M5630
Media Optimization Kit (MS)

Introduction

Determining the proper nutrient formulation for various plant species is still paramount to success in plant tissue culture. It remains one of the more arduous tasks in tissue culture which can require months to years to obtain desired results depending on your species. Mineral nutrient optimization is still necessary regardless of whether a researcher is looking for new formulations in shoot micropropagation, rooting media, or pre-transplant media;

Both Niedz and Reed's research groups have recently presented a concise methodology for media optimization in a series of papers (Reed *et al.* 2013a, Reed *et al.* 2013b, Hand *et al.* 2014, Niedz *et al.* 2014, Poothong and Reed 2014, and Wada *et al.* 2015) with the approach originating back in 2007 (Niedz & Evens 2007). In this method the macronutrients, mesonutrients, micronutrients, and iron are split into different solutions so the amounts of each can be varied over a number of different experiments.

Table 1. This kit contains these mineral nutrients as different solutions (groups) formulated as:

Group	Product No.	Components	Conc. [g/L]	Conc. [mM]	X [MS]	Volume [mL]
I	M5631	NH_4NO_3	165	2061	100	100
II	M5632	KNO_3	95	940	50	2 x 100
III	M5633	$CaCl_2{\cdot}2H_2O$	4.4	29.93	10	1000
		KH_2PO_4	1.7	12.49		
		$MgSO_4{\cdot}7H_2O$	3.7	15.01		
IV	M5634	H_3BO_3	0.62	10.03	100	100
		$CoCl_2{\cdot}6H_2O$	0.0025	0.011		
		$CuSO_4{\cdot}5H_2O$	0.0025	0.010		
		$MnSO_4{\cdot}H_2O$	1.69	10.00		
		$Na_2MoO_4{\cdot}2H_2O$	0.025	0.103		
		KI	0.083	0.500		
		$ZnSO_4{\cdot}7H_2O$	0.86	2.991		
V*	F318	$FeSO_4{\cdot}7H_2O$	2.78	10.00	100	100
		$Na_2EDTA{\cdot}2H_2O$	3.73	10.02		
	M5635	FeNaEDDHA	0.435	1.000	10	1000

*Both F318 and M5635 are provided in the kit so the user may determine the best chelate source for iron.

The most effective way to perform this type of mineral nutrient optimization is in a Design of Experiments (DOE) approach. DOE is preferred to changing concentrations in one-at-a-time

experiments because it requires less total experiments for the same precision in growth response estimation. A good introduction to DOE experiments which goes through these details is the book "DOE Simplified: Practical Tools for Effective Experimentation" by Mark J. Anderson and Patrick J. Whitcomb and is published by CRS Press. There are two main DOE approaches.

1.) 2-Level Factorial approach allows different mineral nutrients to be examined at high and low levels. This is not ideal for optimization, but can provide qualitative information to screen factors (groups).

2.) Response Surface Methods (RSM) can be used to define growth responses in a non-linear fashion with nutrient concentrations. This allows for an optimum concentration of a nutrient to be estimated along the growth response curve.

Inside each of these DOE approaches the most important variables set at the beginning of these experiments are the concentration ranges for use of these solutions. Shown below (Table 2) are the general concentration ranges we are specifying for the example optimizations (cont. on page 3)

Table 2. Concentration ranges used in the DOE example optimizations.

