents of erythrocytes are released into the extracellular compartment. Examples of such components are potassium, haemoglobin, neuro-specific enolase (NSE) or lactate dehydrogenase (LDH). If haemolysis has occurred before the separation of plasma, these compounds can disturb the measurement, thus leading to inaccurate results. For instance, binding av antibodies can be inhibited or masked as a result of this process. However, the significance of interference depends on the type of assay, and degree of haemolysis. It is therefore essential to perform proper collection and handling of the sample (Marques-Garcia, 2020).

5.1.2 Limitations in study design

The study population consists of 306 children aged 7-14 years old, and the samples from these individuals were collected in the period 2016-2017 as a follow-up for the MoBa study where the collection of biological sample material was performed by NEB. By now the individuals that participated in this project have an age range of 13-20 years old. Therefore, the analysis and data for IgG protective levels can only be applied to this particular age group (7-14 years old), as well as other relevant information such as MMR vaccine coverage and prevalence of disease in Norway must comply with the year range the samples were initially collected. Additionally, the sample size is not adequate enough to represent the whole Norwegian population of children, but rather serves as a useful indicator for the status of humoral immune response for the specified age range. In regards of relevant information such as gender, vaccine status and specific age for each individual, this knowledge was unavailable to access due to ethical and privacy reasons and therefore excluded from the data analysis at this point. However, this is important to keep in mind when interpreting the results, since vaccination status and age are closely related.

According to the Norwegian childhood immunization programme, the first dose of the MMR vaccine is given at 15 months of age, and the second dose at 11 years of age. This means that children who have not yet received the second dose (under the age of 11) will have lower IgG antibody levels compared to individuals who have received the second shot (ate the age of 11).

The same principle applies for individuals where a long time has passed since they received the vaccine, because its common that antibody levels decrease over time due to natural antibody waning (Wu, 2021). Consequently, information about age and vaccination status are very important in the assessment of MMR vaccine response, thus leading to a more accurate IgG antibody data interpretation. This is a visible limitation in this thesis and must be taken account to in future analysis due to its overall effect on vaccine induced IgG antibody protective status for measles, mumps, and rubella.

Another concern is the determination of IgG protective levels for measles, and mumps since a correlate of protection for these particular pathogens is not established. However, this issue does not apply for the rubella virus since WHO has established a specific value for protection, where IgG antibody levels >10 IU/ml are considered to equip the majority of individuals with a protective antibody response against rubella (WHO, 2008). In contrast, the current IgG protective levels for measles and mumps (measles; >200 mIU/ml, mumps; >500 AU/ml) were determined in accordance with literature and protocols from commercial methods and previously published work by others and therefore should not be considered as absolute limits. IgG protective levels against measles were determined with the help of a paper published by *Argüelles, M.H. et al (2006),* where an IgG concentration >200 mIU/ml were considered as an accepted protective level. This particular value has been conventionally determined by neutralizing antibodies against MeV which are usually measured *in vitro* by a standard neutralization test and plaque reduction neutralization test. (Argüelles *et al.*, 2006). Neutralizing antibodies are able to hinder a pathogen from entering target cells by blocking viral attachment to the cell surface. Neutralizing antibodies are also considered as binding antibodies, but not all binding antibodies have neutralizing capacities (Zoppi, 2021). Consequently, the results obtained by standard neutralization test can deviate from antibody mechanisms *in vivo.* Nonetheless, neutralizing antibodies have an acceptable correlation with protection from the initial infection, thus making them a useful indicator.

Regarding mumps, two scientific papers by *Plan, P. et al. (2015)* and *Won, H. et al. (2021)* were used as guidance for the determination of IgG protective levels. In the first paper IgG protective antibody titres for mumps were set to be >460 EU (ELISA Enzygnost units)/ml, meaning that individuals with IgG concentrations above this level were considered to be protected against MuV (Plans *et al.*, 2015). In the second paper protective levels were set to be >500 expressed as GMT (geometric mean titres) and determined using ELISA Enzygnost anti-parotitis -virus/IgG method (Won *et al.*, 2021). As the MMR multiplex standard RUBI-1-94

(NIBSC) had been calibrated against an anti-mumps quality control reagent (15/B664-xxx, NIBSC) with the concentration 727.2 EU/ml in Enzygnost Anti Mumps IgG, protective IgG level of >500 AU/ml were assumed as appropriate in the case of this master's thesis. It is also necessary to note that the specified value is relatively high, thus effecting the overall result interpretation of IgG antibody protection status against mumps among the study population. It is quite challenging to specifically determine antibody protective levels for given pathogens due to the complexity of the immune system as well as challenges linked to measurement quantification and method development. Moreover, cellular immunity is also contributing to protection.

5.2 Discussion of results

5.2.1 IgG antibody levels and MMR vaccine response

Data analysis and interpretation of IgG antibody concentration measurements in children's plasma demonstrated an overall high degree of IgG protection against measles, mumps, and rubella among the study population, thus indicating a good MMR vaccine effectiveness. The obtained results also correspond well with vaccine coverage data from SYSVAK (**Figure 1.7**) and disease occurrence data from MSIS (**Figure 1.8**), where the age - and year intervals were correlated with the sample collection period (2016-2017) and age (7-14) for the study population. However, it is important to note that the statistics accessed from MSIS and SYSVAK applies to a much bigger population than the number of children investigated, and therefore must not be directly associated with the obtained results, but instead used as a useful tool for comparison.

According to SYSVAK statistics from 2002-2017, a significantly large proportion of the Norwegian children (two-year olds; 87%-96.8%, nine-year olds; 94%-96.8%) are vaccinated against MeV, MuV and RuV. Accordingly, the MMR disease occurrence for the same age-group and year period are substantially low with occasional outbreaks of MeV and MuV. According to the obtained results regarding IgG antibody protection status (**Figure 4.2D**), a small percentage (2.3%) of the children had extremely low IgG levels present in plasma against either virus. When comparing MMR vaccine coverage percentage and disease occurrence with the proportion of unprotected children, it is clear that the majority of the Norwegian population of children are well equipped with an IgG-specific protective humoral response. Nonetheless, a slight deviation can be observed between the percentage of unprotected children obtained through this study (2.3%) and the proportion of unvaccinated individuals in Norway for the same age-group and timespan reported by SYSVAK (unvaccinated: 3.2% - 13%). A possible reason for this observation can be explained by the principle of herd immunity, which occurs when a sufficient proportion of a given population is immune or protected against a particular disease or an infection through natural or vaccine acquired immunity (**Figure 5.1**). As a consequence, not every single individual must be immune to a specific pathogen to prevent large scale outbreaks within a given population. (Ashby and Best, 2021). The number of cases reported each year can appear lower than what the vaccination statistics predict, since the large number of immune individuals prevent the disease from rapidly spreading throughout the population, thus protecting suspectable or unvaccinated individuals. These finding may also be

reinforced by the MMR disease occurrence statistics from MSIS, where the number of reported MMR cases for the same age group and time period are overall considerably low when compared to vaccine coverage **(Figure 1.8).**

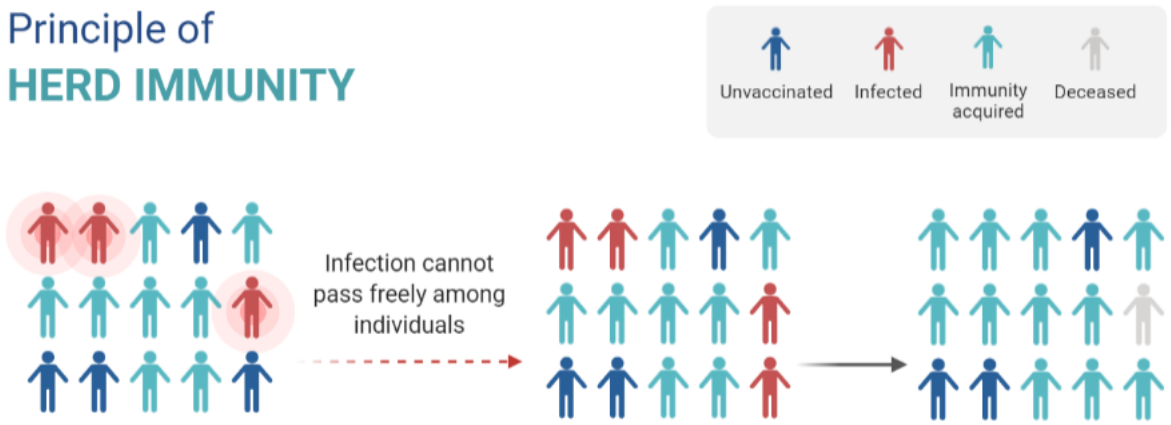


Figure 5.1 The principle of herd immunity. *The figure illustrates how a high vaccination coverage within a population (or naturally acquired immunity), reduce the spread of serious disease. The infection cannot pass freely among individuals due to herd immunity. This illustration is created with BioRender.com.*

As previously mentioned, 2.3% of the children investigated throughout this study were unprotected against all the three antigens, which implies that these individuals are not equipped with a humoral immune response against measles, mumps, and rubella. In order to determine the true cause of a reduced or a non-existent immune response, multiple reasons must be considered since vaccine induced immunity and vaccine status can be influenced by various factors.

