RIDASCREEN® Total Gluten

Art. No. R7041

Validation report

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RIDASCREEN® Total Gluten

Art. No. R7041

Content

1. Method

1.1. General
1.2. Sample preparation (extraction)
1.3. Spike instructions
1.4. Test principle
1.5. Calibration curve

2. Method characteristics

2.1 Specificity
2.2. Interferences and sources of error
2.3. Sensitivity
· Limit of detection (LoD)
· Limit of quantification (LoQ)
2.4. Recovery
2.5. Cross reactivities

3. Method uncertainty

3.1. Precision of extraction
3.2. Repeatability (intra-assay-variation)
3.3. Within-laboratory-reproducibility (inter-assay-variation)
3.4. Robustness
3.5. Stability of the test

4. Conclusion

5. Limitations

6. List of references

7. List of abbreviations

8. Appendix 1 – Quality Assurance Certificate

9. Appendix 2 – Spike instructions

10. Appendix 3 – Equipment

RIDASCREEN® Total Gluten

Art. No. R7041

1. Method

The RIDASCREEN® Total Gluten assay was developed according to the AOAC SMPR® 2017.021. [1], AOAC Appendix M [2] and CLSI guideline EP25-A for stability testing of in-vitro diagnostics [3]. The assay is approved by the AOAC as *Official Method of Analysis* (AOAC *Official Method of Analysis* 2018.15) for determination of gluten in oat and oat products [4].

1.1. General

The RIDASCREEN® Total Gluten is a sandwich enzyme immunoassay for the quantitative determination of intact (non-hydrolyzed) gluten from gluten containing cereals (wheat, rye and barley) in oat and oat products. It is a sandwich ELISA based on a mixture of antibodies against the two protein groups of gluten – the prolamins and glutelins. Hence it determines gluten at all. One of the used antibodies is the R5 antibody. More information on the used antibodies and their target gluten proteins can be found in the test kit instructions.

Since its introduction to the analytical community, the R5 method to quantify gliadin led to a strong improvement of the situation for the food industry and celiac patients. But, during the last years some questions arose on the use of the Codex Alimentarius factor of 2 to convert from prolamins (gliadin) to gluten, an overestimation of rye and barley, inadequate detection of glutelins and the inhomogeneous distribution of gluten in oats. These limitations of the R5 method, especially when measuring oat samples led to AOAC Standard Method Performance Requirement (SMPR®) 2017.021, which was approved by stakeholders in 2017.

The RIDASCREEN® Total Gluten was tested with the following matrices: oat flour, flaked oats, oat cereals, groats, oat cookies and oat porridge.

1.2. Sample preparation (extraction)

The distribution of gluten contaminations from wheat, rye or barley in oat samples can be very inhomogeneous. Furthermore, the samples are difficult to homogenize. Therefore, grind and homogenize well at least 200 g. Hereof, 1 g is used for extraction. The Cocktail (patented) (Art. No. R7006/R7016) is recommended for sample extraction. Tannin and polyphenol containing oat products (e.g. oat products with high content of chocolate, coffee, cocoa, chestnut flour, teff flour, buckwheat, millet and spices) should be extracted with the addition of 1 g skim milk powder. Detailed information can be found in the test kit instruction.

1.3. Spike instructions

Instructions on spiking are described in Appendix 2.

RIDASCREEN® Total Gluten

Art. No. R7041

1.4. Test principle

The wells of the microtiter strips are coated with specific antibodies against the gluten proteins. Present gluten proteins in positive samples will bind to the antibodies. Second antibodies conjugated to peroxidase bind to the antibody-antigen-complex. The bound conjugate converts then a substrate/chromogen into a blue product. Its absorbance is measured photometrically after addition of stop solution and is proportional to the gluten concentration of the sample.

1.5. Calibration curve

The RIDASCREEN® standard material for measuring the calibration curve is a total gluten extract from four wheat varieties. The results with this standard material are traceable to the oat samples described in the AOAC SMPR® 2017.021. [1]
The calculation should be done by use of a 4-parameter function. A typical calibration curve for RIDASCREEN® Total Gluten (Art. No. R7041) is shown in Appendix 1. The lot-specific course of the standard curve is shown in the Quality Assurance Certificate enclosed in the test kit.

2. Method characteristics

2.1 Specificity

Target analytes of the new RIDASCREEN® Total Gluten are all gluten fractions from wheat, rye and barley with the exception of D-hordein from barley. These are in detail: intact (non-hydrolyzed) gliadins and related proteins from wheat, rye and barley, High-Molecular Weight (HMW) Glutenin-Subunits (GS) from wheat, HMW-secalins from rye and Low-Molecular-Weight (LMW)-GS from wheat. Further information on the specificity of the four different antibodies can be found in the test kit instruction.

2.2. Interferences and sources of error

Tannins and polyphenols may impact the assay result and above mentioned recommendation regarding addition of skim milk powder during extraction should be followed.
Apple fibers may impact gluten recovery. In experiments with pure apple fibers recovery was below 30 %. Due to the inhomogeneity of samples, it can be necessary to further increase the weighted sample (>1 g).

RIDASCREEN® Total Gluten

Art. No. R7041

2.3. Sensitivity

Limit of detection (LoD)

It is difficult to get natural oat samples that are really free of any contamination by wheat, rye or barley. However, a true blank sample can be prepared by particular purification procedures. Such a specially prepared sample was included in a collaborative study done with the RIDASCREEN® Total Gluten (see tables 11 and 12; sample 4). The data were presented during an AOAC Expert Review Panel (ERP) meeting in December 2018 in the course of the AOAC OMA approval. Due to the difficulty of getting natural true blank samples the ERP recommended to use these sample data for estimation of LoD. The mean value of all laboratories for the sample was 0.9 mg/kg gluten with a standard deviation of 0.96 mg/kg. According to this, a value of 4 mg/kg gluten was estimated as the LoD (approx. mean + 3 x SD). This value is mentioned in the test kit instruction.