			X [MS]		Conc. [mg/L]		Conc. [mM]	
Group	Product No.	Components	Low-end Range	High-end Range	Low-end Range	High-end Range	Low-end Range	High-end Range
I	M5631	NH_4NO_3	0.125	1.5	206.25	2475	2.58	30.92
II	M5632	KNO_3	0.5	1.5	950	2850	9.40	28.19
III	M5633	$CaCl_2{\cdot}2H_2O$	0.5	1.65	220	726	1.50	4.94
		KH_2PO_4			85	280.5	0.62	2.06
		$MgSO_4{\cdot}7H_2O$			185	610.5	0.75	2.48
IV	M5634	H_3BO_3	0.5	2	3.1	12.4	0.05	0.20
		$CoCl_2{\cdot}6H_2O$			0.0125	0.05	5.25E-05	2.10E-04
		$CuSO_4{\cdot}5H_2O$			0.0125	0.05	5.01E-05	2.00E-04
		$MnSO_4{\cdot}H_2O$			8.45	33.8	0.05	0.20
		$Na_2MoO_4{\cdot}2H_2O$			0.125	0.5	5.17E-04	2.07E-03
		KI			0.415	1.66	2.50E-03	0.01
		$ZnSO_4{\cdot}7H_2O$			4.3	17.2	0.01	0.06
V	F318	$FeSO_4{\cdot}7H_2O$	0.25	1.5	6.95	41.7	0.02	0.11
		$Na_2EDTA{\cdot}2H_2O$			9.325	55.95	0.03	0.20
	M5635	FeNaEDDHA	0.25	1.5	10.875	65.25	0.03	0.15

provided in this manual. It should be noted that some species may prefer micronutrient concentrations up to 3X of that used in MS (Barbara Reed and Randall Niedz, personal communication). Additionally, some species may also prefer mesonutrient concentrations up to 2.5X of that used in MS. If these nutritional deficiencies are suspected, individual experiments to determine this may need to be performed.

Before performing a mineral nutrient optimization, it is useful to consider the species and composition of established media (Table 3) that have been developed. Murashige & Skoog's (MS) medium (Product No. M524, M519) has become the benchmark for tissue culture media because of the vast number of terrestrial plant species it can support in culture (Murashige and Skoog, 1962). Gamborg's B5 medium (Product No. G398) medium drastically reduced the amount of NH_4^+, and supplemented more K^+ in its place. The total NO_3^- on a molar basis is approximately half that of MS (Gamborg *et al.* 1968). Schenk & Hildebrandt's (S&H) medium is very similar to Gamborgs, but contains very low levels of Na^+ (Schenk and Hildebrandt, 1972)

Table 3. Established Plant Tissue Culture media and the species they were developed with.

Medium	Plant Species Developed with	Publication Year
Murashige & Skoog (MS)	Tobacco	1962
Gamborg B5	Soybean	1968
Schenk & Hildebrandt (S&H)	Various monocots & dicots	1972
Quoirin & Lepoivre (Q&L)	Prunus sp. (plums, cherries, etc.)	1977
Lloyd & McCown (WPM)	Mt. Laural (ericaceous)	1981
Driver & Kuniyuki (DKW)	Walnut	1984

Quoirin & Lepoivre (Q&L) (Product No. Q673) use roughly double the amount of NH_4^+ compared to Gamborg's and S&H, but still only 1/3 of the amount of ammonium (NH_4^+) relative to MS (Quoirin and Lepoivre 1977). Q&L however supplemented with higher amounts of calcium (Ca^{2+}). In Lloyd and McCown's medium (Woody Plant Medium, WPM, Product No. L154) potassium nitrate was eliminated and replaced with potassium sulfate (Lloyd and McCown, 1981). The levels of ammonium nitrate are ¼x compared to MS. The Ca^{2+} levels are similar to MS on a molar basis, but the fractional ion content of Ca^{2+} is similar to Q&L because WPM is a lighter medium in terms of total g/L.

The most concentrated of each of these media at 1X in terms of g/L, is Driver and Kuniyuki's (DKW) (Product No. D190). DKW media is triple the Ca^{2+} content of MS, and almost double that of Q&L (Driver and Kuniyuki 1984). DKW media also contains nearly 1.5X the amount of sulfate (SO_4^{2-}) over that of WPM.

Of these media that have been developed and have been successful for years in culturing plant species, 97-99% of the ions on a molar basis which constitute these media are from the macro and mesos (See Appendix for % ions on a molar basis for each of these media). These ion components in the macros and mesos are K^+, NH_4^+, Ca^{2+}, Mg^{2+}, NO_3^-, SO_4^{2-}, Cl-, and PO_4^{3-}. Of these 8 critical ions, Group I, II,

and III account for 6 of these ions (K^+, NH_4^+, Ca^{2+}, Mg^{2+}, NO_3^-, and PO_4^{3-}). Table 4 shows that the percentages of these 6 ions range from 78-97% in these established media.