One of the most obvious reasons for low IgG antibody levels among the proportion of unprotected children can be explained by their vaccine status, meaning that its possible these individuals did not receive the MMR vaccine due to personal or medical reasons. It has been reported that some communities have a higher scepticism towards the use of vaccines due to a lack of trust in the healthcare system, which can lead to a reduced vaccine coverage and risk of sudden outbreaks. According to a paper published by *Jenness et al.*, (2021) the children of Somali immigrants resident in Norway have a generally low measles vaccine coverage, thus leading to sporadic outbreaks within the community. One of the largest outbreaks of measles in Norway since 1997 occurred in 2011 as a consequence of unvaccinated children within a Somali community in Oslo (Jenness *et al.*, 2021). The outbreak was also reported to MSIS and illustrated with a distinct peak in the number of measles cases in 20011 among children aged 0-19 (NIPH, 2022) (**Figure 1.8**). The MeV outbreak started with an infected case from Ethiopia

and managed to spread to 18 other individuals through unvaccinated Somali children. The virus also spread to unvaccinated Norwegian children through emergency clinics, where five of the individuals were under the age of first-dose MMR vaccine recommendation. The reason for under-vaccination within the community was related to the myth that the MMR vaccine causes autism (Jenness *et al.*, 2021). It is uncertain whether or not the 2.3% of unprotected children are somewhat related to the unvaccinated cases within the Somali community since their status for the participation in the MoBa study is unknown since we did not have access to their epidemiological data t the time of this study. However, persistent vaccine scepticism in distinct communities is an important concern factor, which can affect the rest of the population especially susceptible or vulnerable individuals. To obtain a high level of disease protection, it is essential to reduce misinformation and scepticism related to vaccines and restore the individuals trust in the healthcare system. In addition, we also cannot rule out that a low IgG titre does not necessarily implicate no protection from the disease as there might still be a successful $CD4^+$ and $CD8^+$ response (Chen *et al.*, 2021).

The unprotected children could also be immunocompromised, and therefore unable to receive the MMR vaccine. Individuals who are immunocompromised have a supressed or genetically weakened immune system. This includes people with a genetic mutation or a disease such as HIV which leads to a loss of proper immune function, or individuals taking medications to actively supress their immune system due to a transplant or as treatment for autoimmune diseases (Macmillan, 2022). It is also commonly recommended that live-attenuated vaccines such as the MMR vaccine, should not be administered to individuals with altered or compromised immune systems. There have been observations where particular live vaccines have caused severe complications among immunocompromised individuals. For instance, persons with a HIV infection are at high risk for developing complications if infected with MeV (CDC, 2022).

Immune response to vaccination can also be influenced by other aspects, which in turn can diminish the effectiveness of a vaccine by reducing specific antibody production in certain individuals exposed or susceptible to such stressors. A review published by *Zimmermann and Curtis* (2019) have discussed various causes affecting both humoral and cellular immune response to vaccination, including environmental, behavioural, nutritional, perinatal, and extrinsic-and intrinsic host factors. The extent of influence is also dependent on the type of vaccine administered to an individual as well as the form of immune response. Some factors will affect vaccine induced humoral response more than the cellular response and vice versa.

One example of an intrinsic host factor related to vaccine effectiveness is age for when the actual vaccine is administered to an individual. In this case, the measles vaccine (as part of the MMR vaccine) is the best studied example (Zimmermann and Curtis, 2019).

A meta-analysis of 20 studies was performed and the findings indicated that GMTs (geometric mean titres) were lower in children who received their first MMR dose before the age of 9 months in comparison to children who received the shot later. Moreover, the number of antibodies decreased significantly faster when administrating of the first dose was given to the infant before 9 months. Additionally, antibody avidity also decreased among infants who received the vaccine before 6 months of age compared to individuals vaccinated with the first dose at 9 or 12 months. No age driven effects were observed in regard to vaccine induced cellular response against measles (Zimmermann and Curtis, 2019). The principle of antibody avidity was unfortunately not assessed as a part of this master's project, but it plays an important role when determining the strength of humoral immune response to vaccines. Avidity can be described as the binding strength between an antibody and it's specific target antigen (Bauer, 2021). The production of specific antibodies induced through either a natural infection or a vaccine, involves the process of somatic hypermutation and affinity maturation of B-cells. Antibody avidity can therefore be used as a useful tool to assess functional maturation of the humoral immune response and therefore should also be considered when assessing the effectiveness of vaccine (Alam *et al.*, 2013).

Recently it has been discovered that early-life exposure to different toxicants can lead to a reduced immune response to specific vaccines (Zimmermann and Curtis, 2019). A paper published by *Raqib et al.*, (2017) investigated the effects of prenatal and early-life arsenic exposure to MMR vaccine response in school-aged children in rural Bangladesh. It was discovered that an increased arsenic exposure led to a decrease in mumps-specific IgG antibody levels (Raqib *et al.*, 2017). It has also been reported that prenatal exposure to PCBs (polychlorinated biphenyls) can be associated with decreased antibody levels against measles and mumps in children at pre-school age (Weisglas-Kuperus *et al.*, 2000). Other factors influencing vaccine response discussed in the review paper include sex, genetics, microbiota, vaccine schedule and lifestyle related aspects (Zimmermann and Curtis, 2019). Such factors were not investigated during the course of the master's thesis but can be addressed in future assessment and research related to vaccine effectiveness and immune response in children.

When assessing MMR vaccine induced humoral response for each antigen separately, it was discovered that a larger proportion of children had IgG antibody levels below protective level

against mumps in comparison to measles and rubella. From the total population of children 36.9% had IgG concentrations below "protective level" against mumps, while only 2.9% and 10.8% were below the same threshold for measles and rubella respectively (**Figure 4.2**) These findings are somewhat supported by *The Childhood Immunization Programme: Report for 2019 and 2020* published by NIPH, which clearly states that vaccine induced protection against mumps is expected to be lower in comparison to measles and rubella. Additionally, the duration of antibody protection against MuV is also expected to be shorter, meaning that MuV-specific antibodies decrease more rapidly compared to the other two antigens (NIPH, 2021b). Even though this observation is to be expected, the extensiveness of the number of unprotected children against mumps is significantly higher than what is measured for measles and rubella. Nonetheless, it is possible that this outcome is somewhat influenced by the determination of MuV "protective levels" since the correlate of protection for MuV is not established. If the specified IgG protective levels for MuV are high, a larger number of individuals will appear to have IgG concentrations below "protective level" and thus interpreted as "unprotected". Also, the term "unprotected" in regard to vaccine induced immune response should be used vaguely, considering the fact that protection against disease also involves cellular immunity. The results can also be influenced by children's age, meaning that a significant proportion of children who seem to be unprotected against mumps, may not have received their second dose of the MMR vaccine (under the age of 11) or a long time has passed since receiving the second shot.

Nonetheless, multiple scientific papers have investigated the same phenomenon and also reported a decreased humoral immunity to mumps in children and adolescents after immunization with the MMR vaccine. An assessment of MMR vaccine efficacy in young Kuwaitis was done by Madi et al., (2020). IgG antibody titres were measured with a commercial immune-assay against measles, mumps, and rubella in 1000 serum samples collected from children aged 5-20 years. Among the population of children, the highest seropositivity was measured towards measles (94.6%), whereas mumps had the highest seronegativity (29%). In addition, 47% of the 1000 individuals were seropositive against all the three pathogens, while only 2% had no detectable IgG antibody protection to measles, mumps or rubella (Madi *et al*., 2020).

This particular case also demonstrates a significantly high seronegativity towards mumps among young children and adolescents thus reinforcing the results observed during the course of the thesis. It is possible that these findings indicate a need for a possible third dose of the MMR vaccine or a mumps-booster to replenish the decline of mumps-specific antibodies in a

given population. Nonetheless, various factors must be considered to determine this decision and the level of seriousness appears to be minimal in populations with high MMR vaccine coverage and low occurrence of MuV. The idea of a third MMR vaccine dose is possibly most relevant for older individuals, so that the protection is restored in case another outbreak due to imported cases appear. It is also essential to acknowledge that a decrease in mumps-specific antibody levels does not necessarily correlate to an infective vaccine due to protection delivered by cellular immunity and other factors involved. For instance, vaccine effectiveness cannot directly be associate with the level of specific antibodies produced by the immune system. A vaccinated individual can still be at risk for infection, however the level of disease seriousness is much lower in comparison to unvaccinated individuals. Therefore, immunization is one of the most effective ways to prevent the development of serious illness and complications associated with it (Oran and Topol, 2021).