Limit of quantification (LoQ)

As described above, it was not possible to obtain true blank samples for oat flours, flakes and cereals. It was therefore decided to verify the LoQ by a spiking experiment at low level of 4 mg/kg gluten. The experiment was repeated in a second lot. As shown in table 1, mean values for blank samples were as expected from the LoD experiment. When spiking 4 mg/kg gluten, the mean recoveries (corrected for blank samples) ranged from 64 % up to 104 %. CVs were at or below 15 %. This shows that quantification at the 5 mg/kg gluten level is possible with acceptable precision.

Table 1: Verification of LoQ in oat flakes, cereals and flour by extracting 10 replicates of the blank samples and spiked in n=10 at a 4 mg/kg gluten level; for each matrix and test kit lot the mean concentration, standard deviation and coefficient of variation is calculated; the recovery is calculated by using the mean concentration values (corrected for blank samples).

		Blank samples			Samples spiked with 4 mg/kg			
Sample	Kit	Mean (mg/kg)	SD (mg/kg)	CV (%)	Mean (mg/kg)	SD (mg/kg)	CV (%)	Recovery (%)
Flour	Lot 1	3.14	0.64	20.4	5.68	0.64	11.2	**64**
	Lot 2	2.41	0.62	25.8	6.17	0.47	7.7	**87**
Flakes	Lot 1	2.67	0.13	4.7	5.67	0.85	15.0	**82**
	Lot 2	1.87	0.10	5.3	6.01	0.58	9.6	**104**
Cereals	Lot 1	5.50	0.28	5.1	8.85	1.23	13.9	**84**
	Lot 2	3.60	0.27	7.4	6.97	0.75	10.8	**84**

RIDASCREEN® Total Gluten

Art. No. R7041

2.4. Recovery

Recovery using well-defined, spiked samples

To characterize for traceability and trueness, recovery of well-defined samples has been analyzed. For this purpose, the reference materials mentioned in AOAC SMPR® 2017.021 [1] were analyzed with 10 replicates in three different lots (table 2). The materials contain two levels of gluten (10 mg/kg and 20 mg/kg).

Table 2: Analysis of the oat reference materials mentioned in AOAC SMPR® 2017.021 to characterize the RIDASCREEN® Total Gluten for trueness at gluten levels of 10 mg/kg and 20 mg/kg respectively (10 replicates of each sample tested with three different kit lots).

			Lot 1 mg/kg	Lot 2 mg/kg	Lot 3 mg/kg
Wheat in oats	10 mg/kg	Mean of 10 replicates	10.5	9.7	11.5
		Mean recovery (%)	105	97	115
		SD	1.69	1.58	1.42
		CV (%)	16.1	16.3	12.3
	20 mg/kg	Mean of 10 replicates	19.3	18.7	19.9
		Mean recovery (%)	97	94	100
		SD	1.32	1.25	1.52
		CV (%)	6.8	6.7	7.6
Rye in oats	10 mg/kg	Mean of 10 replicates	15.0	17.4	16.5
		Mean recovery (%)	150	174	165
		SD	1.51	2.54	1.87
		CV (%)	10.1	14.6	11.3
	20 mg/kg	Mean of 10 replicates	27.3	30.7	27.7
		Mean recovery (%)	137	154	139
		SD	2.84	2.97	2.89
		CV (%)	10.4	9.7	10.4
Barley in oats	10 mg/kg	Mean of 10 replicates	10.3	13.1	12.6
		Mean recovery (%)	103	131	126
		SD	2.62	2.01	1.58
		CV (%)	25.4	15.3	12.5
	20 mg/kg	Mean of 10 replicates	17.6	21.2	19.9
		Mean recovery (%)	88	106	100
		SD	4.09	5.11	3.27
		CV (%)	23.2	24.1	16.4

At the 10 mg/kg gluten level, recoveries from 97 % up to 115 % were found for wheat contaminated oat flour while for barley materials the recoveries were somewhat higher between 103 % and 131 %.
The highest recoveries between 150 % and 174 % were found for the rye contaminated oat flour.
At the 20 mg/kg gluten level, recoveries from 88 % up to 106 % were found for wheat and barley contaminated oat flour while for rye recoveries between 137 % and 154 % were found. All recoveries were within the requirements of the AOAC SMPR® 2017.021[1].

RIDASCREEN® Total Gluten

Art. No. R7041

Recovery using naturally incurred and processed oat samples

For characterization of recovery from naturally incurred and processed samples, oat cookies (baked for 21 minutes at 175 °C) and oat-based porridge (cooked for 5 min at 100 °C) were produced using oat flour that was contaminated at gluten levels of 2000 mg/kg. The contaminating wheat, rye, and barley were identical to the ones that were used to produce the AOAC reference material.

Table 3: Results obtained for oat-based cookies incurred with two different levels of gluten from wheat or rye or barley; mean concentrations and standard deviations derived from three kit lots.

Sample	Mean (mg/kg)	SD (mg/kg)	CV (%)	Recovery (%)
Wheat gluten 11.5 mg/kg	7.47	2.31	30.9	65
Rye gluten 11.5 mg/kg	11.5	1.62	14.2	100
Barley gluten 11.5 mg/kg	14.0	1.73	12.4	122
Wheat gluten 46 mg/kg	25.1	2.42	9.70	55
Rye gluten 46 mg/kg	38.0	3.88	10.2	83
Barley gluten 46 mg/kg	39.2	4.17	10.6	85

It seems that wheat gluten is more susceptible to heat treatment than the other two gluten containing grains, rye and barley. Nevertheless, all recoveries clearly are within the performance criteria laid down in AOAC SMPR® 2017.021 [1] (50 - 200 %).