Table 4. Percent ion concentration of K^+, NH_4^+, Ca^{2+}, Mg^{2+}, NO_3^-, and PO_4^{3-} in established media.

Medium	% Ions Macros and Mesos in Groups I-III
Murashige & Skoog (MS)	91.1%
Gamborg B5	92.5%
Schenk & Hildebrandt (S&H)	92.4%
Quoirin & Lepoivre (Q&L)	96.9%
Lloyd & McCown (WPM)	78.1%
Driver & Kuniyuki (DKW)	85.1%

Some woody plant media (WPM, DKW) contain significant amounts of sulfate (SO_4^{2-}) on a % ion basis in the media (see Appendix). In the event that the user is working with a woody plant, the user may want to consider preparing a potassium sulfate (Product No. P854) solution at 10 g/L (roughly 10X the concentration used in WPM) and introducing it as a factor (or group) to the DOE.

Two different iron chelates are provided for the researcher to experiment with as well. EDDHA has been found to be a more effective uptake chelator with iron over EDTA in some plant species, so both have been provided with this kit. Prior to performing the following example optimization, the user may want to decide in preliminary experiments which chelate performs best for their species.

Example Optimizations

The two example optimizations provided in this manual are:

1.) 5-factor (group), 2-level design at ½ fractional uses 16 treatments, which uses the 2-Level Factorial approach. This allows for high and low ends of the concentration range to be evaluated (Table 5).
2.) 3-factoral RSM design for the macros and mesos (Group I, II, and III). This design allows for a much more comprehensive look at a number of concentrations within each factor (group). Since the established media outlined here contain 78-97% macros and mesos (ions on a molar basis in Group I-III), it implies that optimizing the concentration of these three groups would initially provide more information on what different plant species may prefer.

The contents in this kit allow for either the 5-factor (group) 2-Level design at ½ fractional or the 3-factoral RSM design to be performed. If additional nutrients are required for additional studies these solutions can be purchased individually. In DOE approaches the different variables are often referred to

as factors. In the examples provided here we will refer to them as groups, since the different solutions are already categorized as that.

Table 5. 5-factor (group), 2-Level design at ½ fractional design.

Design points	Groups (all values are expressed in strength, X of MS media)				
	I	II	III	IV	V
1	1.5	0.5	1.65	2	0.25
2	1.5	1.5	1.65	0.5	0.25
3	0.125	1.5	1.65	0.5	1.5
4	1.5	1.5	0.5	0.5	1.5
5	0.125	0.5	1.65	2	1.5
6	1.5	0.5	1.65	0.5	1.5
7	1.5	1.5	1.65	2	1.5
8	1.5	1.5	0.5	2	0.25
9	1.5	0.5	0.5	0.5	0.25
10	1.5	0.5	0.5	2	1.5
11	0.125	1.5	0.5	0.5	0.25
12	0.125	0.5	0.5	0.5	1.5
13	0.125	0.5	0.5	2	0.25
14	0.125	1.5	1.65	2	0.25
15	0.125	0.5	1.65	0.5	0.25
16	0.125	1.5	0.5	2	1.5

Protocol to prepare Design Point 1 in the 5-factor (group), 2-Level design at ½ fractional design

An Example Calculation of how to determine the volume of each nutrient is in the Appendix (Table A1)

1.) Obtain a container large enough to prepare 0.5L of the medium in
2.) Add 7.5 mL of Group I (NH_4NO_3) 100X MS [Product No. M5631]
3.) Add 5 mL of Group II (KNO_3) 50X MS [Product No. M5632]
4.) Add 82.5 mL of Group III Mesonutrients 10X MS [Product No. M5633]
5.) Add 10 mL of Group IV Micronutrients 100X MS [Product No. M5634]
6.) Depending on the chelate source you choose:
    a. Add 1.25 mL of Iron Sulfate/Chelate Solution [Product No. F318] or
    b. Add 12.5 mL of Group V FeNaEDDHA [Product No. M5635]
7.) Add any carbohydrates, plant growth regulators, gelling agents, or other supplements as necessary
    a. If gellan gum (e.g. G434, G3251) is used, the final Ca^{2+} and Mg^{2+} combined concentrations should be between 3-12 mM to form rigid gels
8.) Add additional tissue culture grade water to bring the medium to the final volume of 0.5L.