The results illustrated in **Figure 4.2D** also report that a moderate proportion of the studied subjects have a partial IgG antibody protection against the MMR viruses, where 31.4% of the children were protected against 2 antigens and 6.2% were protected against 1 antigen. There is a possibility that the number of individuals protected against only one antigen were initially unvaccinated but had acquired immunity through natural infection. Additionally, the majority of 31.4% individuals protected against 2 antigens, are most likely unprotected against mumps since MuV had the highest proportion of subjects below "protective level" in comparison to MeV and RuV.

Since the immune system is a complex network consisting of many cells and functions, it is fundamental to acknowledge that humoral immune response alone does not represent the extensiveness of the whole immune system. Cellular immunity is as important as humoral response during immunization but is much less convenient and more labour demanding to perform compared to the assessment of antibody levels. Therefore, evaluation of humoral immune response to vaccines should be used as an indicator for the status of protection, and not a direct correlate. Ideally, one should analyse both the cellular-and humoral response simultaneously when determining vaccine efficacy within a given population.

5.2.2 MMR Multi-Plex intra-assay precision assessment

According to the evaluation of MMR Multi-Plex intra-assay precision, the results (**Table 4.2**) showed low CV% values (CV%<10) between the duplicates (n=306) for measles, mumps, and rubella for almost all of the analysed samples (measles: 94.0%, mumps: 96.7%, rubella: 95.8%).

Additionally, the mean of all the CV% values was also very good for all the three antigens (measles 3.9%, mumps 3.0% and rubella 3.5%). This means that the measurements performed within the assay had a high degree of precision. However, a small percentage of the CV% were between 20-30% (mumps:0.2%, rubella:0.3%) or above 30 (mumps: 0.3%, rubella: 0.2%), thus indicating a low source of errors. These samples were further investigated, and none had unusually high IgG concentrations. For this reason, the deviations in CV% values were most certainly caused by an insufficient pipetting technique between the duplicates performed during the laboratory procedure.

5.2.3 MMR Multi-plex inter-operator reproducibility assessment

IgG antibody concentrations for the same study population (n=306) were measured by two different operators, thus giving the possibility to investigate the agreement for the measured IgG antibody levels between operator 1 and operator 2. The results for agreement between the two operators were quantified and illustrated with correlation plots, Lin's CCC and Bland - Altman plots (**Figure 4.3**). According to correlation plots and Lin's CCC values and 95% CI (measles: 0.924, 95% CI=0.905-0.938, mumps: 0.976, 95% CI=0.969-0.981, rubella: 0.894, 95% CI=0.873-0.911) the concordance between the IgG concentration values for all the three antigens measured by operator 1 and operator 2 is very good. Bland-Altman plots also illustrate a good agreement between the two operators since the mean difference for IgG antibody measurements is near zero for the majority of concentrations measurement against measles, mumps, and rubella. However, a higher degree of dispersion around the mean is observed for IgG values measured against the mumps antigen, especially at higher concentrations (>~5000 AU/ml), thus indicating a lesser agreement between the operators. To solve this particular issue, it could be useful to add another dilution (besides 1:200 and 1:1000) for the samples with higher IgG concentrations against mumps during the laboratory procedure to see if the agreement was improved.

To pinpoint the exact explanation for this deviation is challenging, due to the fact that many factors have the ability to influence the agreement between operators. There is a possibility that the reduced agreement at higher concentrations measured against MuV is more sensitive in regard to the pureness of the antigen (inactivated virus vs. purified or recombinant viral protein) coupled to the magnetic beads (**Appendix A**). The type of mumps antigen chosen for the coupling procedure was an UV-inactivated MuV A (Ender strain). Given the speculation that the viral particle remains intact after inactivation, attachment of the whole viral particle to the beads can occupy more surface-space in comparison to a purified protein. As a result of this,

fewer IgG antibody molecules are able to bind to the surface, creating a significant issue at higher concentrations. Since the measles antigen chosen for the coupling procedure is also not entirely pure (UV-inactivated measles virus: Edmonston strain), a similar issue should be observed for IgG levels measured against MeV at higher concentrations. A potential explanation for why this was not an issue for measles could have something to do with the diameter of the viral structure. If the MeV coupled to the bead-surface is smaller in size compared to MuV antigen, less space is occupied, and more IgG molecules are able to bind at higher concentrations.

Nonetheless, if the UV-inactivation has changed the morphology of the viral structure, leading to leakage and degradation of genetical material (Bono *et al.*, 2021), you end up with a mixture of MuV components and leaked proteins where some have the ability to bind IgG antibodies, and others don't. The viral particles which are not able to bind specific antibodies, will take up unnecessary space on the surface of the bead, thus giving less available space for proteins who are actually able to bind antibodies. However, the mentioned arguments can only be assumed as speculation since research related to this particular issue is unspecific and limited. According to Bland-Altman plots in regard to measles and mumps, the agreement between the operators was good throughout the whole set of IgG antibody concentrations, and only a few datapoints display random dispersion, which are most certainly outliers.

During further assessment of the MMR Multi-Plex Immunoassay, where specific samples were selected for re-analysis due to a high CV% between operator 1 and operator 2, it was observed that measles antigen had the highest number of samples which had to be re-analysed (34 from 306). A possible explanation for this deviations could be the measles antigen used for the development of the method, thus making it more sensitive for minor deviations in laboratory performance. Overall, the re-analysis of the problem samples reduced the high CV% values for the majority of the samples, meaning that the number of technical errors decreased as a result of re-analysis and ensures that the measured IgG values become more similar between the two operators. The decrease in technical errors when performing the laboratory procedure during re-analysis can for instance be explained by the gain of experience. The more experienced the technicians performing the MMR Multi-Plex analysis, the better the results and the higher the reproducibility of the method due to the reduction of unwanted errors. Another factor to consider is the placement of problematic samples on the 96-well plate, as well as the time of the analysis. Considering the fact that the problematic samples were mostly placed on the same plates and run at the same day, the IgG antibody measurements had a slight increase in accuracy.

Since the method is overall very sensitive towards errors, especially caused during the laboratory procedure, each step must be performed precise and as similar as possible between the operators. Logging and quality check of the control samples also ensure that the MMR Multi-Plex Immunoassay analysis is performed right on a daily basis. Additionally, coupling of the magnetic beads to their respective antigens can also be influenced by technical differences in laboratory procedure practiced by to different operators, and thus leading to deviations in antibody measurements. Overall, it is safe to conclude that the agreement between IgG concentrations measured by operator 1 and operator 2 is fairly good and that MMR Multi-Plex Immunoassay has a good reproducibility estimate.

5.3 Future perspectives

To further investigate and acquire a deeper understanding of factors influencing humoral immune response to MMR vaccine, more detailed information about the subjects participating in the current study is required. First and foremost, age and vaccine status of the individual subjects are very important in order to correlate the measured IgG antibody levels with the number of MMR vaccine doses received by each child. As previously mentioned, the first dose of the MMR vaccine is given at 15 months of age, and the second dose at 11 years of age. This means that children who have not yet received the second dose (under the age of 11) will have lower IgG antibody levels compared to individuals who have received the second shot (ate the age of 11). The same principle applies for individuals where a long time has passed since they received the vaccine, because its common that antibody levels decrease over time. Therefore, interpretation of possible deviations in IgG antibody levels among the individuals for measles, mumps and rubella would be more accurate in correlation with age and vaccine status. Additionally, a research group within the toxicology department at the NIPH had also measured levels of PFAS in the blood taken from the same study population of children, and therefore it could be interesting to link IgG levels with PFAS data to investigate the effects of environmental toxicants on vaccine response. However, this can only be done precisely if IgG levels are correlated with children's age and MMR vaccine status.

Another factor to consider in regards of further analysis, is to potentially use a more sophisticated statistical method for the comparison of IgG antibody protection among the three antigens. In a paper published by *Rasheed et al., 2019,* MMR vaccine induced humoral immune response against measles, mumps and rubella was compared using Index Standard Ratio (ISR) (Rasheed *et al.*, 2019). This statistical analysis could also be applied for the IgG antibody results measured during the course of this thesis.

It would also be of great interest to obtain more information about the children from the study population and their parents, since the participants in the MoBa project have also answered questionnaires regarding information on diet, allergies, social status, activity, smoking habits during pregnancy, type of birth and previous infection in the early life of the child. In this way, it would be possible to investigate the effects of numerous factors in regards of vaccine response among the Norwegian population of children, and to obtain a much better understanding of the immune system and mechanisms of action involved in vaccine response.

