**pentavalent composition in 1004a, 1004b and 1004c formulations**.

| Formul[n] | Process loss | | | | | | Stability log loss per 30 days | | | | | Cumulative log loss per 30 days | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | P1 | | G1 | G2 | G3 | G4 | P1 | G1 | G2 | G3 | G4 | P1 |
| **1004a** | 0.18 | 0.06 | 0 | 0.03 | 0 | **37 °C** | 0.10 | 0 | 0 | 0 | 0 | 0.28 | 0.06 | 0 | 0 | 0 |
| | | | | | | **2-8 °C** | 0.01 | 0 | 0 | 0 | 0.02 | 0.19 | 0.06 | 0 | 0 | 0 |
| **1004b** | 0.01 | 0.11 | 0.16 | 0 | 0.08 | **37 °C** | 0.20 | 0 | 0.03 | 0.14 | 0.07 | 0.21 | 0.11 | 0.19 | 0.14 | 0.15 |
| | | | | | | **2-8 °C** | 0.17 | 0 | 0.03 | 0.05 | 0.04 | 0.18 | 0.11 | 0.19 | 0.05 | 0.12 |
| **1004c** | 0 | 0.14 | 0 | 0.06 | 0 | **37 °C** | 0.23 | 0.02 | 0.06 | 0.06 | 0.08 | 0.23 | 0.16 | 0.06 | 0.12 | 0.08 |
| | | | | | | **2-8 °C** | 0.15 | 0 | 0 | 0.02 | 0.02 | 0.15 | 0.14 | 0 | 0.09 | 0.02 |

*Study 2: Longitudinal thermostability and reproducibility for formulation 1004c in vials.*

The exemplified formulation 1004c is tested for longitudinal stability and process reproducibility at clinically relevant concentrations of rotavirus, under the storage conditions of 37°C ± 2°C (RH 75% ± 5%) and 2°C to 8°C for 8 weeks. The formulation is incorporated with all the five strains at the log concentrations per dose per ml as G1: 6.81; G2: 6.92; G3: 6.91; G4: 6.78 and P1: 6.83 (1x concentrations). A 54 h freeze drying cycle as mentioned in Example 2 of Study 1, is used for preparation of this formulation and is found to support freeze drying of full load in the freeze dryer (> 680 vials per batch). The total solid content is 13g/100ml in aqueous liquid feed.

Process loss is calculated for three independent manufacturing batches (N=1, 2, 3), as a difference of potency between samples before and after freeze drying.

Stability loss (log loss) at 37°C ± 2°C with %RH 75% ± 5% is calculated by linear regression of potency data of samples incubated at 37°C ± 2°C and %RH 75% ± 5% as shown in Table 13. The moisture content of all the three manufacturing batches are below 2.0% (<2.0%).

**Table 13: Summary of the process loss, stability loss and the cumulative loss of pentavalent composition in 1004c formulations in three independent**

**manufacturing batches**

| Formulatn | Process loss | | | | | | Stability log loss per 8 weeks | | | | | Cumulative log loss per 8 weeks | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | P1 | | G1 | G2 | G3 | G4 | P1 | G1 | G2 | G3 | G4 | P1 |
| N=1 | 0 | 0.14 | 0 | 0.06 | 0 | 37°C | 0.43 | 0.04 | 0.11 | 0.11 | 0.14 | 0.43 | 0.18 | 0.11 | 0.17 | 0.14 |
| | | | | | | 2-8 °C | 0.29 | 0 | 0 | 0.04 | 0.04 | 0.29 | 0 | 0 | 0.10 | 0.04 |
| N=2 | 0.11 | 0.12 | 0.12 | 0.15 | 0.12 | 37°C | 0.11 | 0.21 | 0.12 | 0.02 | 0.16 | 0.22 | 0.33 | 0.24 | 0.18 | 0.28 |
| | | | | | | 2-8 °C | 0.03 | 0.06 | 0 | 0 | 0 | 0.14 | 0.17 | 0 | 0 | 0 |
| N=3 | 0.10 | 0.07 | 0.07 | 0.12 | 0.13 | 37 °C | 0.21 | 0.18 | 0.16 | 0.10 | 0.13 | 0.31 | 0.25 | 0.23 | 0.22 | 0.27 |
| | | | | | | 2-8 °C | 0.07 | 0.17 | 0.04 | 0.08 | 0.03 | 0.17 | 0.24 | 0.11 | 0.21 | 0.17 |

*Study 3: Titer scale up, Longitudinal studies and Reproducibility for 1004c formulation in vials.*

The composition for the formulation is same as that used in **Example 2 of Study 1** except that the initial human-bovine rotavirus serotype concentrations is increased 5 times (5x) with the log values per ml per 5 doses as G1: 7.51; G2: 7.62; G3: 7.61; G4: 7.47; P1: 7.52 in vials. The total solid content is 13g/100ml in aqueous liquid feed for all the three independent manufacturing batches N=1, 2 and 3. The solution pH is adjusted to 6.10 ± 0.1. The 54 h freeze drying cycle (**similar to Study 1 of Example 2)** is used for preparation of this formulation in Type-I glass vials with 3 mL capacity.