9.) While stirring, determine the pH. If necessary, adjust the medium to the desired pH using potassium hydroxide to raise the pH or hydrochloric acid to lower the pH. A pH of 5.6 to 5.8 is typically recommended for most plants.

10.) Sterilize the medium in a validated autoclave or pressure 9+cooker at 1 kg/cm2, 121 °C (15 psi, 250 ° F), for the time period described under "Sterilization of Media" below.

11.) Allow medium to cool prior to use.

To perform the 3-factorial RSM design to look at the macros and mesos (Table 6.), the micros and iron concentration should be set throughout the experiment. Some preliminary experiments should be performed to ascertain what the micronutrient and iron solution concentrations should be set to. Micronutrient optimization is very complex because the interactions of the elements vary with the plant species tested, but a wide range to test from would be 0.5 to 3X the MS levels.

Table 6. 3-factorial (group) RSM Design.

Design points	Groups (all values are expressed in strength, X of MS media)		
	I	II	III
1	0.125	1.5	0.5
2	0.8125	0.5	1.65
3	0.8125	1.5	1.65
4	0.125	0.5	0.5
5	0.8125	1	0.7875
6	1.5	1	1.65
7	1.5	0.5	0.5
8	1.5	1.5	0.5
9	0.125	1	1.65
10	1.5	1.5	1.65
11	0.8125	1	0.7875
12	0.8125	0.5	0.5
13	0.125	1.5	0.5
14	0.125	0.5	1.075
15	0.125	0.5	1.075
16	1.5	1.5	0.5
17	1.5	0.5	0.5
18	0.125	1	0.5
19	1.5	0.5	1.65
20	0.125	1.5	1.65

Protocol to prepare Design Point 2 in the 3-factorial (group) RSM design

An Example Calculation of how to determine the volume of each nutrient is in the Appendix (Table A2)

1.) Obtain a container large enough to prepare 0.5L of the medium in
2.) Add 4.06 mL of Group I (NH_4NO_3) 100X MS [Product No. M5631]
3.) Add 5 mL of Group II (KNO_3) 50X MS [Product No. M5632]
4.) Add 82.5 mL of Group III Mesonutrients 10X MS [Product No. M5633]
5.) Add set volume of Group IV Micronutrients 100X MS [Product No. M5634]
6.) Depending on the chelate source you choose:
    a. Add set volume of Iron Sulfate/Chelate Solution [Product No. F318] or
    b. Add set volume of Group V FeNaEDDHA [Product No. M5635]
7.) Add any carbohydrates, plant growth regulators, gelling agents, or other supplements as necessary
    c. If gellan gum (e.g. G434, G3251) is used, the final Ca^{2+} and Mg^{2+} combined concentrations should be between 3-12 mM to form rigid gels
8.) Add additional tissue culture grade water to bring the medium to the final volume of 0.5L.
9.) While stirring, determine the pH. If necessary, adjust the medium to the desired pH using potassium hydroxide to raise the pH or hydrochloric acid to lower the pH. A pH of 5.6 to 5.8 is typically recommended for most plants.
10.) Sterilize the medium in a validated autoclave or pressure cooker at 1 kg/cm^2, 121 °C (15 psi, 250 ° F), for the time period described under "Sterilization of Media" below.
11.) Allow medium to cool prior to use.

Note: There are many other 2-Level and RSM factorial designs that can be performed with this kit; only a couple of examples are provided here. **A spreadsheet which codes the actual X of MS for each solution in 2, 3, 4, and 5-factorial (group) RSM design is available on the phytotechlab.com website. This spreadsheet will also allow the user to change the concentration ranges for each nutrient within these designs**.