6 Conclusion

The main purpose of this study was to investigate IgG antibody levels measured in plasma against measles, mumps, and rubella, as an indicator for humoral immune response to MMR vaccine among the Norwegian population of children. An additional goal was to perform quality assessment of the MMR Multi-Plex Immunoassay by analysing intra-assay precision and inter-operator reproducibility.

The assessment of IgG antibody levels by MMR Multi-Plex Immunoassay revealed a good intra-assay precision, as well as a substantial degree of inter-operator reproducibility. The results showed a high degree of IgG antibody protection against measles, mumps, and rubella among the study population of children measured by high IgG antibody concentrations, thus indicating a good humoral immune response and a substantial MMR vaccine effectiveness. Still 2.3% of the study population had IgG antibody concentrations below protective level for all the three antigens.

Furthermore, a larger proportion of children were unprotected against mumps in comparison to measles and rubella. These findings are somewhat supported by The Childhood Immunization Programme: Report for 2019 and 2020 published by NIPH, as well as multiple scientific papers, thus indicate a need for a possible third dose of the MMR vaccine or a mumps-booster. However, various factors must be considered to determine this decision and the level of seriousness appears to be minimal in populations with high vaccine coverage and low occurrence of MuV. Cellular immunity is also considered to be important for protection; therefore, measuring of antibody levels alone cannot be used to conclude whether a person is protected or not.

References

Adlersberg, J.B. (1997) 'The immunoglobulin hinge (interdomain) region.', *La Ricerca in clinica e in laboratorio*, 6(3), pp. 191–205.

Alam, M.M. *et al.* (2013) 'Antibody Avidity in Humoral Immune Responses in Bangladeshi Children and Adults following Administration of an Oral Killed Cholera Vaccine', *Clinical and Vaccine Immunology*, 20(10), pp. 1541–1548. doi:10.1128/CVI.00341-13.

Ando, R., Cox, C., Houser, B. and Moriarty, L. (2020) *Expert Coffee Chat Webinar: Bio-Plex Multiplex Immunoassays in Vaccine Reserach and Development*, *Bio-Rad*. Available at: https://www.bio-rad.com/en-no/life-science-research/support/webinars/expert-coffee-chat-webinar-bio-plex-multiplex-immunoassays-vaccine-research-development?ID=1640135008680 (Accessed: 20 April 2022).

Angel, A., Vaillant, J., Jamal, Z. and Ramphul, K. (2022) *Immunoglobulin*, *StatPearls*. Available at: https://www.ncbi.nlm.nih.gov/books/NBK513460/.

Angeloni, S., Cordes, R., Dunbar, S., Garcia, C., Martin, C. and Stone, V. (2016) *Luminex xMAP Cookbook*. Third edit.

Argüelles, M.H. *et al.* (2006) 'Measles Virus-Specific Antibody Levels in Individuals in Argentina Who Received a One-Dose Vaccine', *Journal of Clinical Microbiology*, 44(8), pp. 2733–2738. doi:10.1128/JCM.00980-05.

Aryal, S. (2021) *Indirect ELISA - Introduction, Steps, Advantages and Protocol*, *Microbe Notes*. Available at: https://microbenotes.com/indirect-elisa-introduction-steps-advantages-and-protocol/ (Accessed: 30 December 2021).

Ashby, B. and Best, A. (2021) 'Herd immunity', *Current Biology*, 31(4), pp. R174–R177. doi:10.1016/j.cub.2021.01.006.

Bauer, G. (2021) 'The potential significance of high avidity immunoglobulin G (IgG) for protective immunity towards SARS-CoV-2', *International Journal of Infectious Diseases*, 106, pp. 61–64. doi:10.1016/j.ijid.2021.01.061.

Berild, J., Bruun, T., Christensen, A., Dorenbarg, D. and Dvergsdal, E. (2021) *The Childhood Immunization Programme 2019-2020*.

Bland, J.M. and Altman, D.G. (1986) 'Statistical methods for assessing agreement between two methods of clinical measurement.', *Lancet (London, England)*, 1(8476), pp. 307–10.

Bono, N., Ponti, F., Punta, C. and Candiani, G. (2021) 'Effect of UV Irradiation and TiO2-Photocatalysis on Airborne Bacteria and Viruses: An Overview', *Materials*, 14(5), p. 1075. doi:10.3390/ma14051075.

Braunschweig, D. (2017) *Multiplex Immunoassays: Five Tips for Optimizing Your Experiments*, *Bioradiations*. Available at: https://www.bioradiations.com/multiplex-immunoassays-five-tips-for-optimizing-your-experiments/ (Accessed: 24 April 2022).

Brgles, M. *et al.* (2016) 'Identification of mumps virus protein and lipid composition by mass spectrometry', *Virology Journal*, 13(1), p. 9. doi:10.1186/s12985-016-0463-0.

Bruce, A., Johnson, A., Lewis, J., Morgan, D., Raff, M., Roberts, K. and Walter, P. (2014) 'The

Innate and Adaptive Immune Systems', in *Molecular Biology of the Cell*. 6th edn. Garland Science, Taylor & Francis Group, pp. 1297–1337.

CDC (2014) *Combination Vaccines - Information for Parents*.

CDC (2022) *Altered Immunocompetence:, Centers for Disease Control and Prevention*. Available at: https://www.cdc.gov/vaccines/hcp/acip-recs/general-recs/immunocompetence.html (Accessed: 2 May 2022).

Chen, J. *et al.* (2021) 'Decline in neutralising antibody responses, but sustained T-cell immunity, in COVID-19 patients at 7 months post-infection', *Clinical & Translational Immunology*, 10(7). doi:10.1002/cti2.1319.

Cruse, J. and Lewis, R. (2010) *Atlas of Immunology*. Third edit. CRP Press.

Das, P.K. and Kielian, M. (2021) 'Molecular and Structural Insights into the Life Cycle of Rubella Virus', *Journal of Virology*. Edited by T.C. Pierson, 95(10). doi:10.1128/JVI.02349-20.

DeStefano, F. and Shimabukuro, T.T. (2019) 'The MMR Vaccine and Autism', *Annual Review of Virology*, 6(1), pp. 585–600. doi:10.1146/annurev-virology-092818-015515.

Ebrahimi, E. (2018) *Re: What are the acceptable values for the percetage deviation (DEV%) and the coefficient of variance (CV)?*, *ResearchGate*. Available at: https://www.researchgate.net/post/What_are_the_acceptable_values_for_the_percentage_deviation_DEV_and_the_coefficient_of_variance_CV (Accessed: 6 May 2022).

Expasy (2009) *Rubulavirus*, *ViralZone*. Available at: https://viralzone.expasy.org/88 (Accessed: 29 March 2022).

Gair, J. and Molnar, C. (2015a) 'Adaptive Immune Response', in *Concepts of Biology -1st Canadian Edition*. 1st edn. BCcampus.

Gair, J. and Molnar, C. (2015b) 'Innate Immune Response', in *Concepts of Biology -1st Canadian Edition*. 1st edn. BCcampus.

Galiza, E. and Heath, P. (2021) 'Immunization', *Medicine*, 49(10), pp. 618–623. doi:10.1016/j.mpmed.2021.07.004.

Janeway, A, C., Travers, P., Walport, M. and Shlomchik, J, M. (2001) 'The structure of aq typical antibody molecule', in *Immunobiology*. 5th edn. New York, NY: Garland Science.

Janeway, C., Travers, P., Walport, M. and Shlomchik, M. (2001) 'The Humoral Immune Response', in *Immunbiology: The Immune System in Health and Disease.* 5th edn. New York, NY: Garland Science.

Jenness, S.M., Aavitsland, P., White, R.A. and Winje, B.A. (2021) 'Measles vaccine coverage among children born to Somali immigrants in Norway', *BMC Public Health*, 21(1), p. 668. doi:10.1186/s12889-021-10694-z.

Jenson, H. and Leach, T, C. (2012) *Measles*, *pediatricweb*. Available at: https://www.pediatricweb.com/webpost/iframe/MedicalConditions_433.asp?tArticleId=172 (Accessed: 22 February 2022).

Kubota, M., Matsuoka, R., Suzuki, T., Yonekura, K., Yanagi, Y. and Hashiguchi, T. (2019) 'Molecular Mechanism of the Flexible Glycan Receptor Recognition by Mumps Virus', *Journal of Virology*. Edited by R.E. Dutch, 93(15). doi:10.1128/JVI.00344-19.

Lambert, N., Strebel, P., Orenstein, W., Icenogle, J. and Poland, G.A. (2015) 'Rubella', *The Lancet*, 385(9984), pp. 2297–2307. doi:10.1016/S0140-6736(14)60539-0.