Table 4: Results obtained for oat-based porridge incurred with two different levels of gluten from wheat or rye or barley; mean concentrations and standard deviations derived from three kit lots.

Sample	Mean (mg/kg)	SD (mg/kg)	CV (%)	Recovery (%)
Wheat gluten 8 mg/kg	7.86	1.35	17.2	82
Rye gluten 8 mg/kg	8.54	0.80	9.40	104
Barley gluten 8 mg/kg	9.39	3.63	38.6	121
Wheat gluten 32 mg/kg	24.1	3.21	13.3	70
Rye gluten 32 mg/kg	27.3	4.71	17.3	86
Barley gluten 32 mg/kg	25.7	2.87	11.2	84

In case of porridge, the recoveries for all three contaminating grains were more comparable. The recoveries for rye and barley in porridge are almost identical to those in the cookies, whereas recoveries for wheat are higher in porridge, probably due to the lower level of heat treatment. All recoveries stated in table 4 are within the performance criteria laid down in AOAC SMPR® 2017.021 [1].

RIDASCREEN® Total Gluten

Art. No. R7041

Recovery using spiked oat containing samples

In addition to incurred samples, oat flour, oat flakes and oat cereals were spiked with gluten from wheat over the whole calibration range (7 mg/kg up to 77 mg/kg).

Spiking was performed twice for each concentration level using two independent spiking solutions. The samples' gluten concentration (target) does not only include gluten from the spike but also gluten that is already present in the blank samples due to a low level of gluten contamination.

Results are presented in table 5 for three different test kit lots and each concentration analyzed in duplicate. Mean recoveries (not shown in the table) over all lots and concentration levels were 84 % in flour, 81 % in flakes and 75 % in cereals.

Table 5: Results from spiking experiments of oat flour, oat flakes and oat cereals at 4 different levels with two replicates per concentration and tested lot; gluten from wheat was used for this experiment; for recovery calculation, the analyzed result was compared with the target concentration (sample base level plus spike concentration).

	Lot 1			Lot 2			Lot 3		
Spike mg/kg	Target* (mg/kg)	Result (mg/kg)	Recovery (%)	Target* (mg/kg)	Result (mg/kg)	Recovery (%)	Target* (mg/kg)	Result (mg/kg)	Recovery (%)
		Flour			Flour			Flour	
0	–	2.60	–	–	1.56	–	–	3.51	–
	–	2.85	–	–	1.56	–	–	3.54	–
7	9.7	7.63	78	8.6	7.45	87	10.5	8.42	80
	9.7	5.96	61	8.6	5.56	65	10.5	7.08	67
17	19.7	17.38	88	18.6	17.50	94	20.5	19.78	96
	19.7	16.65	84	18.6	17.34	93	20.5	19.11	93
37	39.7	31.37	79	38.6	33.39	87	40.5	35.45	87
	39.7	33.01	83	38.6	34.71	90	40.5	36.64	90
77	79.7	64.07	80	78.6	70.66	90	80.5	72.19	90
	79.7	60.89	76	78.6	66.19	84	80.5	66.97	83
		Flakes			Flakes			Flakes	
0	–	2.77	–	–	2.01	–	–	3.08	–
	–	3.35	–	–	1.85	–	–	3.45	–
7	10.1	6.68	66	8.9	5.92	66	10.3	7.24	71
	10.1	6.05	60	8.9	5.38	60	10.3	7.70	75
17	20.1	17.59	88	18.9	16.86	89	20.3	19.11	94
	20.1	17.54	87	18.9	16.89	89	20.3	19.27	95
37	40.1	33.83	84	38.9	34.42	88	40.3	36.11	90
	40.1	35.79	89	38.9	35.72	92	40.3	36.93	92
77	80.1	59.03	74	78.9	60.20	76	80.3	65.34	81
	80.1	58.32	73	78.9	60.14	76	80.3	65.34	81
		Cereals			Cereals			Cereals	
0	–	3.74	–	–	2.90	–	–	4.24	–
	–	5.08	–	–	2.74	–	–	4.50	–
7	11.4	6.77	59	9.8	7.25	74	11.4	7.97	70
	11.4	6.43	56	9.8	5.49	56	11.4	6.77	60
17	21.4	16.03	75	19.8	17.10	86	21.4	18.22	85
	21.4	16.39	77	19.8	17.23	87	21.4	18.42	86
37	41.4	35.86	87	39.8	37.21	93	41.4	38.62	93
	41.4	29.89	72	39.8	30.94	78	41.4	33.38	81
77	81.4	54.40	67	79.8	58.03	73	81.4	61.47	76
	81.4	55.21	68	79.8	56.80	71	81.4	60.74	75

* Target concentration: sample base level plus spike concentration

RIDASCREEN® Total Gluten

Art. No. R7041

Recovery with original wheat species

To test reactivity of original wheat species like einkorn or emmer, gluten has been extracted from different varieties of four original wheat species. The extracts have been diluted and tested for recovery. Results are shown in table 6. Good recoveries were found with all varieties.

Table 6: Mean recoveries from the analysis of the gluten content of different varieties of Emmer, Einkorn, Durum wheat, and Spelt.