Sterilization of Media

Plant tissue culture media are generally sterilized by autoclaving at 121°C and 1.05 kg/cm^2 (15 psi). This high temperature not only kills bacteria and fungi, but also their heat-resistant spores. Media can be sterilized in either an autoclave or pressure cooker with similar results. The time required for sterilization depends upon the volume of medium, type of vessel, and the type of autoclave. The suggested minimum times required for sterilization of different media volumes are listed below. It is advisable to dispense medium in small aliquots whenever possible as many media components are broken down by prolonged exposure to heat.

Volume of Medium per Vessel [mL]	Suggested Minimum Autoclaving Time [mL]
25	15-20
50	25
100	28
250	31
1000	40
2000	48
4000	63

Please Note: Minimum autoclaving time includes the time required for the liquid volume to reach the sterilizing temperature (121°C) and 15 minutes at 121°C (Burger, 1988). Times may vary due to differences in autoclaves. Validation with your autoclave or pressure cooker is recommended. Use of a product such as the Diack® Sterilization Indicators [Product No. S748] is useful for this purpose.

Acknowledgements:

We wish to thank Randall Niedz and Barbara Reed for their input of how DOE should be incorporated into a general kit.

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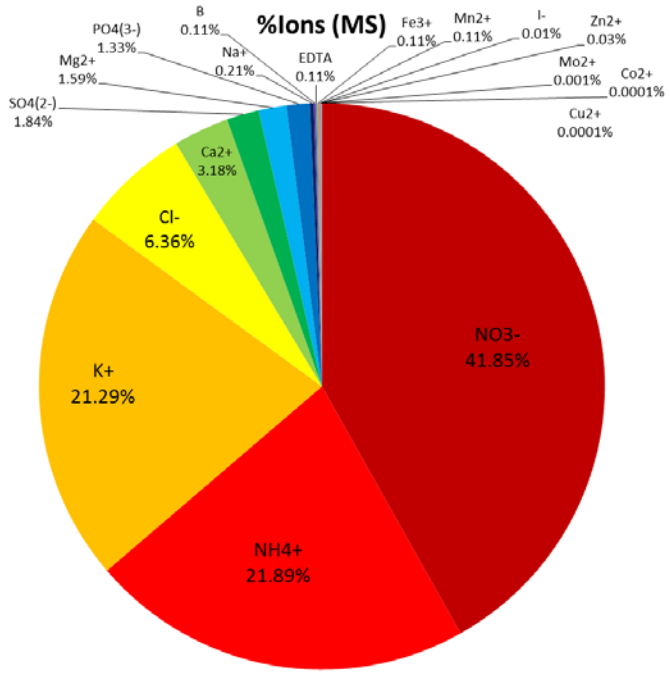
Appendix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K+
21.29%
Cl-
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Ca2+
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SO4(2-)
1.84%
Mg2+
1.59%
PO4(3-)
1.33%
B
0.11%
Na+
0.21%
EDTA
0.11%
Fe3+
0.11%
Mn2+
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I-
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Co2+
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Cu2+
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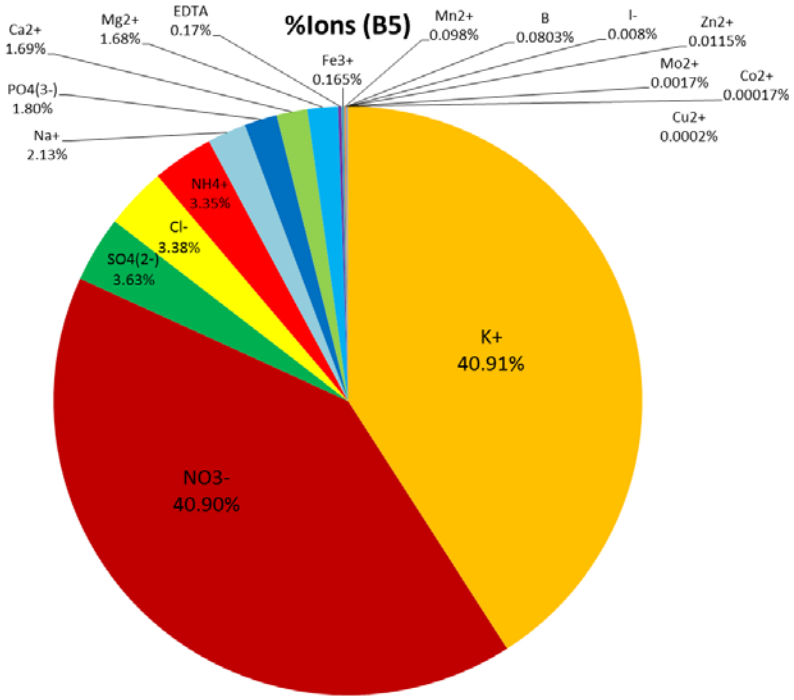
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SO4(2-)
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NH4+
3.35%
Na+
2.13%
PO4(3-)
1.80%