Lee, J.-Y. and Bowden, D.S. (2000) 'Rubella Virus Replication and Links to Teratogenicity', *Clinical Microbiology Reviews*, 13(4), pp. 571–587. doi:10.1128/CMR.13.4.571-587.2000.

Liang, Y., Yao, J. and Gillam, S. (2000) 'Rubella Virus Nonstructural Protein Protease Domains Involved in trans - and cis -Cleavage Activities', *Journal of Virology*, 74(12), pp. 5412–5423. doi:10.1128/JVI.74.12.5412-5423.2000.

Ludbrook, J. (1997) 'SPECIAL ARTICLE COMPARING METHODS OF MEASUREMENT', *Clinical and Experimental Pharmacology and Physiology*, 24(2), pp. 193–203. doi:10.1111/j.1440-1681.1997.tb01807.x.

Macmillan, C. (2022) 'What Does It Mean To Be "Immunocompromised"?', *Yale Medicine*.

Madi, N., Altawalah, H., Alfouzan, W., Al-Nakib, W., Al-Roumi, E. and Jeragh, A. (2020) 'Assessment of immune status against measles, mumps, and rubella in young Kuwaitis: MMR vaccine efficacy', *Journal of Medical Virology*, 92(8), pp. 963–970. doi:10.1002/jmv.25665.

Maldonado, Y.A. and Shetty, A.K. (2018) 'Rubeola Virus:Measles and Subacute Sclerosing Panencephalitis', in *Principles and Practice of Pediatric Infectious Diseases*. Elsevier, pp. 1169-1176.e3. doi:10.1016/B978-0-323-40181-4.00227-9.

Marques-Garcia, F. (2020) 'Methods for Hemolysis Interference Study in Laboratory Medicine - A Critical Review.', *EJIFCC*, 31(1), pp. 85–97.

Mathew, J., Sankar, P. and Varacallo, M. (2022) *Physiology, Blood Plasma, StatPearls*.

Mirzaei, H.R. (2020) 'Adaptive Immunity', in *Reference Module in Biomedical Sciences*. Elsevier. doi:10.1016/B978-0-12-818731-9.00028-8.

Moss, W.J., Griffin, D.E. and Feinstone, W.H. (2009) 'Measles', in *Vaccines for Biodefense and Emerging and Neglected Diseases*. Elsevier, pp. 551–565. doi:10.1016/B978-0-12-369408-9.00030-5.

MSIS (2022a) *Measles outbreak statistics, The Norwegian Surveillance System for Communicable Diseases*. Available at: http://www.msis.no/ (Accessed: 22 March 2022).

MSIS (2022b) *Mumps outbreak statistics, The Norwegian Surveillance System for Communicable Diseases*. Available at: http://www.msis.no/ (Accessed: 22 March 2022).

MSIS (2022c) *Rubella outbreak statistics, The Norwegian Surveillance System for Communicable Diseases*. Available at: http://www.msis.no/ (Accessed: 22 March 2022).

NIPH (2010a) *Measles (morbilli) -guidance for healthcare professionals, Norwegian Institute of Public Health*. Available at: https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/meslinger-morbilli---veileder-for-h/ (Accessed: 22 March 2022).

NIPH (2010b) *Mumps-guidance for healthcare professionals, Norwegian Institute of Public Health*. Available at: https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/kusma---veileder-for-helsepersonell/ (Accessed: 22 March 2022).

NIPH (2010c) *Rubella - guidance for healthcare professionals, Norwegian Institute of Public Health*. Available at: https://www.fhi.no/nettpub/smittevernveilederen/sykdommer-a-a/rubella-rode-hunder---veileder-for-/ (Accessed: 22 March 2022).

NIPH (2017) *About The Norwegian Surveillance System for Communicable Diseases (MSIS), The Norwegian Institute of Public Health*. Available at: https://www.fhi.no/hn/helseregistre-og-registre/msis/meldesystemet-for-smittsomme-sykdommer/.

NIPH (2018) *NON - PROTECTED. The impact of perfluorinated toxicants and the gut microbiome on vaccine responses in children - project description*.

NIPH (2020a) *Measles vaccination coverage (NHC), Norhealth*. Available at: https://www.norgeshelsa.no/norgeshelsa/index.jsp?headers=AAR&AARslice=2020_2020&stubs=GEO&stubs=ALDER&stubs=VAKSINE&measure=common&virtualslice=Rate_value&ALDERslice=2_2&GEOslice=0&layers=virtual&GEOsubset=0&study=http%3A%2F%2F10.1.5.31%3A80%2Fobj%2FfStud (Accessed: 22 March 2022).

NIPH (2020b) *Mumps vaccination coverage (NHC), Norhealth*. Available at: https://www.norgeshelsa.no/norgeshelsa/index.jsp?headers=AAR&AARslice=2020_2020&stubs=GEO&stubs=ALDER&stubs=VAKSINE&measure=common&virtualslice=Rate_value&ALDERslice=2_2&GEOslice=0&layers=virtual&GEOsubset=0&study=http%3A%2F%2F10.1.5.31%3A80%2Fobj%2FfStud (Accessed: 22 March 2022).

NIPH (2020c) *Rubella vaccination coverage (NHC), Norhealth*. Available at: https://www.norgeshelsa.no/norgeshelsa/index.jsp?headers=AAR&AARslice=2020_2020&stubs=GEO&stubs=ALDER&stubs=VAKSINE&measure=common&virtualslice=Rate_value&ALDERslice=2_2&GEOslice=0&layers=virtual&GEOsubset=0&study=http%3A%2F%2F10.1.5.31%3A80%2Fobj%2FfStud (Accessed: 22 March 2022).

NIPH (2020d) *Vaccine coverage - Childhood Immunization Programme, annual values for large municipalities, Norhealth*. Available at: https://khs.fhi.no/webview/ (Accessed: 30 April 2022).

NIPH (2021a) *About The Norwegian Environmental Biobank, Norwegian Institute of Public Health*. Available at: https://www.fhi.no/en/studies/norwegian-environmental-biobank/om-miljobiobanken/ (Accessed: 20 December 2021).

NIPH (2021b) *Childhood Immunization Programme in Norway: Report for 2019 and 2020*.

NIPH (2022) *MSIS statistics for the occurance of measles, mumps and rubella, The Norwegian Surveillance System for Communicable Diseases*. Available at: http://www.msis.no/ (Accessed: 30 April 2022).

O'Connor, P., Yankovic, D., Zimmerman, L., Ben Mamou, M. and Reef, S. (2021) 'Progress Toward Rubella Elimination — World Health Organization European Region, 2005–2019', *MMWR. Morbidity and Mortality Weekly Report*, 70(23), pp. 833–839. doi:10.15585/mmwr.mm7023a1.

Oran, D. and Topol, E. (2021) 'The Crucial Vcaccine Benefit We're Not Talking about Enough', *Scientific American*.

Parham, P. (2014) *The Immune System*. 4th edn. Garland Science, Taylor & Francis Group.

Parkman, P.D. (1996) *Togaviruses: Rubella Virus, Medical Microbiology*.

Piao, L., Park, H. and Jo, C.H. (2017) 'Theoretical prediction and validation of cell recovery rates in preparing platelet-rich plasma through a centrifugation', *PLOS ONE*. Edited by J.H.-C. Wang, 12(11), p. e0187509. doi:10.1371/journal.pone.0187509.

Plans, P. *et al.* (2015) 'Prevalence of anti-rubella, anti-measles and anti-mumps IgG antibodies

in neonates and pregnant women in Catalonia (Spain) in 2013: susceptibility to measles increased from 2003 to 2013', *European Journal of Clinical Microbiology & Infectious Diseases*, 34(6), pp. 1161–1171. doi:10.1007/s10096-015-2339-4.

Plitnick, L.M. (2013) 'Global Regulatory Guidelines for Vaccines', in *Nonclinical Development of Novel Biologics, Biosimilars, Vaccines and Specialty Biologics*. Elsevier, pp. 225–241. doi:10.1016/B978-0-12-394810-6.00009-5.

Pollard, A.J. and Bijker, E.M. (2021) 'A guide to vaccinology: from basic principles to new developments', *Nature Reviews Immunology*, 21(2), pp. 83–100. doi:10.1038/s41577-020-00479-7.

Racaniello, V. (2016) *Rubella virus and birth defects, Virology blog*. Available at: https://www.virology.ws/2016/02/17/rubella-virus-and-birth-defects/ (Accessed: 29 March 2022).

Raqib, R. *et al.* (2017) 'Humoral Immunity in Arsenic-Exposed Children in Rural Bangladesh: Total Immunoglobulins and Vaccine-Specific Antibodies', *Environmental Health Perspectives*, 125(6), p. 067006. doi:10.1289/EHP318.