Emmer		Einkorn		Durum wheat		Spelt	
Variety	Mean recovery	Variety	Mean recovery	Variety	Mean recovery	Variety	Mean recovery
CC1E-0405 8/01	77 %	8.108/04	64 %	Wintergold	109 %	Badenkrone	97 %
Heulholzer Kolben	106 %	M-04018/01	75 %	Lunadur	102 %	Badenstern	95 %
Osiris	111 %	Monlis	94 %	Logidur	98 %	Franckenkorn	100 %
Ramses	114 %	Terzino	78 %	Elsadur	104 %	Oberkulmer Rotkorn	88 %
Teutonia	126 %	Tifi	99 %	Auradur	105 %	Zollernspelz	85 %

2.5. Cross reactivities

The cross reactivity panel (table 7) is slightly modified but still according to Koerner et al. [5] and represents the opinion of the AOAC Allergen community. Some commodities were added to the list due to long lasting experiences of the manufacturer. In total, 83 commodities were tested.

The commodities were extracted once with Cocktail (patented) and 80 % ethanol and tested in the RIDASCREEN® Total Gluten. Some commodities were tested by adding skim milk powder to mask the interference by polyphenols.

Table 7 clearly shows that no cross reactivities (OD of sample > OD of standard 2) exists against the 83 tested commodities. A lot of these commodities are used to compose alternative food for CD patients.

Table 7: Compounds tested for cross reactivities and results of OD readings of these extracts compared to standard 1 (0 mg/kg gluten) and standard 2 (5 mg/kg gluten); compounds marked with an asterisk were extracted with addition of 1 g of gluten-free skim milk powder to mask polyphenols and other interfering substances.

	OD standard 1	OD standard 2	OD sample	Result (mg/kg)
Cereals				
Amaranth	0.143	0.414	0.112	< LoQ
Arrowroot	0.187	0.444	0.116	< LoQ
Buckwheat, flour*	0.143	0.414	0.225	< LoQ
Chestnut, flour*	0.187	0.444	0.114	< LoQ
Corn starch	0.106	0.429	0.255	< LoQ
Millet, flour*	0.161	0.426	0.159	< LoQ
Quinoa, flour	0.161	0.426	0.177	< LoQ
Rice flour, sweet	0.187	0.444	0.141	< LoQ
Rice flour, white	0.187	0.444	0.243	< LoQ
Tapioca, flour	0.228	0.445	0.265	< LoQ
Teff, flour*	0.160	0.443	0.238	< LoQ

RIDASCREEN® Total Gluten

Art. No. R7041

	OD standard 1	OD standard 2	OD sample	Result (mg/kg)
Beans/lentils/peas				
Black bean, flour	0.143	0.414	0.130	< LoQ
Chick peas	0.106	0.429	0.214	< LoQ
Fava beans	0.143	0.414	0.234	< LoQ
Garbanzo beans	0.187	0.444	0.242	< LoQ
Guar gum	0.161	0.426	0.186	< LoQ
Green beans	0.161	0.426	0.152	< LoQ
Green pea, flour	0.161	0.426	0.151	< LoQ
Lentil, flour	0.161	0.426	0.153	< LoQ
Lima bean, flour	0.161	0.426	0.151	< LoQ
Lupine, flour	0.161	0.426	0.178	< LoQ
Pea flour, yellow	0.161	0.426	0.173	< LoQ
Peanut, raw	0.161	0.426	0.168	< LoQ
Peanut, roasted	0.161	0.426	0.173	< LoQ
Romano bean, flour	0.161	0.426	0.192	< LoQ
Sorghum, flour	0.111	0.390	0.113	< LoQ
Soya, flour	0.161	0.426	0.201	< LoQ
Soya milk	0.228	0.445	0.238	< LoQ
Soya protein	0.228	0.445	0.219	< LoQ
White bean, flour	0.111	0.390	0.212	< LoQ
Seeds				
Carob, seedlings	0.143	0.414	0.200	< LoQ
Flax seed	0.143	0.414	0.269	< LoQ
Pistachio	0.161	0.426	0.164	< LoQ
Poppy seed	0.161	0.426	0.176	< LoQ
Sesame, flour	0.161	0.426	0.218	< LoQ
Sunflower kernel	0.228	0.445	0.255	< LoQ
Nuts				
Almond, raw	0.143	0.414	0.124	< LoQ
Almond, roasted	0.143	0.414	0.132	< LoQ
Cashew, raw	0.143	0.414	0.176	< LoQ
Hazelnut, flour	0.161	0.426	0.130	< LoQ
Hazelnut, raw	0.161	0.426	0.149	< LoQ
Hazelnut, roasted	0.161	0.426	0.147	< LoQ
Walnut, raw	0.228	0.445	0.219	< LoQ
Macadamia, raw	0.161	0.426	0.144	< LoQ
Meat				
Beef and pork hash	0.143	0.414	0.139	< LoQ
Chicken	0.143	0.414	0.176	< LoQ
Sausage	0.161	0.426	0.215	< LoQ
Turkey han	0.228	0.445	0.211	< LoQ