Ca2+
1.69%
Mg2+
1.68%
EDTA
0.17%
Fe3+
0.165%
Mn2+
0.098%
B
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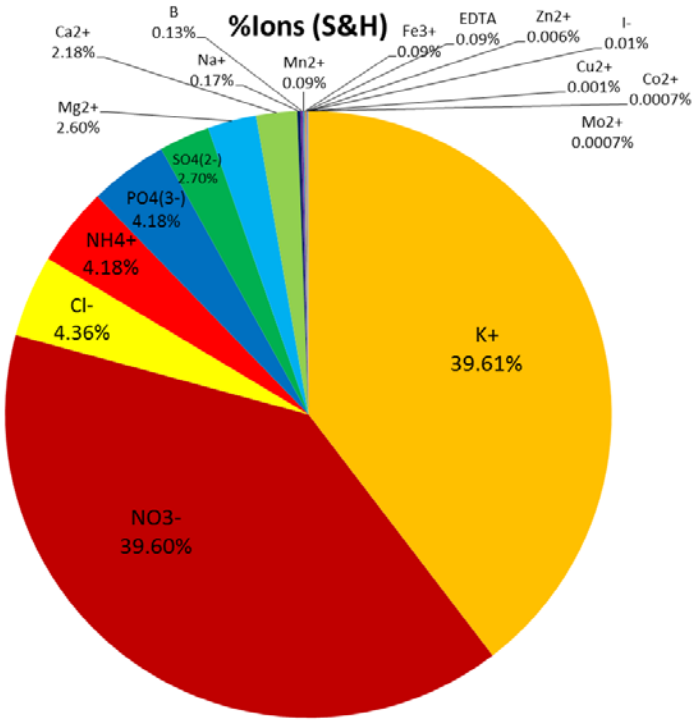
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Na+ 0.17%
Mn2+ 0.09%
Fe3+ 0.09%
EDTA 0.09%
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I- 0.01%
Cu2+ 0.001%
Co2+ 0.0007%
Mo2+ 0.0007%
Mg2+ 2.60%
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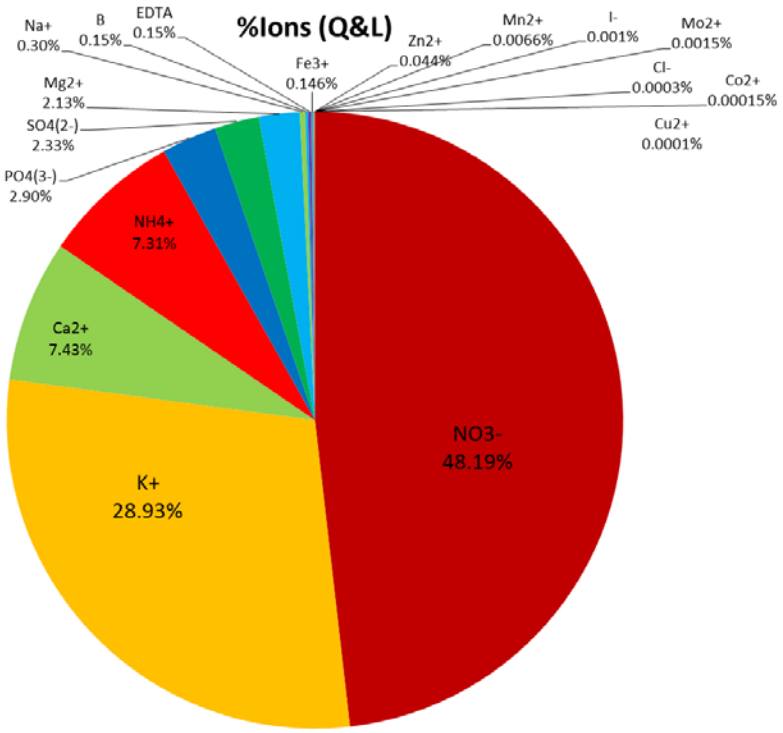
Cl- 4.36%
K+ 39.61%
NO3- 39.60%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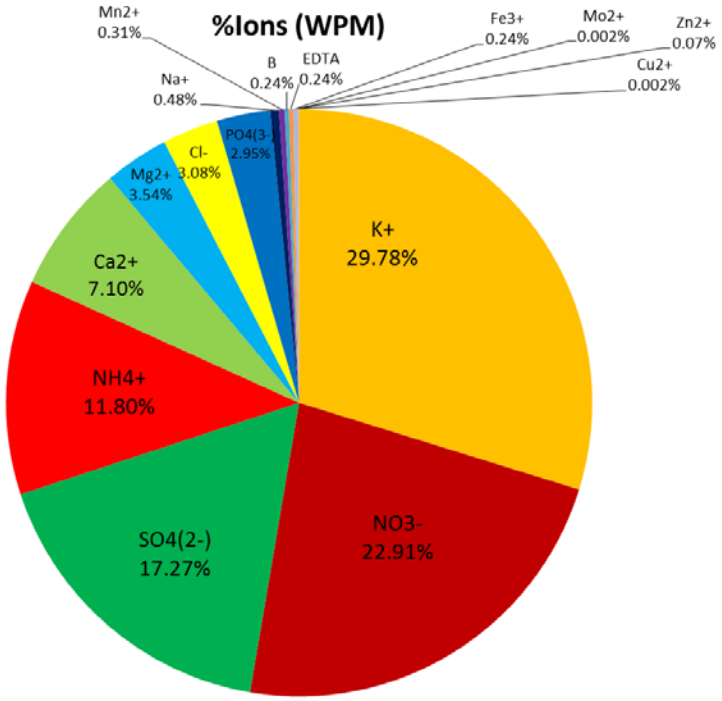
Zn2+ 0.044%
Mn2+ 0.0066%
I- 0.001%
Mo2+ 0.0015%
Cl- 0.0003%
Co2+ 0.00015%
Cu2+ 0.0001%
Mg2+ 2.13%
SO4(2-) 2.33%
PO4(3-) 2.90%
NH4+ 7.31%
Ca2+ 7.43%
K+ 28.93%
NO3- 48.19%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Fe3+ 0.24%
Mo2+ 0.002%
Zn2+ 0.07%
Cu2+ 0.002%
B 0.24%
EDTA 0.24%
Na+ 0.48%
PO4(3-) 2.95%
Cl- 3.08%
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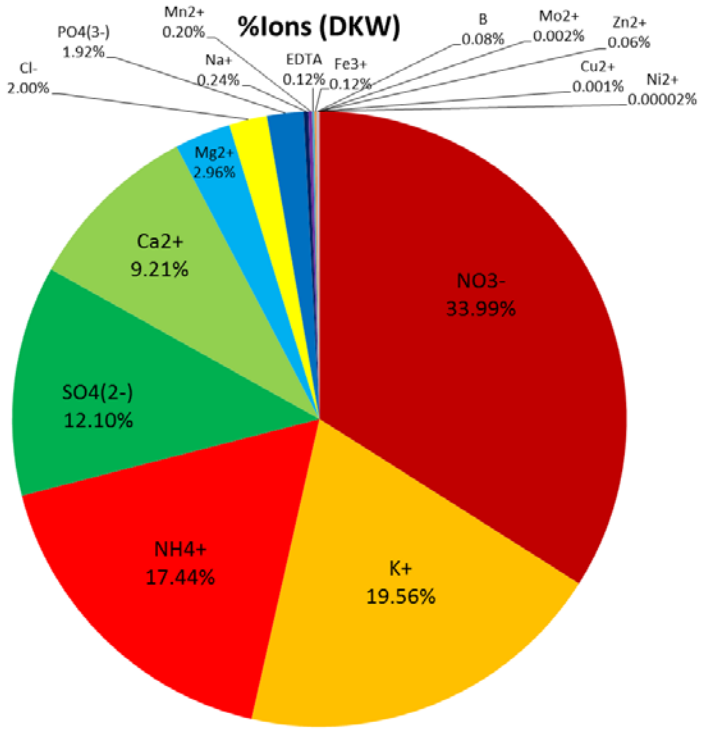
Ca2+ 7.10%
NH4+ 11.80%
K+ 29.78%
SO4(2-) 17.27%
NO3- 22.91%