Rasheed, M.A.U. *et al.* (2019) 'Decreased humoral immunity to mumps in young adults immunized with MMR vaccine in childhood', *Proceedings of the National Academy of Sciences*, 116(38), pp. 19071–19076. doi:10.1073/pnas.1905570116.

Reslova, N., Michna, V., Kasny, M., Mikel, P. and Kralik, P. (2017) 'xMAP Technology: Applications in Detection of Pathogens', *Frontiers in Microbiology*, 8. doi:10.3389/fmicb.2017.00055.

Rønningen, K.S. *et al.* (2006) 'The biobank of the Norwegian mother and child cohort Study: A resource for the next 100 years', *European Journal of Epidemiology*, 21(8), pp. 619–625. doi:10.1007/s10654-006-9041-x.

Rubin, S., Eckhaus, M., Rennick, L.J., Bamford, C.G. and Duprex, W.P. (2015) 'Molecular biology, pathogenesis and pathology of mumps virus', *The Journal of Pathology*, 235(2), pp. 242–252. doi:10.1002/path.4445.

Schreuder, P. and Alsaker, E. (2014) 'The Norwegian Mother and Child Cohort Study (MoBa) – MoBa recruitment and logistics', *Norsk Epidemiologi*, 24(1–2). doi:10.5324/nje.v24i1-2.1754.

Schroeder, H.W. and Cavacini, L. (2010) 'Structure and function of immunoglobulins', *Journal of Allergy and Clinical Immunology*, 125(2), pp. S41–S52. doi:10.1016/j.jaci.2009.09.046.

Smits, G.P., van Gageldonk, P.G., Schouls, L.M., van der Klis, F.R.M. and Berbers, G.A.M. (2012) 'Development of a Bead-Based Multiplex Immunoassay for Simultaneous Quantitative Detection of IgG Serum Antibodies against Measles, Mumps, Rubella, and Varicella-Zoster Virus', *Clinical and Vaccine Immunology*, 19(3), pp. 396–400. doi:10.1128/CVI.05537-11.

Steens, A., Stefanoff, P., Daae, A., Vestrheim, D.F. and Riise Bergsaker, M.A. (2020) 'High overall confidence in childhood vaccination in Norway, slightly lower among the unemployed and those with a lower level of education', *Vaccine*, 38(29), pp. 4536–4541. doi:10.1016/j.vaccine.2020.05.011.

Su, S.-B., Chang, H.-L. and Chen, K.-T. (2020) 'Current Status of Mumps Virus Infection: Epidemiology, Pathogenesis, and Vaccine', *International Journal of Environmental Research and Public Health*, 17(5), p. 1686. doi:10.3390/ijerph17051686.

Veneti, L. *et al.* (2018) 'Large outbreak of mumps virus genotype G among vaccinated students in Norway, 2015 to 2016', *Eurosurveillance*, 23(38). doi:10.2807/1560-7917.ES.2018.23.38.1700642.

Weisglas-Kuperus, N., Patandin, S., Berbers, G.A., Sas, T.C., Mulder, P.G., Sauer, P.J. and Hooijkaas, H. (2000) 'Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children.', *Environmental Health Perspectives*, 108(12), pp. 1203–1207. doi:10.1289/ehp.001081203.

Wellington, K. and Goa, K.L. (2003) 'Measles, mumps, rubella vaccine (Priorix; GSK-MMR): a review of its use in the prevention of measles, mumps and rubella.', *Drugs*, 63(19), pp. 2107–26. doi:10.2165/00003495-200363190-00012.

WHO (2008) *The Immunological Basis for for Immunization Series: Module 11:Rubella*. Geneva.

WHO (2020a) *WHO Immunological Basis for Immunization Series: Module 16: Mumps*. Geneva.

WHO (2020b) *WHO Immunological Basis for Immunization Series: Module 7:Measles*. Geneva.

WHO (2021a) *Measles-containing -vaccine first dose (MCV1) immunization coverage among 1 -year-olds (%), World Health Organization*. Available at: https://www.who.int/data/gho/data/indicators/indicator-details/GHO/measles-containing-vaccine-first-dose-(mcv1)-immunization-coverage-among-1-year-olds-(-) (Accessed: 22 March 2022).

WHO (2021b) *Mumps reported cases WHO region, World Health Organization*. Available at: https://apps.who.int/gho/data/view.main.1520_53?lang=en (Accessed: 22 March 2022).

WHO (2021c) *WHO-EpiBrief:A report on the epidemiology of selected vaccine-preventable diseases in the European region*.

Won, H. *et al.* (2021) 'Cross-neutralization between vaccine and circulating wild-type mumps viruses in Korea', *Vaccine*, 39(13), pp. 1870–1876. doi:10.1016/j.vaccine.2021.01.039.

Wu, K. (2021) 'The Good Part About "Waning" Immunity', *The Atlantic*.

Yadav, D.K., Yadav, N. and Khurana, S.M.P. (2014) 'Vaccines', in *Animal Biotechnology*. Elsevier, pp. 491–508. doi:10.1016/B978-0-12-416002-6.00026-2.

Zaiontz, C. (2022) *Lin's Concordance Correlation Coefficient, Real statistics using Excel*. Available at: https://www.real-statistics.com/reliability/interrater-reliability/lins-concordance-correlation-coefficient/ (Accessed: 14 May 2022).

Zimmermann, P. and Curtis, N. (2019) 'Factors That Influence the Immune Response to Vaccination', *Clinical Microbiology Reviews*, 32(2). doi:10.1128/CMR.00084-18.

Zoppi, L. (2021) 'What are Neutralizing Antibodies?', *News Medical Life Sciences*.

Appendices

Appendix A: Materials

Table A.1: Reagents used in the MMR Bio-Plex Immunoassay

Product name	Lot No. / Catalog No.	Supplier
Sulfo-NHS (N-hydroxysulfosuccinimide)	UJ283657	Thermo Fisher Scientific, NY, USA
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)	RA228748	Thermo Fisher Scientific, NY, USA
0.1% Tween-20	P1379	Sigma-Aldrich, Oslo Norway
3% Bovine Serum Albumin (BSA)	SLBQ3713V	Sigma-Aldrich, Oslo, Norway
70% Isopropyl	N/A	Sigma-Aldrich, Oslo, Norway
Sodium hypochlorite (bleach)	N/A	Sigma-Aldrich, Oslo, Norway
Bio-Plex Amine Coupling	171406001	NIPH, Oslo, Norway
Bio-Plex Calibration Kit	171203060	Bio-Rad Laboratories, Oslo, Norway
20x Waaler PBS	N/A	Bio-Rad Laboratories, Oslo, Norway
1x Waaler PBS	N/A	NIPH, Oslo, Norway
Anti-human IgG R-Phycorythrin conjugate from goat, P8047	SLCC5339	NIPH, Oslo, Norway Sigma-Aldrich, Oslo, Norway
XMAP Reagent MagPlex Microspheres 1 ml 1,25 x 107 beads/ml (Region 026)	B59990	Bio-Rad Laboratories, Oslo, Norway
XMAP Reagent MagPlex Microsphere 1 ml 1,25 x 107 beads/ml (Region 029)	B46417	Bio-Rad Laboratories, Oslo, Norway
XMAP Reagent MagPlex Microsphere 1 ml 1,25 x 107 beads/ml.	B35120	Bio-Rad Laboratories, Oslo, Norway

Table A.2: Antigens used in MMR Multi-Plex bead-coupling procedure

Antigen	Product code	Lot No.	Inactivated	Supplier
Native Measles Virus (Edmonston strain) 2.26 mg/ml	PIP013	A20070801	UV-inactivation	Bio-Rad Laboratories, Oslo, Norway

Mumps Virus A Antigen (Enders strain) 1 mg/ml	MBS5303605	A20070801	UV -inactivation	MyBioSource, San Diego, USA
Rubella Antigen (HPV-77) Native Protein (Zinc finger and BTB- domain containing protein 5) 500 µg/ml	GWB-HG4389	M24271014	UV-inactivation	GenWay Biotech Inc, San Diego, USA

Table A.3: Standards and controls used in MMR Multi-Plex Immunoassay, including testing of coupled beads

Standard/Control	REF	Lot No.	Supplier
Standard:			
Anti-Rubella Immunoglobulin, WHO International standard RUBI-1-94 50 µl, 1600 IU/ml *	N/A	N/A	NIBSC, Ridge, UK
Commercial controls:			
Anti-measles QC1 100µl	GCRMEASQC1	15/B667-04	NIBSC, Ridge, UK
Anti-mumps QC1 100µl	GCRMUMPSQC1	15/B664-03	NIBSC, Ridge, UK
Anti-rubella QC1 100µL	QCRRUBQC	14/B654-03	NIBSC, Ridge, UK
In-house control:			
Positive control MIA MM3 R-PLEX 200µl**	N/A	N/A	NIPH, Oslo, Norway
In-house negative control: Rubella QC 1:10 ADHS (antibody depleted human) 100µl **	N/A	N/A	NIPH, Oslo, Norway

***** The International WHO standard for rubella was calibrated against measles and mumps (performed in-house).

** Developed and prepared in-house

Appendix B: Instruments and laboratory equipment

Table B.1: Overview of instruments used in MMR Multi-Plex Immunoassay, including bead-coupling procedure.