RIDASCREEN® Total Gluten

Art. No. R7041

	OD standard 1	OD standard 2	OD sample	Result (mg/kg)
Spices				
Anise*	0.187	0.444	0.240	< LoQ
Basil*	0.106	0.429	0.325	< LoQ
Caraway*	0.187	0.444	0.208	< LoQ
Cinnamon*	0.143	0.414	0.202	< LoQ
Cloves*	0.143	0.414	0.246	< LoQ
Coriander *	0.187	0.444	0.147	< LoQ
Curcuma*	0.143	0.414	0.315	< LoQ
Curry*	0.106	0.429	0.149	< LoQ
Fennel*	0.106	0.429	0.154	< LoQ
Garlic*	0.143	0.414	0.287	< LoQ
Ginger*	0.187	0.444	0.147	< LoQ
Spices				
Majoram*	0.160	0.460	0.220	< LoQ
Mustard powder*	0.161	0.426	0.165	< LoQ
Mustard *	0.161	0.426	0.150	< LoQ
Nutmeg*	0.161	0.426	0.145	< LoQ
Paprika*	0.161	0.426	0.184	< LoQ
Pepper*	0.161	0.426	0.168	< LoQ
Salt*	0.161	0.426	0.228	< LoQ
Other				
Apple fibre	0.143	0.414	0.141	< LoQ
Apricot, dried fruit	0.143	0.414	0.143	< LoQ
Carrageen	0.143	0.414	0.176	< LoQ
Casein	0.143	0.414	0.177	< LoQ
Cacao*	0.143	0.414	0.144	< LoQ
Coconut	0.143	0.414	0.225	< LoQ
Coffee*	0.143	0.414	0.235	< LoQ
Egg powder	0.143	0.414	0.208	< LoQ
Fig, dried fruit	0.106	0.429	0.133	< LoQ
Orange juice	0.161	0.426	0.142	< LoQ
Pineapple, dried fruit	0.161	0.426	0.181	< LoQ
Papaya, dried fruit	0.161	0.426	0.171	< LoQ
Potato, flour	0.161	0.426	0.175	< LoQ
Skim milk powder	0.106	0.429	0.172	< LoQ
Stabilizer xanthan	0.228	0.445	0.208	< LoQ
Sugar beet sirup	0.228	0.445	0.166	< LoQ
Tea, black*	0.143	0.414	0.191	< LoQ

* Extracted with addition of 1 g gluten-free skim milk powder

RIDASCREEN® Total Gluten

Art. No. R7041

3. Method uncertainty

3.1. Precision of extraction

To characterize the part of measurement uncertainty which is attributable to the extraction, a precision of extraction was performed. Three different samples were extracted. Each sample was extracted six times independently.

Table 8: Determination of extraction precision by analysis of three different incurred samples in one test kit lot by one person on three different days; each sample was extracted as six-fold replicate.

	Wheat in oats 20 mg/kg		Barley in oats 20 mg/kg		Snack B 41 mg/kg	
	Replicate	mg/kg	Replicate	mg/kg	Replicate	mg/kg
Day 1	1	18.0	1	19.8	1	30.5
	2	18.3	2	17.2	2	33.8
	3	13.8	3	17.0	3	37.5
	4	15.8	4	14.9	4	35.2
	5	23.2	5	13.3	5	34.5
	6	25.9	6	15.2	6	33.1
Day 2	1	17.1	1	19.8	1	30.3
	2	16.9	2	16.9	2	30.7
	3	17.0	3	18.9	3	32.9
	4	12.5	4	20.0	4	33.5
	5	11.4	5	18.2	5	29.1
	6	15.5	6	19.0	6	29.8
Day 3	1	15.9	1	17.3	1	29.9
	2	27.4	2	19.7	2	29.8
	3	20.6	3	20.2	3	30.3
	4	17.6	4	17.7	4	32.1
	5	18.5	5	24.7	5	29.6
	6	18.0	6	23.5	6	31.7
Mean		18.0		18.5		31.9
SD		4.17		2.80		2.33
CV (%)		23.2		15.1		7.3

As can be seen in table 8, the precision of extraction is mainly driven by the type of sample. Snack B is based on corn and was chosen for this study since its degree of homogeneity was well characterized [6]. Consequently, the CV for this sample is 7.3 % when the sample was extracted in n=6 and analyzed by one technician in one test kit lot on three days. As expected, oat-based samples led to higher CVs of 15.1 % and 23.2 % for barley in oats and wheat in oats, respectively. The higher CVs are probably due to the lower level of homogeneity of oat-based samples. These samples are the AOAC Reference Materials as mentioned in AOAC SMPR® 2017.021 [1] (oat flour containing wheat or barley gluten at a concentration of 20 mg/kg).

RIDASCREEN® Total Gluten

Art. No. R7041

3.2. Repeatability (intra-assay-variation)

To estimate the variation between technical duplicates on a plate, three different sample extracts were analyzed in n=6 wells in each run. The experiment was repeated on two more days. Table 9 shows that the variation is not higher than 9.1 %. The analysis of the wheat extract on day 2 is judged to be an outlier.

Table 9: Determination of repeatability precision by analysis of three different extracts in one test kit lot by one person on three different days; each sample was pipetted as a six-fold replicate on the plate.

		Concentration (mg/kg)		
Sample		Day 1	Day 2	Day 3
F-series wheat (20 mg/kg gluten)	Mean	16.6	16.8	17.5
	SD	0.74	3.44	0.70
	CV (%)	4.5	20.6	4.0
F-series rye (20 mg/kg gluten)	Mean	19.1	19.5	19.5
	SD	1.44	0.71	0.98
	CV (%)	7.5	3.6	5.0
Snack B (41 mg/kg gluten)	Mean	34.9	37.2	35.9
	SD	3.17	2.52	2.27
	CV (%)	9.1	6.8	6.3

RIDASCREEN® Total Gluten

Art. No. R7041

3.3. Within-laboratory-reproducibility (inter-assay-variation)

Three samples were used to characterize the laboratory-internal reproducibility (also called intermediate precision). Each sample was extracted six times each by three different persons and tested in three different lots on three different days (six extractions x three different samples x three different analysts = 54 data points in total). The measured concentrations of each extract were calculated. Mean, standard deviation and coefficient of variation were calculated for each day and for total of all runs. The coefficients of variation (all persons, lot 1 - 3) were between 10.8 % and 16.0 % with the lowest value for the snack B sample that was not oat-based but corn-based. All coefficients of variation were within the acceptable range.

Table 10: Determination of laboratory-internal reproducibility by analysis of three different incurred samples in three different test kit lots by three different persons on three different days; each sample was extracted six times by each person.