A negligible process loss is observed when compared to the pre- freeze drying liquid control. The longitudinal studies are conducted under the storage conditions of 2°C - 8°C, 37°C ± 2°C (RH 75% ± 5%) and 45°C ± 2°C (RH 75% ± 5%). The data is depicted in Fig. 1, Fig. 2 and Fig. 3 respectively. Based on the linear regression of this data, it is observed that the freeze dried rotavirus formulation is stable at all these storage conditions for almost 252 days (09 months). Slope values for the samples stored at 2°C to 8°C showed no loss. Arrhenius kinetics simulations of the data indicated that expected time period for stability, as indicated by $Log_{10}$=0.5 potency loss for any of the five serotypes within this formulation, at 37°C ± 2°C (RH 75% ± 5%) can be 23

***WE CLAIM:***

1. A thermostable freeze dried rotavirus vaccine formulation wherein said vaccine formulation comprises of
   - at least one rotavirus serotype, and
   - at least one excipient comprising of a lyoprotectant, a first class of buffering agent, a salt, an ion, a bulking agent, a dispersant, an activating agent, either alone or in combination thereof,

   wherein
   - said lyoprotectant is selected from sucrose, trehalose, sorbitol or lactose, in the range of 1.2% w/v to 6.0% w/v;
   - said first class of buffering agent is selected from HEPES, Tris, Phosphate, citrate or Histidine, in the range of 10 mM to 50 mM;
   - said salt is selected from Sodium chloride, Magnesium chloride, Potassium chloride, in the range of 0mM-50mM;
   - said ion is a divalent cation selected from Zn (II) and Ca (II), in the range of 0 mM to 2 mM;
   - said bulking agent is selected from Polyvinylpyrrolidone (PVP), Dextran or Mannitol, in the range of 2% to 6 % w/v;
   - said dispersant is selected from Tween® 20 and Tween® 80, in the range of 0 to 0.02%w/v;
   - said activating agent is selected from L-Arginine, Glycine; in the range of 10 to 100mM either alone or in combination,,

   wherein said vaccine formulation does not exceed potency losses exceeding $Log_{10}$ = [0.5] when stored at 5 ± 3ºC up to 2 years and when stored up to 45ºC for at least 18 weeks.

2. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein
   - said lyoprotectant is Sucrose, Trehalose or combination thereof
   - said first class buffering agent is HEPES;

- said salt is Sodium Chloride (NaCl);
- said divalent cation is $Ca^{+2}$;
- said first class of bulking agent is PVP;
- said dispersant is Tween® 20; and
- said activating agent is L-Arginine and/or Glycine.

3. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said rotavirus serotype is selected from at least one rotavirus strains selected from bovine, rhesus, human, ovine, rhesus/human reassortants, bovine/human reassortants and other monovalent or multivalent rotavirus.

4. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said rotavirus serotype is selected from G1, G2, G3, G4 and P1 Human- Bovine reassortants either alone or in combination.

5. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said rotavirus serotype having Log10 concentration as G1: 6.81, G2: 6.92, G3: 6.91, G4: 6.78 and P1: 6.83 per dose.

6. An improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1, said process comprising the steps of:

(a) selecting at least one rotavirus serotype;

(b) adding said selected rotavirus serotype of step (a) to said at least one excipient to prepare a liquid feed with optimal potency retention;

(c) freeze drying said aqueous liquid feed of step (b) to obtain a freeze dried formulation;

(d) milling said freeze dried formulation obtained from step (c) by using suitable milling methods at temperatures ranging from $0^{o}C$ to $25^{o}C$ to obtain a milled dried formulation; and

(e) blending of said milled dried formulation obtained from step (d) with a

blending agent individually or in combination with a second class of buffering agent having acid neutralizing capacity to obtain said thermostable freeze dried rotavirus vaccine formulation for appropriate packaging of monodose or multidose,

-wherein the said process result in a process loss not exceeding $Log_{10}$ = [0.2],

wherein said blending agent is selected from PVP K25, PVP K40, sucrose, mannitol, maltodextrin with dextrose equivalent ranging from 4 to 20, fructose, glucose, lactose either alone or in the combinations providing D90> 250 µm to milled blended freeze dried powder and the moisture content of final freeze-dried rotavirus vaccine formulation is below 3.0%.

7. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said liquid feed is having solid contents in the range of 10g/100ml to 25g/100ml, more preferably 13g/100ml (13%).

8. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said freeze drying process is performed either at clinically relevant specification (1x) or at higher (5x) rotavirus concentrations.

9.The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said liquid feed is having titre value in the range of 5.0E+06 IU/mL to 6.0E+07 IU/ mL each serotype, preferably 5.0E+06 IU/mL to 4.0E+07 IU/mL each serotype.

10. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said freeze drying process results in cumulative potency loss not exceeding $Log_{10}$ = [0.5] at

temperature in the range of 37°C ± 2°C for at least 30, 60, 90, 180 days and $Log_{10}$ = [0.5] at temperature in the range of 45°C ± 2°C for at least 30, 60, 90, 120 days.

11. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said milled freeze dried powder is obtained by milling methods including Sieve milling, Hammer milling, Ball milling, Conical sieve milling and Roller milling etc. wherein;

- Particle size of said milled freeze dried powder have $D_{90}$ value, is not less than 250 µm;
- the preferred temperature range is in range of 20°C - 22°C; and
- said milling is performed in presence of a dry gas including Nitrogen, Argon, $CO_2$, air or combinations thereof.

12. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said vaccine formulation is in dry form such as freeze dried cake, freeze dried powder, milled freeze dried powder, milled and blended freeze dried powder.

13. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 11 wherein said milled freeze dried powder shows glass transition temperature ($T_g$ ) in the range of 48.0 ± 1.0°C.

14. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said second class of buffering agent being selected from HEPES, Trisodium citrate dihydrate, histidine, calcium carbonate, sodium carbonate, potassium carbonate, sodium bicarbonate, calcium bicarbonate, potassium bicarbonate,

aluminum hydroxide, sodium dihydrogen phosphate monohydrate or magnesium hydroxide or combinations thereof.

15. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said second class of buffering agent when used in conjunction with blending agent results in said acid neutralization capacity of the vaccine in the range of 0.3 mEq/dose to 1.0 mEq/dose of powder.

16. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 wherein said vaccine is to be administered orally with or without a reconstitution buffer or with or without water for injection.

17. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 16 wherein said reconstitution buffer is selected from calcium carbonate, sodium carbonate, potassium carbonate, sodium bicarbonate, calcium bicarbonate, potassium bicarbonate, aluminum hydroxide or magnesium hydroxide, sodium dihydrogen phosphate monohydrate, trisodium citrate dihydrate or combinations thereof, more preferably phosphate-citrate.

18. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 17 wherein said vaccine formulation when reconstituted with reconstitution buffer results in acid neutralizing capacity in the range of 0.3 mEq/dose to 1.0 mEq/dose.

19. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 18 wherein the palatability of said reconstitution buffer is maintained by adding 0- 60% of sugars such as lactose, glucose, sucrose, fructose or the combinations

thereof.

20. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 6 prepared by an improved freeze drying process, said process comprising the steps of:

(a) selecting at least one rotavirus serotype;

(b) adding said selected rotavirus serotype of step (a) to said at least one excipient to prepare a liquid feed with optimal potency retention;

(c) freeze drying said liquid feed of step (b) to obtain a freeze dried formulation ;

(d) milling said freeze dried formulation obtained from step (c) by using suitable milling methods at temperatures ranging from 0°C to 25°C to obtain a milled dried formulation;

(e) blending said milled dried formulation obtained from step (d) with a blending agent individually or in combination with a second class of buffering agent having acid neutralizing capacity to obtain said thermostable freeze dried rotavirus vaccine formulation for appropriate packaging of monodose or multidose; and

(f) packaging of said thermostable freeze dried rotavirus vaccine formulation in a container along with said reconstitution buffer comprising said buffering agent having said acid neutralizing capacity in a separate container for reconstitution prior to oral administration;

wherein the moisture content of final freeze-dried rotavirus vaccine formulation is below 3.0%.

21. The improved freeze drying process of preparing thermostable freeze dried rotavirus vaccine formulation as claimed in claim 17 wherein said vaccine when reconstituted with said reconstitution buffer maintains virus potency losses not exceeding $Log_{10}$ = [0.5] at storage conditions of

$2^{o}C$ - $8^{o}C$ for 48 h and $25^{o}C$ for 8 h.

22. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said vaccine formulation dissolves within 60 ± 5.0 sec in said reconstitution buffer maintained at $23^{o}C \pm 1^{o}C$.

23. The thermostable freeze dried rotavirus vaccine formulation as claimed in claim 1 wherein said formulation is obtained by a process comprising the steps of:

(a) selecting at least one rotavirus serotype;

(b) adding said selected rotavirus serotype of step (a) to said at least one excipient to prepare a liquid feed with optimal potency retention;

(c) freeze drying said aqueous liquid feed of step (b) to obtain a freeze dried formulation with residual moisture content less than 3.0%.

-wherein step (c) is performed in vials.