Instrument	Model	Supplier
Platform shaker	Titramax 1000	Heidolph Instruments, Germany
Ultrasonic bath	XUBA3	Grant Instruments, Cambridge, UK
Microplate wash station	Bio-Plex Pro™	Bio-Rad Laboratories, Oslo, Norway
Rotator	Tube rotator	VWR International AS, Oslo, Norway
Multi-plex Immunoassay System	Bio-Plex 200 Systems LX10021034421 Powered by Luminex xMAP Technology	Bio-Rad Laboratories, Oslo, Norway

Table B.2 Overview of laboratory equipment used in MMR Multi-Plex Immunoassay, including bead-coupling procedure.

Product name	Lot No. / Catalog No.	Supplier
Bio-Plex Pro™ Flat Bottom 96- Well Plates	1711025001	Bio-Rad Laboratories, Oslo, Norway
Eppendorf® Protein LoBind tubes, 2 ml	Z666513-100EA	Sigma-Aldrich, Oslo, Norway
Sealing Tape for 96-Well Plates	SPE164G	Thermo Fisher Scientific, NY, USA
DynaMag™ -2 Magnet	12321ID	Thermo Fisher Scientific, NY, USA
Bio-Plex MCV Plate IV	171203033	Bio-Rad Laboratories, Oslo, Norway
Bio-Plex Reservoir	171203050	Bio-Rad Laboratories, Oslo, Norway

Appendix C: Software

Table C.1 Software used in the processing , statistical analysis, and visualization of data

Software	Version	Supplier
Bio-Plex Manager	6.2	Bio-Rad Laboratories, Oslo, Norway
GraphPad Prism	8.0	GraphPad by Dotmatics, San Diego, USA
Microsoft Office Excel	2016	Microsoft, Redmond, USA

Appendix D: MMR Multi-Plex Immunoassay templates

	Assay 1: Testing of old beads						Assay 2: Testing of new beads					
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1:RUBI-1-94 standard 1:100	Duplicate	Blank	Duplicate	Marta serum 1:1000	Duplicate	S1:RUBI-1-94 standard 1:100	Duplicate	Blank	Duplicate	Marta serum 1:1000	Duplicate
B	S2:RUBI-1-94 standard 1:400	Duplicate	Measles QC 1:200	Duplicate	QC Rubella 1:10 ADHS 1:200	Duplicate	S2:RUBI-1-94 standard 1:400	Duplicate	Measles QC 1:200	Duplicate	QC Rubella 1:10 ADHS 1:200	Duplicate
C	S3:RUBI-1-94 standard 1:1600	Duplicate	Measles QC 1:1000	Duplicate	QC Rubella 1:10 ADHS 1:1000	Duplicate	S3:RUBI-1-94 standard 1:1600	Duplicate	Measles QC 1:1000	Duplicate	QC Rubella 1:10 ADHS 1:1000	Duplicate
D	S4:RUBI-1-94 standard 1:6400	Duplicate	Mumps QC 1:200	Duplicate	Blank	Duplicate	S4:RUBI-1-94 standard 1:6400	Duplicate	Mumps QC 1:200	Duplicate	Blank	Duplicate
E	S5:RUBI-1-94 standard 1:25600	Duplicate	Mumps QC 1:1000	Duplicate	Blank	Duplicate	S5:RUBI-1-94 standard 1:25600	Duplicate	Mumps QC 1:1000	Duplicate	Blank	Duplicate
F	S6:RUBI-1-94 standard 1:102400	Duplicate	Rubella QC 1:200	Duplicate	Blank	Duplicate	S6:RUBI-1-94 standard 1:102400	Duplicate	Rubella QC 1:200	Duplicate	Blank	Duplicate
G	S7:RUBI-1-94 standard 1:409600	Duplicate	Rubella QC 1:1000	Duplicate	Blank	Duplicate	S7:RUBI-1-94 standard 1:409600	Duplicate	Rubella QC 1:1000	Duplicate	Blank	Duplicate
H	S8:RUBI-1-94 standard 1:1638400	Duplicate	Marta serum 1:200	Duplicate	Blank	Duplicate	S8:RUBI-1-94 standard 1:1638400	Duplicate	Marta serum 1:200	Duplicate	Blank	Duplicate

Figure D.1 Template for the set-up of Assay 1 and Assay 2 for the testing procedure of newly antigen-coupled magnetic beads*. The figure illustrates the placement and dilution factor for the standard (blue) and control samples (red) on the Bio-Plex 96-well plate.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1:RUBI-1-94 standard 1:100	Duplicate	Blank	Duplicate	Marta serum 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
B	S2:RUBI-1-94 standard 1:400	Duplicate	Measles QC 1:200	Duplicate	QC Rubella 1:10 ADHS 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
C	S3:RUBI-1-94 standard 1:1600	Duplicate	Measles QC 1:1000	Duplicate	QC Rubella 1:10 ADHS 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
D	S4:RUBI-1-94 standard 1:6400	Duplicate	Mumps QC 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
E	S5:RUBI-1-94 standard 1:25600	Duplicate	Mumps QC 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
F	S6:RUBI-1-94 standard 1:102400	Duplicate	Rubella QC 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
G	S7:RUBI-1-94 standard 1:409600	Duplicate	Rubella QC 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate
H	S8:RUBI-1-94 standard 1:1638400	Duplicate	Marta serum 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:200	Duplicate	#Sample nr. ID nr. 1:1000	Duplicate	Blank	Duplicate

Figure D.2 Template for the set-up of MMR Multi-Plex Immunoassay IgG antibody analysis. *The figure illustrates the placement and dilution factor for the standard (blue), controls (red) and plasma samples (green) on the Bio-Plex 96-well plate.*

Appendix E: FI-values and IgG antibody concentrations for the control samples

Table E.1 Acceptable range of FI-values and corresponding IgG concentrations for the control samples (QC, positive and negative) measured against measles. *The values illustrated in the table were used for daily quality check of each run performed with MMR Multi-Plex Immunoassay*

Measles (IgG mIU/ml)											
	Pos. control (MNBH)	FI (1:200)	Neg. Control (RubQC 1:10, ADHS)	FI (1:200)	QC Measles	FI (1:200)	QC Mumps	FI (1:200)	QC Rubella	FI (1:200)	Blank
Average	2604.6	849.1	115.2	81.4	727.2	374.7	1549.2	618.7	936.6	430.7	31.8
Standard deviation	523.4	341.7	52	14.6	92.2	144.4	249	248.4	199.6	171.7	5.9
CV %	20.1	40.2	45.1	17.9	12.7	38.5	16.1	40.1	21.3	39.9	18.5
2 STD +	3651	1532	219	111	912	663	2047	1115	1336	774	44
2 SD -	1558	166	11	52	543	86	1051	122	537	87	20
3 SD +	4175	1874	271	125	1004	808	2296	1364	1535	946	50
3 SD -	1034.4	-175.9	-40.8	37.6	450.7	-58.4	802.2	-126.5	337.7	-84.3	14.2

Table E.2 Acceptable range of FI-values and corresponding IgG concentrations for the control samples (QC, positive and negative) measured against mumps. *The values illustrated in the table were used for daily quality check of each run performed with MMR Multi-Plex Immunoassay*

Mumps (IgG mIU/ml)											
	Pos. control (MNBH)	FI (1:200)	Neg. Control (RubQC 1:10, ADHS)	FI (1:200)	QC Measles	FI (1:200)	QC Mumps	FI (1:200)	QC Rubella	FI (1:200)	Blank
Average	3911	3942.3	75.5	328.1	1307.7	2106	728.8	1394.9	824.4	1640.8	30.2
Standard deviation	777.1	1408.3	15.4	61	162.3	798.8	108	549.2	133.1	503.4	12.3
CV %	19.9	35.7	20.4	18.6	12.4	37.9	14.8	39.4	16.1	30.7	40.9
2 STD +	5465	6759	106	450	1632	3704	945	2493	1091	2648	55
2 SD -	2357	1126	45	206	983	508	513	296	558	634	5
3 SD +	6242	8167	122	511	1795	4503	1053	3043	1224	3151	67
3 SD -	1579.7	-282.6	29.3	145	820.7	-290.5	404.9	-252.8	425.1	130.6	-6.9