			Wheat in oats 20 mg/kg gluten	Barley in oats 20 mg/kg gluten	Snack B 41 mg/kg gluten
			mg/kg gluten	mg/kg gluten	mg/kg gluten
Person 1	Lot 1 Day 1	Mean	19.2	18.9	36.0
		SD	1.37	1.92	2.27
		CV (%)	7.10	10.1	6.30
Person 2	Lot 2 Day 2	Mean	17.5	17.9	33.3
		SD	3.93	3.21	2.11
		CV (%)	22.4	18.0	6.30
Person 3	Lot 3 Day 3	Mean	21.1	18.3	29.2
		SD	2.75	1.87	2.15
		CV (%)	13.1	10.2	7.40
All persons	**Lot 1 - 3**	**Mean**	**19.3**	**18.4**	**32.8**
		SD	**3.09**	**2.31**	**3.53**
		CV (%)	**16.0**	**12.6**	**10.8**

Between-laboratory-reproducibility (results of a collaborative study)

Following the AOAC guidelines, an international collaborative study was set up to validate the RIDASCREEN® Total Gluten as Official Method of Analysis of AOAC International (OMA) for quantitative gluten measurement in oat and oat-based foods.

21 different samples were analyzed as duplicates in a blinded manner by 19 laboratories in North America, Australia and Europe. The samples included AOAC reference materials, spiked, naturally contaminated and incurred materials. An overview is given in table 11.

RIDASCREEN® Total Gluten

Art. No. R7041

Table 11: Overview about samples used in the collaborative study. Concentration levels for wheat, rye and barley refer to the whole cereal and not to the gluten concentration level.

No.	Sample description	Content*
1	Wheat flour in oat flour (SMPR® reference material)	10 mg/kg gluten
2	Rye flour in oat flour (SMPR® reference material)	10 mg/kg gluten
3	Barley flour in oat flour (SMPR® reference material)	10 mg/kg gluten
4	Oat flour (SMPR® reference material)	Unspiked
5	Wheat flour contaminated processed corn-based snack	82 mg/kg gluten
6	Mixture of corn-based snacks	41 mg/kg gluten
7	Rice flour (naturally contaminated)	Very low gluten level
8	Flaked oats – blank	Unspiked
9	Flaked oats – low	265 mg/kg (B)
10	Flaked oats – high	530 mg/kg (B)
11	Cereal – blank	Unspiked
12	Cereal – low	179 mg/kg (B), 90 mg/kg (W), 90 mg/kg (R)
13	Cereal – medium	269 mg/kg (B), 134 mg/kg (W), 134 mg/kg (R)
14	Cereal – high	358 mg/kg (B), 179 mg/kg (W), 179 mg/kg (R)
15	Flour – blank	Unspiked
16	Flour – low	83 mg/kg (B), 41 mg/kg (W), 41 mg/kg (R)
17	Flour – medium	165 mg/kg (B), 83 mg/kg (W), 83 mg/kg (R)
18	Flour – high	330 mg/kg (B), 165 mg/kg (W), 165 mg/kg (R)
19	Groats – blank (naturally incurred)	na
20	Groats – low (naturally incurred)	na
21	Groats – high (naturally incurred)	na

* (B): barley (W): wheat (R): rye

Extraction was performed with the Cocktail (patented). ELISA was performed according to the test kit instructions. All laboratories supplied the data to the organizer of the collaborative study for statistical analysis. The definition of outliers and the overall statistical analysis was performed according to AOAC guidelines.

The collaborative study showed very good results. The analysis of the three reference materials with gluten contents from wheat or rye or barley of 10 mg/kg resulted in mean values of 10.8 mg/kg for wheat (108 % recovery), 13.7 mg/kg for rye (137 % recovery), and 11.0 mg/kg for barley (110 % recovery).
The relative standard deviation of reproducibility was between 15 and 21 % which is mainly driven by the inhomogeneity of the samples. Therefore it can be concluded that the RIDASCREEN® Total Gluten is not only precise but also accurate.

RIDASCREEN® Total Gluten

Art. No. R7041

Table 12: Overview about the results of the collaborative study.

Part A/sample	1	2	3	4	5	6	7	8	9	10
n Labs	19	18	17	15	18	19	16	19	19	19
N replicates	38	36	34	30	36	38	32	38	38	38
Mean; mg/kg	10.8	13.7	11.0	0.9	62.1	33.4	4.0	10.0	32.8	47.9
s(r); mg/kg	2.29	1.88	1.40	0.96	5.31	4.62	0.79	9.68	5.88	7.12
s(R); mg/kg	2.29	2.05	1.96	0.96	7.35	6.82	2.52	9.99	7.27	7.96
RSD(r), %	21.1	13.7	12.7	103.9	8.5	13.8	19.8	96.7	17.9	14.9
RSD(R); %	21.1	15.0	17.8	103.9	11.8	20.4	63.0	99.8	22.2	16.6

Part B/sample	11	12	13	14	15	16	17	18	19	20	21
n Labs	19	19	19	19	17	19	19	19	19	19	18
N replicates	38	38	38	38	34	38	38	38	38	38	36
Mean; mg/kg	3.1	21.0	15.9	27.2	2.1	6.3	12.9	22.0	7.2	13.5	20.3
s(r); mg/kg	1.51	5.91	3.87	5.80	2.23	2.70	5.34	5.99	1.93	2.86	2.22
s(R); mg/kg	2.20	6.15	4.68	6.88	2.23	3.44	5.79	6.75	2.31	2.92	3.67
RSD(r), %	48.4	28.2	24.3	21.3	107.2	42.6	41.3	27.2	26.6	21.2	10.9
RSD(R); %	70.5	29.3	29.4	25.3	107.2	54.3	44.7	30.6	31.9	21.7	18.1

The precision estimates demonstrate that the ELISA procedure is highly precise since RSD(R) values of 20 % or lower were obtained. Most of the oat-based products showed RSD(R) values at or lower than 30 % with the exception of oat flours that seems to be more inhomogeneous than the other samples. Additional information on the collaborative study is published in the Journal of AOAC [4].

3.4. Robustness

Robustness was checked by slight variation of pipetting volume, incubation time and incubation temperature. The standard assay performance is 100 µL pipetting volume, an incubation scheme of 20 min/20 min/10 min and 23 °C (20 - 25 °C). Pipetting volume and incubation time were varied +/- 10 % and incubation temperature was varied to 20 °C and 25 °C. Five different samples were tested; samples contained wheat, rye or barley. Table 13 shows that there is no consistent tendency or trend in the results when ELISA parameters were varied within an expectable range regarding pipetting volume and incubation time. However, higher incubation temperatures lead to increased results for barley and to a lower degree also for rye. This was confirmed by additional experiments (data not shown). Therefore, it is important not to exceed incubation temperature above 25 °C.

RIDASCREEN® Total Gluten

Art. No. R7041

Table 13: Variation of pipetted volumes for all pipetting steps from 90 µL to 110 µL; variation of incubation time from 18 min to 22 min for the first and second ELISA incubation step combined with variation of incubation time from 9 min to 11 min for the third ELISA incubation step; variation of incubation temperature from 20 °C to 25 °C for the whole ELISA procedure; results are given in mg/kg gluten.

	Control	90 µL	110 µL	18 min	22 min	20 °C	25 °C
Sample A (blank)	2.24	3.29	2.9	2.42	2.9	2.68	2.24
Sample B (wheat)	23.6	28.4	24.5	22.0	24.5	23.3	23.6
Sample C (barley)	31.2	27.7	27.9	27.6	27.9	24.8	31.2
Sample D (wheat)	38.1	39.1	38.1	33.0	38.1	39.6	38.1
Sample E (rye)	33.0	31.9	32.7	29.0	32.7	32.2	33.0

3.5. Stability of the test

The stability of the test is routinely checked by R-Biopharm's quality assurance laboratory after defined storage intervals. Test kits are stored in a cold room at temperatures of 2 - 8 °C. Before testing, the kit components are equilibrated to room temperature (20 - 25 °C). Real time stability of the test is regularly controlled according to the total quality management schedule of the company.

4. Conclusion

With the described ELISA a sensitive and reliable method is available which allows a quantitative and correct determination of gluten or parts of it from wheat, rye and barley in oat and oat products.

5. Limitations

Samples tested negative may contain gluten contamination below the limit of detection of the assay, or they still may contain other components like starch for example.
Due to the multitude of food types, matrix effects cannot be excluded. In processed food (e.g. heat treatment, dehydration, etc.), proteins may be altered or fragmented, this may have an impact on the recovery/cross reactivity. When analyzing a non-validated matrix, it is recommended to verify the results by spiking experiments.
For evaluation of the cross reactivity only one exemplary sample was analyzed, other samples may show a different result. Cross reactivities of the used antibodies have been determined for the pure food (e.g. corn flour). In a composed/processed food (e.g. corn bread), cross reactivities might be different.

Interfering substances (e.g. polyphenols, tannins) can be detected by spike experiments. A false-positive impact of interfering substances may be prevented by addition of skim milk powder (SMP) during sample extraction. Hence it is recommended to use SMP with sample matrices being at risk showing too high

RIDASCREEN® Total Gluten

Art. No. R7041

results by interfering substances (see test kit instruction). However, if a matrix is proven by the user not to be impacted, the addition of SMP can be omitted. In this case, positive results should be interpreted carefully. Exemplary matrices/foods have been used for spiking within the scope of this study.

6. List of references

[1] *Boison J, Allred L, Almy D, Anderson L, Baumert J, Bhandari S, Cebolla A, Chen Y, Crowley E, Diaz-Amigo C, Doi H, Don C, Downs M, Dubiel N, Dyer B, Emerson L, Farrow M, Fritz R, Galera C, Garber E, Godefroy S, Grace T, Hochegger R, Johnson K, Kasturi P, Koerner T, Lacorn M, Massong F, Meinhardt P, Mui T, O'Meara M, Pan SJ, Popping B, Prinster M, Quesada E, Radcliffe S, Scherf K, Sharma G, Shoji M, Stoughton M, Sweeney L, Szpylka J, Taylor S, Tittlemier S, Torgler C, Wehling P, Yeung J, Zweigenbaum J*: Standard Method Performance Requirements (SMPRs®) for Quantitation of Wheat, Rye and Barley Gluten in Oats. J AOAC Int. (2017) 101, 1238-1242.

[2] AOAC International. Appendix M: Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices. AOAC Official Methods of Analysis. Gaithersburg. MD, 2012.

[3] CLSI EP25-A (2009), Evaluation of stability of In-Vitro Diagnostic Reagents, Approved Guideline

[4] *Lacorn M, Weiss T, Wehling P, Arlinghaus M, Scherf K*: Quantification of Wheat, Rye and Barley Gluten in Oat and Oat Products by ELISA RIDASCREEN® Total Gluten: collaborative Study, First Action 2018.15. J AOAC Int. (2019) 102, 1535-1543

[5] *Koerner T, Abbott M, Godefroy SB, Popping B, Yeung JM, Diaz-Amigo C, Roberts J, Taylor SL, Baumert JL, Ulberth F, Wehling P, Koehler P*: Validation procedures for quantitative gluten ELISA methods: AOAC allergen community guidance and best practices. J AOAC Int. (2013) 96:1033.

[6] *Koehler P, Schwalb T, Immer U, Lacorn M, Wehling P, Don C*: AACCI Approved Methods Technical Committee Report: Collaborative Study on the Immunochemical Determination of Intact Gluten Using an R5 Sandwich ELISA. Cereal Foods World (2013) 58, 36-40.

7. List of abbreviations

CV	Coefficient of variation	Rec.	Recovery
LoD	Limit of detection	SD	Standard deviation
LoQ	Limit of quantitation	SMP	Skim milk powder

RIDASCREEN® Total Gluten

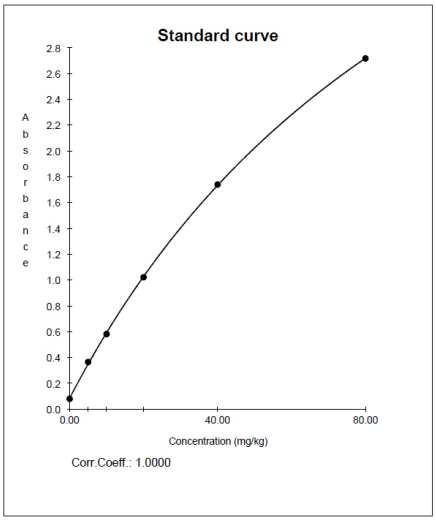
Art. No. R7041

8. Appendix 1 – Quality Assurance Certificate

Quality Assurance Certificate

RIDASCREEN® Total Gluten

REF Art. No.	R7041
LOT Lot	23470
Expiry	2021-08



Standards

Std. Conc.(mg/kg) CV(%)	n mean
Std1 0.00 6.3	3 0.080
Std2 5.00 2.0	3 0.365
Std3 10.00 3.2	3 0.582
Std4 20.00 0.3	3 1.022
Std5 40.00 0.6	3 1.740
Std6 80.00 2.9	3 2.717

	Lot. No.	Expiry
Microtiter plate	22250	2022-05
Buffer	24450	2022-03
Standards	22450	2021-11
Wash buffer	14489	2022-10
Conjugate	21450	2021-08
Substrate/Chromogen (Red Chromogen Pro)	21030	2023-02

	Lot. No.	Expiry
Stop solution	12489	2024-10

IFU (and other accompanying documents where applicable)

We herewith certify that this batch has been approved by the Quality Control Department and has met the release criteria.

Please note: The absorbance for the standards may decrease during the shelf life of the kit. The general shape of the curve will remain similar, while the slope might change slightly. Furthermore refer to product leaflet section 8. Indication of instability or deterioration of reagents.

Quality Control Department

Date: 2020-11-18

Quality Management System certified ISO 9001 • ISO 13485 DQS Medizinprodukte GmbH, Germany

Remark: This document is created electronically and therefore valid without a signature.

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RIDASCREEN® Total Gluten

Art. No. R7041

9. Appendix 2 – Spike instructions

Spike with wheat flour extract

If the exact gluten content of the wheat flour is not known, the following approximation can be used to calculate the gluten content: gluten content (%) = total protein content of the wheat flour (%) * 0.8. Typical gluten contents for wheat flours are 7 - 12 %.

Preparation of the spike solution

- Weigh in 250 mg of wheat flour and add 2.5 ml Cocktail (patented). Vortex thoroughly and incubate for 40 min in a 50 °C (122 °F) water bath.
- Let the vial cool down on the table for five minutes, then add 7.5 ml 80% ethanol.
- Vortex thoroughly and shake for 1 h upside down or by a rotator at room temperature (20 - 25 °C / 68 - 77 °F).
- Centrifuge the extract for 10 min with at least 2500 g at room temperature and filter the supernatant after centrifugation.
- This solution A has a gluten content of 1/40 of the wheat flour (e.g. the solution has a concentration of 0.25 % gluten, equal to 2.5 mg/ml, for a wheat flour with 10 % gluten)

Spiking of samples

- Dilute solution A 1:10 with Total Gluten sample dilution buffer (e.g. 500 µl solution A in 4500 µl buffer) -> solution B with 250 µg gluten / ml in case of a wheat flour with 10 % gluten.
- Weigh in 1 g of the oat containing sample.
- For a 20 mg gluten / kg spike, add 80 µl of solution B (in case of a wheat flour with 10 % gluten).
- Extract the spiked sample according to the instructions for use.
- If the wheat flour contains a different gluten concentration and / or other spike levels are chosen, spike volumes have to be adjusted accordingly. In any case, it is not recommended to pipet less than 50 µl of the spike solution. If necessary, make additional dilutions or change dilution ratios for the spike solution.

RIDASCREEN® Total Gluten

Art. No. R7041

10. Appendix 3 – Equipment

Pipettes:
- 10 - 100 µL Eppendorf Research Plus, Eppendorf
- 20 - 200 µL Eppendorf Research Plus, Eppendorf
- 100 - 1000 µL Eppendorf Research Plus, Eppendorf
- Multichannel: 30 - 300 µL Eppendorf Research Plus, Eppendorf

Tips:
- epT.I.P.S.® Standard/Bulk 2 - 200 µL, Eppendorf
- epT.I.P.S.® Standard 50 - 1000 µL, Eppendorf

Multistepper:
- Multipette® Xstream Eppendorf Research Plus, Eppendorf

Multistepper tips:
- 2.5; 5 and 50 mL Combitips Advanced®, Eppendorf

Serological pipette:
- 5 mL and 25 mL CELLSTAR® Serological Pipette, Greiner Bio-One

Pre-plate:
- Mikrotiter Assembly breakable strip 1x8, Thermo Scientific
- Low binding from Greiner Bio-One Art.-No. 655101

Photometer:
- Tecan Sunrise, Tecan

Wash:
- Dispensette®, Brand
- Brand Tech® 8-channel Manifold, Brand