Example Calculation:

$$V_{ADD} = V_{TOTAL}\left(\frac{X_{TABLE}}{X_{CONC.SOL.}}\right)$$

Where: V_{ADD} = Volume needed to add to media to achieve the X (of MS) listed on the table

V_{TOTAL} = Total volume of media being prepared for the design point

X_{TABLE} = X (of MS) prescribed for the group in the table

$X_{CONC.SOL.}$ = X (of MS) of the concentrated solution

For design point 2 in the 3-factorial (group) RSM Method with Group I

X_{TABLE} = 0.8125X (of MS) prescribed for the group in the table

V_{TOTAL} = 500 mL (0.5L)

$X_{CONC.SOL.}$ = 100X (of MS) for Group I

$$V_{ADD} = 500mL\left(\frac{0.8125X}{100X}\right) = 4.06\ mL\ of\ Group\ I\ to\ 500\ mL\ of\ media$$

Table A1. Volume per design point in 5-factor (group), 2-Level design at ½ fractional design.

Design points	Groups (volume of mL to add to 500 mL)					
	I	II	III	IV	V (EDTA)	or, V (EDDHA)
1	7.5	5	82.5	10	1.25	12.5
2	7.5	15	82.5	2.5	1.25	12.5
3	0.625	15	82.5	2.5	7.5	75
4	7.5	15	25	2.5	7.5	75
5	0.625	5	82.5	10	7.5	75
6	7.5	5	82.5	2.5	7.5	75
7	7.5	15	82.5	10	7.5	75
8	7.5	15	25	10	1.25	12.5
9	7.5	5	25	2.5	1.25	12.5
10	7.5	5	25	10	7.5	75
11	0.625	15	25	2.5	1.25	12.5
12	0.625	5	25	2.5	7.5	75
13	0.625	5	25	10	1.25	12.5
14	0.625	15	82.5	10	1.25	12.5
15	0.625	5	82.5	2.5	1.25	12.5
16	0.625	15	25	10	7.5	75

Table A2. Volume per design point in 3-factorial (group) RSM Design.

Design points	Groups (volume of mL to add to 500 mL)		
	I	II	III
1	0.625	15	25
2	4.0625	5	82.5
3	4.0625	15	82.5
4	0.625	5	25
5	4.0625	10	39.375
6	7.5	10	82.5
7	7.5	5	25
8	7.5	15	25
9	0.625	10	82.5
10	7.5	15	82.5
11	4.0625	10	39.375
12	4.0625	5	25
13	0.625	15	25
14	0.625	5	53.75
15	0.625	5	53.75

16	7.5	15	25
17	7.5	5	25
18	0.625	10	25
19	7.5	5	82.5
20	0.625	15	82.5

Notes:

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