Table E.3 Acceptable range of FI-values and corresponding IgG concentrations for the control samples (QC, positive and negative) measured against rubella. *The values illustrated in the table were used for daily quality check of each run performed with MMR Multi-Plex Immunoassay*

Rubella (IgG mIU/ml)											
	Pos. control (MNBH)	FI (1:200)	Neg. Control (RubQC 1:10, ADHS)	FI (1:200)	QC Measles	FI (1:200)	QC Mumps	FI (1:200)	QC Rubella	FI (1:200)	Blank
Average	245.2	6078.9	3.5	331.5	53.4	2220	59.7	2418.4	35.5	1762.8	47.6
Standard deviation	48.6	1517.5	0.8	119.5	6.6	687.8	8.2	755.6	4.4	540.1	47
CV %	19.8	25	22.4	36	12.4	31	13.7	31.2	12.4	30.6	98.9
2 STD +	342	9114	5	570	67	3596	76	3930	44	2843	142
2 SD -	148	3044	2	93	40	845	43	907	27	683	-47
3 SD +	391	10632	6	690	73	4283	84	4685	49	3383	189
3 SD -	99.3	1526.3	1.1	-26.9	33.5	156.8	35.2	151.7	22.3	142.5	-93.5

Appendix F: Standard curves for MMR antigens

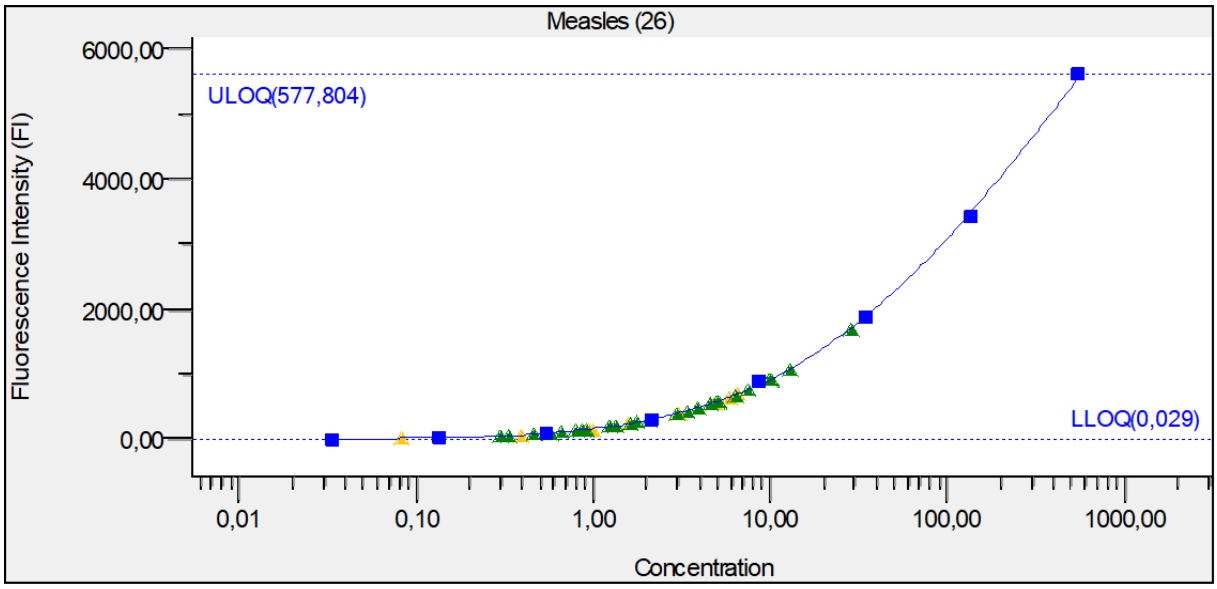


Figure F.1 MeV standard curve generated by Bio-Plex Software Manager.

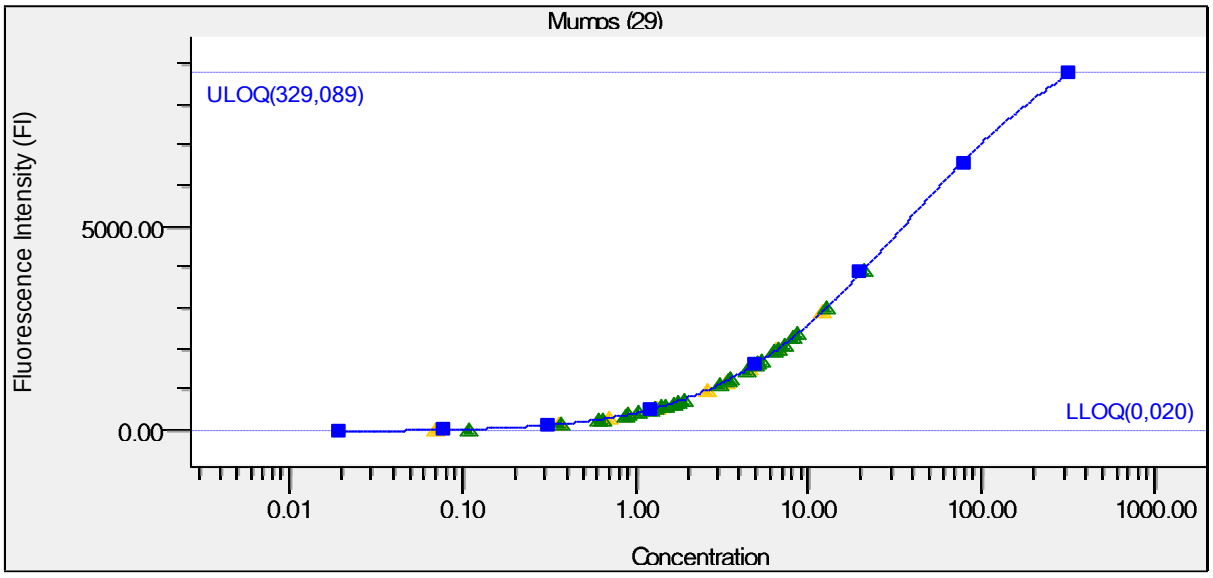


Figure F.2 MuV standard curve generated by Bio-Plex Software Manager.

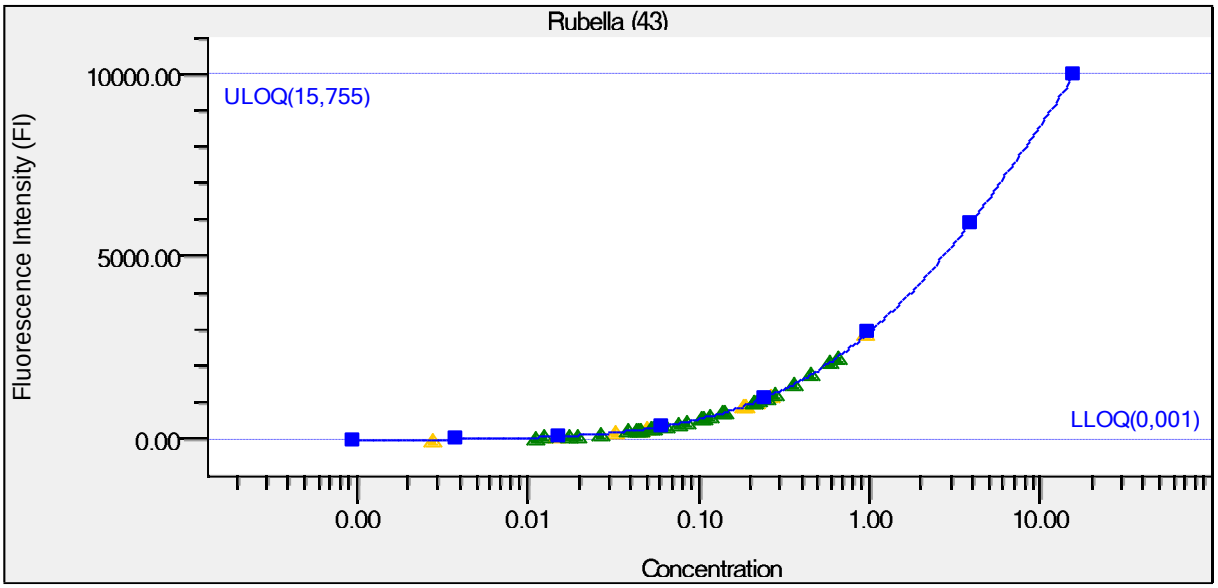


Figure F.3 RuV standard curve generated by Bio-Plex Software Manager.

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway